

# **Design, synthesis, and bioactivity of hydroxybenzoic acid derivatives and their polymer nanocomposites**

By

**KHUMBLANI MNQIWU**

(2050388)

*A thesis submitted in partial fulfilment of the requirements for the degree*

**Doctor of Philosophy**

As applicable in the School of Oral Health Sciences

University of the Witwatersrand

South Africa

Supervisor: Dr. Sharon Moeno

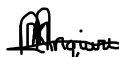
Co-supervisor: Prof. Mrudula Patel

## Declaration

---

I hereby declare that this thesis entitled “design, synthesis, and bioactivity of hydroxybenzoic acid derivatives and their polymer nanocomposites” is my original work and has not been submitted in whole or in part for any other degree or qualification at any other university or institution. All sources used or referred to in this thesis have been accordingly acknowledged. Any assistance received in the preparation of this thesis has been acknowledged. All data, figures, tables, and other information obtained from external sources have been properly cited and referenced. The views and opinions expressed in this thesis are entirely my own and do not necessarily reflect those of the university or institution.

**Name:** \_Khumblani Mnqiwu\_\_\_\_\_

**Signature:**  \_\_\_\_\_

**Date:** \_26 July 2024\_\_\_\_\_

## **Dedication and Acknowledgements**

---

### **Dedication:**

To my late father, whose unwavering love, wisdom, and guidance continue to inspire me every day. You will always be my guiding light, and I dedicate this work to you and our family, who have been my pillars of strength throughout this journey.

### **Acknowledgement:**

I extend my deepest gratitude to my supervisors, Dr. Moeno and Prof. Patel, for their steadfast support, invaluable guidance, and constant encouragement. Your mentorship has been instrumental in shaping my academic path.

To my beloved wife, Lesego Moagi, your unwavering support, love, and understanding have been my source of strength. I am endlessly grateful for your presence by my side throughout this challenging journey.

To my dear children, Sibusiso and Anovuyo, thank you for your patience and understanding during the demanding times of my PhD tenure. Your love and understanding mean the world to me.

I am indebted to my mother and siblings for their unwavering support and encouragement. Your belief in me kept me going, even during the most overwhelming moments.

A heartfelt thank you goes to my dear friend soon-to-be Dr. Wanda Bout. Your companionship, late-night thesis writing sessions, and meaningful conversations were a source of solace and motivation. I couldn't have done it without you.

Special thanks to my best friend, Phumzile Skele, for always being there for me, and offering unwavering support and assistance whenever needed.

I am grateful to Dr. Musa Marimani for his constant support, whether financially, emotionally, or spiritually. Your friendship has been a blessing.

To Mr. George Ralefatane, your unwavering support and assistance during my early days at Wits are deeply appreciated. Thank you for standing by me.

Acknowledgement is also due to Ntombikayise Xelwa, Mulalo Mulaudzi, and Themban Tshiteme for their emotional support and belief in me.

A heartfelt message to Sibongile Langa, whose expertise in microbiology has been invaluable. Your guidance and mentorship have been transformative.

Last but not least, my heartfelt gratitude to Dr. Phumlani Tetyana and his wife, Dr. Zikhona Tetyana, for their unwavering support and assistance. Your generosity and kindness have been truly humbling.

To all those whose contributions I may not have mentioned, thank you for being part of my journey and for your invaluable support. This work would not have been possible without your collective efforts.



## Publications and conferences

---

### List of conferences

- “Synthesis of gallic acid-based intermediate compounds for potential application as anticancer and antimicrobial agents” – Poster presentation at the 9<sup>th</sup> Wits University cross-faculty symposium at Wits, South Africa. September 2018.
- “Virtual screening of benzoic acid derivatives as novel DNA Topoisomerase I inhibitors: In silico study” Oral presentation at the 10th Wits University cross-faculty symposium at Wits, South Africa. September 2019. **WON 3rd PRIZE**
- “In-silico design of bioactive compounds and polymeric nanoparticles: for use in anticancer application” Oral presentation at SANi NYRS held at University of Pretoria, South Africa. November 2019
- “Virtual Screening of Benzoic Acid Derivatives as Novel DNA Topoisomerase I Inhibitors: In Silico Study” Oral presentation at the Virtual Conference on Computational Science (VCCS-2019) held during August 2019 virtually and hosted by the University of Mauritius, Mauritius.
- “Resilience of Hydroxybenzoic acid derivatives against Camptothecin resistant DNA topoisomerase I: In-silico study” Oral presentation at the 2nd Wits PhD seminar at Wits, South Africa. September 2021.
- “Hydroxybenzoic acid amide derivatives and their in-silico molecular interactions with DNA topoisomerase I proteins” Oral presentation 12th Wits University cross-faculty symposium at Wits, South Africa. October 2021.

### Publications in progress

Mnqiwu, K., Thetyana, P., Patel, M., Langa, S., Moeno, S. Synthesis, cytotoxicity and *in-vitro* antimicrobial activity of hydroxybenzoic acid derivatives. Submitted to Chemistry & Biology Journal.

Mnqiwu, K., Ngoepe, M., Moeno, S. Hydroxybenzoic acid derivatives as potential DNA topoisomerase I and human multidrug resistance ABCG2 inhibitors: In-silico study. Submitted to Journal of Computational Biology and Chemistry.

Mnqiwu, K., Langa, S., Marimani, M., Patel, M., Moeno, S. Antivirulence effects of hydroxybenzoic acid derivatives and their polymer nanocomposites. To be submitted to Frontiers in Cellular and Infectious Microbiology.

## Abstract

---

Cancer remains a global health challenge, necessitating a multifaceted approach integrating prevention, early detection, diagnosis, treatment, and ongoing research. Recent advancements in understanding the molecular intricacies of cancer have led to the development of targeted therapies and immunotherapy.

However, the weakened immunity in cancer patients poses additional risks, with secondary opportunistic infections becoming a significant concern. Addressing this dual challenge requires innovative strategies, including *in-silico* methods to expedite drug discovery. In this study, novel compounds were designed and assessed for potential antimicrobial and anticancer properties through computational modeling. While *in-silico* predictions suggested promising candidates, experimental validation revealed discrepancies, emphasizing the complexity of predicting bioactivity solely through computational models. The computational method, however, showed that tri-substituted compounds are active and it was proven by experimental work. Compound C4 (benzene-1,3,5-triyl tris(3-hydroxybenzoate)) which is also a tri-substituted compound derived from 3-hydroxybenzoic acid showed great promise instead of the predicted compound C13 (benzene-1,3,5-triyl tris(3,4,5-trihydroxybenzoate)). Even though, compound C1 (3-acetylphenyl 3-hydroxybenzoate) was the leading candidate among other ester and amide compounds. Most of the compounds were non-toxic both *in silico* and *in vitro* corroborating the experimental and computational findings.

Nonetheless, the synthesized compounds showed potential for further optimization as drug precursors. To enhance their activity and delivery, novel polymer nanoparticles were employed as carriers, resulting in improved efficacy in antimicrobial and anticancer assays. These findings underscore the importance of experimental validation and highlight the potential of nanomaterial-based drug delivery systems in addressing the dual challenge of cancer and microbial infections.

## Table of contents

---

### Table of Contents

|                                                                                     |      |
|-------------------------------------------------------------------------------------|------|
| Declaration.....                                                                    | i    |
| Dedication and Acknowledgements .....                                               | ii   |
| Publications and conferences.....                                                   | iv   |
| Abstract.....                                                                       | vi   |
| Table of contents.....                                                              | vii  |
| List of Figures .....                                                               | xi   |
| List of Tables .....                                                                | xv   |
| List of Abbreviations .....                                                         | xvii |
| Chapter 1: Introduction .....                                                       | 1    |
| 1. Background and introduction.....                                                 | 1    |
| 1.1 Cancer prevalence.....                                                          | 1    |
| 1.2 Cancer-targeted therapy.....                                                    | 2    |
| 1.3 DNA topoisomerase I enzyme.....                                                 | 3    |
| 1.4 Problem statement .....                                                         | 3    |
| 1.5 Justification.....                                                              | 4    |
| 1.6 Novelty of the study .....                                                      | 4    |
| 1.7 Hypothesis .....                                                                | 5    |
| Chapter 2: Literature Review.....                                                   | 6    |
| 2.1 Cancer.....                                                                     | 6    |
| 2.1.1 Global prevalence of cancer .....                                             | 6    |
| 2.1.2 Treatment modalities of cancer .....                                          | 7    |
| 2.1.3 Shortfalls of the current treatment modalities .....                          | 15   |
| 2.1.4 Cancer target therapy.....                                                    | 16   |
| 2.1.5 The search for new chemotherapeutic agents.....                               | 21   |
| 2.1.6 Hydroxybenzoic acid (HBA).....                                                | 21   |
| 2.2 Microbial infections .....                                                      | 28   |
| 2.2.1 Clinical strains .....                                                        | 29   |
| 2.2.2 Microbial strains that affect cancer patients .....                           | 33   |
| 2.2.3 Antimicrobial agents used to treat cancer patients and their shortfalls ..... | 34   |
| 2.2.4 Antimicrobial agents that bear hydroxyl moieties or hydroxybenzoic acid.....  | 38   |
| 2.2.5 Important HBA compounds for antimicrobial activity.....                       | 38   |

|                                                                                                    |    |
|----------------------------------------------------------------------------------------------------|----|
| 2.3 <i>In silico</i> drug design .....                                                             | 39 |
| 2.4 Drug delivery systems (DDS) .....                                                              | 42 |
| 2.4.1 Mechanism of DDS .....                                                                       | 43 |
| 2.4.2 Nanocarriers.....                                                                            | 44 |
| 2.5 Conclusion and present work .....                                                              | 50 |
| Chapter 3: Aims and objectives, thesis outline and research design .....                           | 51 |
| 3.1 Aims and Objectives.....                                                                       | 51 |
| 3.2 Thesis outline.....                                                                            | 51 |
| 3.3 Study design .....                                                                             | 54 |
| Chapter 4: <i>In-silico</i> drug design.....                                                       | 55 |
| 4.1 Introduction .....                                                                             | 55 |
| 4.2 Computational methods.....                                                                     | 57 |
| 4.2.1 Tools used.....                                                                              | 57 |
| 4.2.2 Preparation of ligands and proteins .....                                                    | 58 |
| 4.2.3 Molecular docking.....                                                                       | 58 |
| 4.2.4 Molecular dynamic simulations.....                                                           | 59 |
| 4.2.5 Absorption, distribution, metabolism, excretion (ADME)/Toxicity properties .....             | 59 |
| 4.2.6 Density Functional Theory (DFT) calculations.....                                            | 60 |
| 4.3 Results .....                                                                                  | 60 |
| 4.3.1 Docking Scores Analysis.....                                                                 | 64 |
| 4.3.2 Protein-ligand interactions:.....                                                            | 66 |
| 4.3.3 Density Functional Theory (DFT) calculations - Frontier molecular orbitals (FMOs): .....     | 68 |
| 4.3.4 Molecular Dynamics (MD) .....                                                                | 72 |
| 4.4 Discussion .....                                                                               | 75 |
| 4.4.1 <i>In silico</i> drug-likeness and toxicity studies.....                                     | 76 |
| 4.4.2 Physicochemical properties .....                                                             | 78 |
| 4.4.3 Molecular docking and protein-ligand interaction .....                                       | 79 |
| 4.5 Conclusion.....                                                                                | 80 |
| Chapter 5: Synthesis and characterization of hydroxybenzoic acid amide and ester derivatives ..... | 81 |
| 5.1 Introduction .....                                                                             | 81 |
| 5.2 Materials and methods .....                                                                    | 82 |
| 5.2.1 Materials and characterization.....                                                          | 82 |
| 5.2.2 Synthesis of ester derivative compounds.....                                                 | 83 |

|                                                                                             |     |
|---------------------------------------------------------------------------------------------|-----|
| 5.2.3 Synthesis of amide/anilide compounds .....                                            | 89  |
| 5.3 Results .....                                                                           | 94  |
| 5.3.1 Elucidation of ester compounds using FTIR spectroscopy .....                          | 94  |
| 5.3.2 Elucidation of amide compounds using FTIR spectroscopy .....                          | 97  |
| 5.3.3 Elucidation of ester compounds using <sup>1</sup> H-NMR spectroscopy .....            | 99  |
| 5.3.4 Elucidation of amide compounds using NMR spectroscopy.....                            | 112 |
| 5.3.5 Structural confirmation of amide and ester compounds from mass spectroscopy (MS)..... | 125 |
| 5.4 Discussion .....                                                                        | 129 |
| 5.4.1 FTIR, NMR and MS structural elucidation of ester compounds.....                       | 129 |
| 5.4.2 FTIR, NMR and MS structural elucidation of amide compounds .....                      | 135 |
| 5.5 Conclusion.....                                                                         | 137 |
| Chapter 6: Synthesis and characterization of nanoparticles .....                            | 140 |
| 6.1 Introduction .....                                                                      | 140 |
| 6.2 Materials and Methods.....                                                              | 141 |
| 6.2.1 Materials .....                                                                       | 141 |
| 6.2.2 Methods .....                                                                         | 142 |
| 6.3 Results .....                                                                           | 143 |
| 6.3.1 FTIR results of the blank polymer and loaded nanoparticles .....                      | 143 |
| 6.3.2 TEM results of blank nanoparticles.....                                               | 145 |
| 6.3.3 TEM results of loaded nanoparticles .....                                             | 146 |
| 6.3.4 Dynamic light scattering (DLS) results of blank and loaded nanoparticles .....        | 147 |
| 6.4 Discussion .....                                                                        | 147 |
| 6.4.1 Fourier transform infrared (FTIR) spectroscopy .....                                  | 147 |
| 6.4.2 Transmission electron microscopy (TEM) .....                                          | 148 |
| 6.4.3 Dynamic light scattering (DLS) .....                                                  | 151 |
| 6.5 Conclusion.....                                                                         | 153 |
| Chapter 7: Antimicrobial assay and activity .....                                           | 154 |
| 7.1. Introduction .....                                                                     | 154 |
| 7.2. Methods and materials .....                                                            | 154 |
| 7.2.1. Test compounds .....                                                                 | 154 |
| 7.2.2. Test organisms .....                                                                 | 156 |
| 7.2.3. MIC and MBC/MFC assay .....                                                          | 156 |
| 7.3. Results .....                                                                          | 156 |
| 7.3.1. MIC/MBC results of Amides.....                                                       | 156 |

|                                                                                        |     |
|----------------------------------------------------------------------------------------|-----|
| 7.3.2. MIC/MFCs results of Amides.....                                                 | 160 |
| 7.3.3. MIC/MBC of Ester derivatives.....                                               | 164 |
| 7.3.4. MIC/MFC of Ester derivatives .....                                              | 169 |
| 7.3.5. MIC/MBC of nanoparticles loaded with Ester and Amide derivatives .....          | 174 |
| 7.3.6. MIC/MFC of nanoparticles loaded with Ester and Amide derivatives .....          | 177 |
| 7.3.7. MIC/MBC/MFC of Combination of Amide and Ester derivatives .....                 | 180 |
| 7.4. Discussion .....                                                                  | 184 |
| 7.4.1. Antibacterial activity of Amides and Ester derivatives .....                    | 184 |
| 7.4.2. Antifungal activity of Amides and Ester derivatives.....                        | 187 |
| 7.4.3. Combination of amide and ester compounds.....                                   | 188 |
| 7.4.4. Antimicrobial activity of nanoparticle-loaded Amides and Ester derivatives..... | 189 |
| 7.5 Conclusions.....                                                                   | 191 |
| Chapter 8: Cell Viability and Proliferation.....                                       | 192 |
| 8.1 Introduction .....                                                                 | 192 |
| 8.2. Methods and materials .....                                                       | 193 |
| 8.2.1 Cell Culture: .....                                                              | 193 |
| 8.2.2 Test Compounds:.....                                                             | 193 |
| 8.2.3 Cytotoxicity Assay .....                                                         | 193 |
| 8.3 Results .....                                                                      | 194 |
| 8.3.1 Cytotoxicity and anticancer activity of the amide compounds.....                 | 194 |
| 8.3.2 Cytotoxicity and anticancer activity of ester compounds.....                     | 199 |
| 8.3.3 Cytotoxicity and anticancer activity of blank and loaded nanoparticles .....     | 204 |
| 8.4 Discussion .....                                                                   | 207 |
| 8.4.1 Cytotoxicity and anticancer activity of the amide compounds.....                 | 207 |
| 8.4.2 Cytotoxicity and anticancer activity of the ester compounds.....                 | 208 |
| 8.4.3 Cytotoxicity and anticancer activity of the nanoparticles .....                  | 210 |
| 8.5 Conclusion.....                                                                    | 211 |
| Chapter 9: General Discussion.....                                                     | 213 |
| Chapter 10: Overall conclusion and recommendations .....                               | 220 |
| 10.1 Conclusion.....                                                                   | 220 |
| 10.2 Limitations of the study.....                                                     | 221 |
| 10.3 Recommendations of the study .....                                                | 221 |
| References.....                                                                        | 223 |
| Appendices.....                                                                        | 248 |

## List of Figures

---

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |    |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Figure 1.1:</b> Number of new cases in 2020, both sexes and all ages (WHO, 2020) .....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | 2  |
| <br>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |    |
| <b>Figure 2. 1:</b> The legend includes the national ranking of cancer as a cause of death among individuals under the age of 70 in 2019. It also provides the number of countries represented in each ranking group. Adopted from Sung et al. <sup>14</sup> .....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 7  |
| <b>Figure 2. 2:</b> Unwinding of the supercoiled DNA by TOP1-mediated DNA cleavage complex (adopted from <sup>75</sup> ). (a) DNA supercoiling caused by DNA replication, transcription, and chromatin remodelling <sup>75</sup> . (b) Relaxation of the supercoiled DNA through an intermediate TOP1 cleavage complex step. (c) The intermediate TOP1-cleaved complex step were three processes takes place i.e. Nicking which is where the DNA single-strand breaks (nicks), controlled rotation when the TOP1 provides swivel points that enable the rotation of the intact DNA strand around the break and facilitate DNA relaxation, and the third step religation which is where the TOP1 realign and seals the broken strand. (d) By misaligning the ends of the broken DNA, drugs and DNA lesions prevent religation causing different cell death mechanisms. .... | 18 |
| <b>Figure 2. 3:</b> The FDA approved CPT analogues adopted from <sup>81</sup> .....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | 20 |
| <b>Figure 2. 4:</b> The other camptothecin analogs adopted from <sup>81</sup> .....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | 20 |
| <b>Figure 2. 5:</b> Selected hydroxybenzoic acids for this study. ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | 22 |
| <b>Figure 2. 6:</b> Natural sources of gallic acid and its derivatives.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 24 |
| <b>Figure 2. 7:</b> Naturally occurring gallic acid and derivatives.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | 25 |
| <b>Figure 2. 8:</b> Scanning electron microgram (SEM) of <i>S. aureus</i> showing spherically shaped bacterium and clinical manifestations of <i>S. aureus</i> . Images adopted from <sup>133</sup> .....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | 30 |
| <b>Figure 2. 9:</b> The images shows a gram-stained of facultative anaerobic, rod-shaped coliform bacterium ( <i>Escherichia coli</i> ) viewed under light microscope and symptoms of <i>E. coli</i> . Images were obtained from <sup>137</sup> . ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | 31 |
| <b>Figure 2. 10:</b> The image on the left shows an extracellular bacteria made up of non-motile and non-sporing cocci (round cells) that tend to form chains. The image on the right shows a patient with a sore throat caused by <i>P. aeruginosa</i> . The images were adopted from centre for disease control (CDC) retrieved from ( <a href="https://www.cdc.gov/streplab/groupa-strep/index.html">https://www.cdc.gov/streplab/groupa-strep/index.html</a> ). ....                                                                                                                                                                                                                                                                                                                                                                                                   | 32 |
| <b>Figure 2. 11:</b> Some of the antifungal agents used to treat infections in cancer patients.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | 35 |
| <b>Figure 2. 12:</b> Some of the Gram-negative antibacterial drugs used to treat infections in cancer patients.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | 36 |
| <b>Figure 2. 13:</b> Some of the Gram-positive antibacterial drugs used to treat infections in cancer patients. ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 36 |
| <b>Figure 2. 14:</b> Classification and mechanism of action of antibacterial drugs. Inhibition of bacterial cell wall synthesis, enhanced permeability of bacterial cell membranes, interference with bacterial protein synthesis, and inhibition of bacterial nucleic acid replication and transcription are the four major mechanisms. Adopted with modification from Gao and co-workers <sup>161</sup> .....                                                                                                                                                                                                                                                                                                                                                                                                                                                            | 37 |



|                                                                                                                                                                                                                                                                   |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Figure 2. 15:</b> Structure-based drug design (SBDD) and ligand-based drug design (LBDD) traditional workflow reprinted from <sup>180</sup> via license: Creative Commons Attribution 4.0 International. ....                                                  | 41  |
| <b>Figure 2. 16:</b> The structures illustrating (a) single-walled carbon nanotubes and (b) multi-walled carbon nanotubes. Adopted from <sup>105</sup> . ....                                                                                                     | 45  |
| <b>Figure 2. 17:</b> Schematic illustration of key physicochemical characteristics of nanomaterials that affect their potential for use in biomedicine. Adopted from Navya and co-workers <sup>220</sup> . .                                                      | 46  |
| <b>Figure 2. 18:</b> Physicochemical properties of an ideal PNPs and their characteristics based size, surface and morphology. Adopted from Elsabahy and Wooley <sup>224</sup> .....                                                                              | 47  |
| <b>Figure 2. 19:</b> Schematic diagram of nanoparticles that are responsive to acidic pH of the tumor tissue <sup>232</sup> .....                                                                                                                                 | 49  |
|                                                                                                                                                                                                                                                                   |     |
| <b>Figure 4. 1:</b> 2D structures of the designed hydroxybenzoic acid amide derivative compounds .....                                                                                                                                                            | 56  |
| <b>Figure 4. 2:</b> 2D structures of the designed hydroxybenzoic acid ester derivative compounds .....                                                                                                                                                            | 57  |
| <b>Figure 4. 3:</b> Three dimensional X-ray crystal structures of the selected DNA topoisomerase I and ABCG2 proteins obtained from the protein data bank (PDB) with their PDB ID's (1K4T and 7NEZ).....                                                          | 65  |
| <b>Figure 4. 4:</b> 2D docking protein-ligand interactions of C13 (b & d) and reference compound TTC (a & c) against 1K4T and 7NEZ. ....                                                                                                                          | 68  |
| <b>Figure 4. 5:</b> Frontier molecular orbital descriptors of amide compounds (a) KAM 4 which showed the best LUMO value and high $\Delta E$ value, (b) KAM 2 which showed the lowest $\Delta E$ value and (c) KAM 11 which showed the best HOMO eigenvalue. .... | 71  |
| <b>Figure 4. 6:</b> Frontier molecular orbital descriptors of ester compounds (a) C8 which showed the lowest $\Delta E$ value and (b) C13 which showed the best HOMO and LUMO values and also the highest $\Delta E$ value.....                                   | 72  |
| <b>Figure 4. 7:</b> Root-mean-square deviation (RMSD) graph of compound C13 (CE12) and TTC complexes with 1K4T and 7NEZ proteins.....                                                                                                                             | 73  |
| <b>Figure 4. 8:</b> H-bonds graph of compound C13 (CE12) and TTC complexes with 1K4T and 7NEZ proteins.....                                                                                                                                                       | 74  |
| <b>Figure 4. 9:</b> The root-mean-square fluctuation (RMSF) graph of compound CE12 and TTC complexes with 1K4T and 7NEZ proteins.....                                                                                                                             | 75  |
|                                                                                                                                                                                                                                                                   |     |
| <b>Figure 5. 1:</b> FTIR graph of 3-acetylphenyl 3-hydroxybenzoate ( <b>C1</b> ).....                                                                                                                                                                             | 96  |
| <b>Figure 5. 2:</b> FTIR graph of N,N'-(1,3-phenylene)bis(3-hydroxybenzamide) ( <b>KAM 1</b> ).....                                                                                                                                                               | 98  |
| <b>Figure 5. 3:</b> Classifications of ester according to their substitutions (a) mono-ester from acetophenone, (b) mono-ester from N-phenylacetamide, (c) di-esters, and (d) tri-esters. ....                                                                    | 99  |
| <b>Figure 5. 4:</b> NMR spectrum of 3-acetylphenyl 3,4,5-trihydroxybenzoate ( <b>C11</b> ) in DMSO- $d_6$ (a) $^1H$ at 500 MHz and (b) $^{13}C$ at 100 MHz.....                                                                                                   | 102 |
| <b>Figure 5. 5:</b> 2D NMR of 3-acetamidophenyl 3,4,5-trihydroxybenzoate (a) COSY and (b) HMBC in DMSO- $d_6$ .....                                                                                                                                               | 105 |
| <b>Figure 5. 6:</b> NMR spectrum of 5-acetyl-1,3-phenylene bis(3,4,5-trihydroxybenzoate) ( <b>C12</b> ) in DMSO- $d_6$ (a) $^1H$ at 500 MHz and (b) $^{13}C$ at 100 MHz.....                                                                                      | 108 |

|                                                                                                                                                                                                                                                                           |     |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Figure 5. 7:</b> NMR spectrum of benzene-1,3,5-triyl tris(3,5-dihydroxybenzoate) ( <b>C9</b> ) in DMSO-d <sub>6</sub> (a) <sup>1</sup> H at 500 MHz and (b) <sup>13</sup> C at 100 MHz .....                                                                           | 111 |
| <b>Figure 5. 8:</b> Classifications of amide compounds according to their substitutions (a) mono-amides from aniline, (b) mono-amides from 3-aminophenol (c) di-amides from 1,3-diaminobenzene, and (d) di-amides from 2,4-diaminophenol. ....                            | 112 |
| <b>Figure 5. 9:</b> NMR spectrum of 3,4,5-trihydroxy-N-phenylbenzamide ( <b>KAM 9</b> ) in DMSO-d <sub>6</sub> (a) <sup>1</sup> H at 500 MHz and (b) <sup>13</sup> C at 100 MHz. ....                                                                                     | 115 |
| <b>Figure 5. 10:</b> NMR spectrum of 3,4,5-trihydroxy-N-(3-hydroxyphenyl)benzamide ( <b>KAM 5</b> ) in DMSO-d <sub>6</sub> (a) <sup>1</sup> H at 500 MHz and (b) <sup>13</sup> C at 100 MHz. ....                                                                         | 118 |
| <b>Figure 5. 11:</b> NMR spectrum of N,N'-(1,3-phenylene)bis(3,4,5-trihydroxybenzamide) ( <b>KAM 6</b> ) in DMSO-d <sub>6</sub> (a) <sup>1</sup> H at 500 MHz and (b) <sup>13</sup> C at 100 MHz. ....                                                                    | 121 |
| <b>Figure 5. 12:</b> NMR spectrum of N,N'-(4-hydroxy-1,3-phenylene)bis(3,4,5-trihydroxybenzamide) ( <b>KAM 11</b> ) in DMSO-d <sub>6</sub> (a) <sup>1</sup> H at 500 MHz and (b) <sup>13</sup> C at 100 MHz .....                                                         | 124 |
| <b>Figure 5. 13:</b> Mass spectrum of 3-acetylphenyl 3-hydroxybenzoate ( <b>C1</b> ) fragments .....                                                                                                                                                                      | 126 |
| <b>Figure 5. 14:</b> Mass spectrum of 3,5-dihydroxy-N-phenylbenzamide ( <b>KAM 10</b> ) fragments .....                                                                                                                                                                   | 128 |
|                                                                                                                                                                                                                                                                           |     |
| <b>Figure 6. 1:</b> Fourier transform infrared spectra of pristine NIPAM (red), pristine TCA (blue) and blank nanoparticles (black). ....                                                                                                                                 | 144 |
| <b>Figure 6. 2:</b> Fourier transform infrared spectra of loaded nanoparticles (KAM1-L (blue), C1-L (red)) and blank nanoparticles (black). ....                                                                                                                          | 144 |
| <b>Figure 6. 3:</b> TEM images of blank nanoparticles for (a-c) 30:70 monomer (NIPAM: TCA) concentration, 50:50 monomer (NIPAM: TCA), and 70:30 monomer (NIPAM: TCA) respectively and histogram micrographs of (d-e) 30:70 monomers and 50:50 monomers respectively. .... | 145 |
| <b>Figure 6. 4:</b> TEM images of (a-b) C1-loaded and KAM1-loaded nanoparticles respectively and histogram of (c-d) C1-loaded and KAM1-loaded nanoparticles respectively. ....                                                                                            | 146 |
|                                                                                                                                                                                                                                                                           |     |
| <b>Figure 7. 1:</b> 2D structures of the designed hydroxybenzoic acid amide derivative compounds .....                                                                                                                                                                    | 155 |
| <b>Figure 7. 2:</b> 2D structures of the designed hydroxybenzoic acid amide derivative compounds .....                                                                                                                                                                    | 155 |
|                                                                                                                                                                                                                                                                           |     |
| <b>Figure 8. 1:</b> Effect of amide compounds (KAM1 to KAM 12) on HEK 293 cells (a) % cell viability vs. concentration curve and (b) cell cytotoxicity at half-maximum (CC <sub>50</sub> ) of amide compounds against HEK-293 cells. ....                                 | 195 |
| <b>Figure 8. 2:</b> Effect of amide compounds (KAM1 to KAM 12) on Mia-PaCa- 2 cells (a) % inhibition vs. concentration curve and (b) inhibition concentration at half maximum (IC <sub>50</sub> ) of amide compounds against MiaPaCa-2 cells. ....                        | 197 |
| <b>Figure 8. 3:</b> Effect of ester compounds (C1 to C15) on HEK-293 cells (a) % cell viability vs. concentration curve and (b) CC <sub>50</sub> bar graph of ester compounds against HEK-293 cells. ....                                                                 | 200 |
| <b>Figure 8. 4:</b> Effect of ester compounds (C1 to C15) on Mia-PaCa-2 cells (a) % inhibition vs. concentration curve and (b) IC <sub>50</sub> bar graph of ester compounds against MiaPaCa-2 cells. ..                                                                  | 202 |
| <b>Figure 8. 5:</b> Graphs representing (a) % cell viability vs. concentration curve and (b) IC <sub>50</sub> bar graph of blank and loaded nanoparticles against HEK 293 cells. ....                                                                                     | 205 |

**Figure 8. 6:** The graphs representing (a) % inhibition vs. concentration curve and (b) the IC<sub>50</sub> bar graph of blank and loaded nanoparticles against MiaPaCa-2 cells. ....206

**Figure 9. 1:** Summarized flowchart representation of the study .....219

## List of Tables

---

|                                                                                                                                                                                         |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Table 2. 1:</b> Types of chemotherapy used in the treatment of cancer and their descriptions <sup>21</sup> .8                                                                        | 8   |
| <b>Table 2. 2:</b> Antimetabolite targets and their commonly used inhibitors <sup>21</sup> .9                                                                                           | 9   |
| <b>Table 2. 3:</b> Different classification of alkylating agents and their commonly used drugs <sup>33</sup> ..10                                                                       | 10  |
| <b>Table 2. 4:</b> Subgroups of plant alkaloids and the commonly known and used alkaloids .....10                                                                                       | 10  |
| <b>Table 2. 5:</b> Different types of biotherapy inhibitors and their mechanism of action .....11                                                                                       | 11  |
| <b>Table 2. 6:</b> Hormone therapy target inhibitors and their functions <sup>55</sup> .....14                                                                                          | 14  |
|                                                                                                                                                                                         |     |
| <b>Table 4. 1:</b> Toxicity and bioavailability properties of the designed HBA amide derivatives.60                                                                                     | 60  |
| <b>Table 4. 2:</b> Toxicity and bioavailability properties of the designed HBA ester derivatives ...62                                                                                  | 62  |
| <b>Table 4. 3:</b> SwissADME predicted physicochemical properties of the designed amide compounds (Lipinski's RO5).....63                                                               | 63  |
| <b>Table 4. 4:</b> Swiss predicted physicochemical properties of the designed ester compounds (Lipinski's RO5) .....64                                                                  | 64  |
| <b>Table 4. 5:</b> Docking results for the designed compounds against DNA-topoisomerase I and ABCG2 proteins.....66                                                                     | 66  |
| <b>Table 4. 6:</b> Selected lead compounds protein-ligand interaction H-bond residues.....67                                                                                            | 67  |
| <b>Table 4. 7:</b> Frontier molecular orbitals results of the selected amide compounds (KAM 1, KAM 2, KAM 3, KAM 4, KAM 6, and KAM 11).....70                                           | 70  |
| <b>Table 4. 8:</b> Frontier molecular orbitals results of the selected ester compounds (C4, C8, C9, C12, and C13).....70                                                                | 70  |
|                                                                                                                                                                                         |     |
| <b>Table 5. 1:</b> Characteristic peaks of ester compounds ( <b>C1-C15</b> ) .....96                                                                                                    | 96  |
| <b>Table 5. 2:</b> Summary of the characteristic peaks of amide compounds (KAM 1-KAM 12)..98                                                                                            | 98  |
|                                                                                                                                                                                         |     |
| <b>Table 6. 1:</b> Dynamic light scattering (DLS) physicochemical characteristic of blank (50:50 molar ratio) and loaded nanoparticles.....147                                          | 147 |
|                                                                                                                                                                                         |     |
| <b>Table 7. 1:</b> Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentrations (MBC) of Amide derivatives against gram-positive and gram-negative bacteria.....157  | 157 |
| <b>Table 7. 2:</b> Summary of MIC/MBC results of Amide derivatives against Gram-negative and Gram-positive bacteria.....159                                                             | 159 |
| <b>Table 7. 3:</b> Minimum inhibitory concentrations (MIC) and Minimum fungicidal concentrations (MFC) of Amide derivatives against Candida species. ....161                            | 161 |
| <b>Table 7. 4:</b> Summary of MIC/MFC results of Amide derivatives .....163                                                                                                             | 163 |
| <b>Table 7. 5:</b> Minimum inhibitory concentrations (MIC) and Minimum Bactericidal concentrations (MBC) of Ester derivatives against Gram-negative and Gram-positive bacteria .....165 | 165 |
| <b>Table 7. 6:</b> Summary of MIC/MBC results of esters derivatives.....167                                                                                                             | 167 |
| <b>Table 7. 7:</b> Minimum inhibitory concentrations and Minimum fungicidal concentrations of Ester derivatives .....170                                                                | 170 |

|                                                                                                                                                             |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Table 7. 8:</b> Summary of MIC/MFC results of ester derivatives.....                                                                                     | 172 |
| <b>Table 7. 9:</b> Minimum inhibitory concentrations and Minimum bactericidal concentrations of nanoparticles loaded with Amide and Ester derivatives. .... | 175 |
| <b>Table 7. 10:</b> Summary of MIC/MBC results of nanoparticles loaded with Amide and Ester derivatives .....                                               | 176 |
| <b>Table 7. 11:</b> Minimum inhibitory concentrations and Minimum fungicidal concentrations of nanoparticles loaded with Amide and Ester derivatives. ....  | 178 |
| <b>Table 7. 12:</b> Summary of MIC/MFC results of nanoparticles loaded with Amide and Ester derivatives .....                                               | 179 |
| <b>Table 7. 13:</b> Minimum inhibitory concentrations and Minimum bactericidal concentrations of combination of Amide and Ester derivatives .....           | 181 |
| <b>Table 7. 14:</b> Minimum inhibitory concentrations and Minimum fungicidal concentrations of combination of Amide and Ester derivatives .....             | 183 |
|                                                                                                                                                             |     |
| <b>Table 8. 1:</b> Summary of the cytotoxicity, anticancer, and selectivity index (SI) of the amide compounds .....                                         | 198 |
| <b>Table 8. 2:</b> Summary of the cytotoxicity, anticancer, and selectivity index (SI) of the ester compounds .....                                         | 203 |
| <b>Table 8. 3:</b> Summary of the cytotoxicity, anticancer, and selectivity index (SI) of the loaded and blank nanoparticles.....                           | 207 |

## List of Abbreviations

---

|                     |                                                   |
|---------------------|---------------------------------------------------|
| °C                  | degrees Celsius                                   |
| <sup>13</sup> C NMR | carbon-13 nuclear magnetic resonance spectroscopy |
| <sup>1</sup> H NMR  | proton nuclear magnetic resonance spectroscopy    |
| br                  | broad                                             |
| COSY                | homonuclear correlation spectroscopy              |
| d                   | doublet                                           |
| dd                  | doublet of doublets                               |
| ddd                 | doublet of doublet of doublets                    |
| dddd                | doublet of doublet of doublet of doublets         |
| DMF                 | dimethylformamide                                 |
| DMSO                | dimethyl sulfoxide                                |
| DMSO-d <sub>6</sub> | deuterated dimethyl sulfoxide                     |
| EtOAc               | ethyl acetate                                     |
| EtOH                | ethanol                                           |
| FID                 | free induction decay                              |
| FTIR                | Fourier transform infrared                        |
| h                   | hour                                              |
| HMBC                | heteronuclear multiple bond correlation           |
| HRMS                | high resolution mass spectrometry                 |
| COSY                | Correlation Spectroscopy                          |
| Hz                  | hertz                                             |
| IR                  | infrared                                          |
| μM                  | micromolar                                        |
| m                   | multiplet                                         |
| MBC                 | minimum bactericidal concentration                |
| MeOH                | methanol                                          |
| MHA                 | Mueller-Hinton agar                               |
| MHz                 | megahertz                                         |
| min                 | minutes                                           |
| mL                  | millilitre                                        |

|                  |                                              |
|------------------|----------------------------------------------|
| mmol             | millimole                                    |
| mp               | melting point                                |
| PNPs             | Polymer nanoparticles                        |
| DCM              | Dichloromethane                              |
| NMP              | <i>N</i> -methylpyrrolidine                  |
| MIC              | Minimum inhibitory concentration             |
| CC <sub>50</sub> | Cytotoxic concentration at half-maximum      |
| IC <sub>50</sub> | Inhibition concentration at half-maximum     |
| RT               | RT room temperature                          |
| s                | singlet                                      |
| t                | triplet                                      |
| td               | triplet of doublets                          |
| THF              | tetrahydrofuran                              |
| TLC              | thin layer chromatography                    |
| TMS              | tetramethylsilane                            |
| TMSCl            | trimethylsilyl chloride                      |
| DNA TOP1         | Deoxyribonucleic acid topoisomerase I enzyme |
| ABCG2            | ATP-Binding Cassette G-2                     |
| DLS              | Dynamic light scattering                     |

## Chapter 1: Introduction

---

### 1. Background and introduction

The healthcare landscape is currently facing a dual challenge: the rapid spread of infectious diseases and the rising incidence of chronic diseases <sup>1</sup>. Infectious diseases once thought to be largely controllable, have re-emerged as a significant threat to human health. This resurgence is partly due to the increasing resistance to existing antimicrobial drugs, which has rendered many treatments ineffective and allowed infections to spread more rapidly and widely than before. The World Health Organization (WHO) has highlighted antimicrobial resistance as one of the top ten global public health threats, emphasizing the urgent need for new and effective treatments.

Simultaneously, chronic diseases are on the rise, contributing significantly to the global disease burden. Conditions such as diabetes, heart disease, and cancer are increasing in prevalence, driven by a combination of aging populations, lifestyle factors, and improved diagnostic techniques <sup>2</sup>. Cancer, in particular, remains one of the most deadly diseases worldwide, second only to cardiovascular disease<sup>3</sup>. The incidence and diagnosis rates of cancer are rising worldwide, which is putting tremendous strain on healthcare systems and highlighting the urgent need for innovative treatment strategies.

The convergence of these two trends; rising antimicrobial resistance and the growing burden of chronic disease highlights an urgent need to develop new drugs. Innovative treatments are essential to address the complexities of modern healthcare challenges, improve patient outcomes, and reduce the overall impact of these diseases on society.

There is an evident need for new drugs to treat chronic and infectious diseases. This rapid spread of infectious and chronic diseases has emerged as one of the greatest potential threats to human health, partly due to the aforementioned development of resistance to available antimicrobial and anticancer drugs <sup>4</sup>. The local prevalence is described in detail in the section below.

#### 1.1 Cancer prevalence

Cancer is one of the most prevalent diseases and it has been a global public health concern for centuries. According to the Cancer Association of South Africa (CANSA), cancer is a leading



cause of death in both developed and developing countries, including South Africa. In South Africa cancer has killed over 56 802 people with the new cases estimated to be 108 162 and the five-year number of prevalent cases being 262 455 (WHO, 2020). The below figure shows the most prevalent types of cancer in South Africa and the number of new cases for 2020.

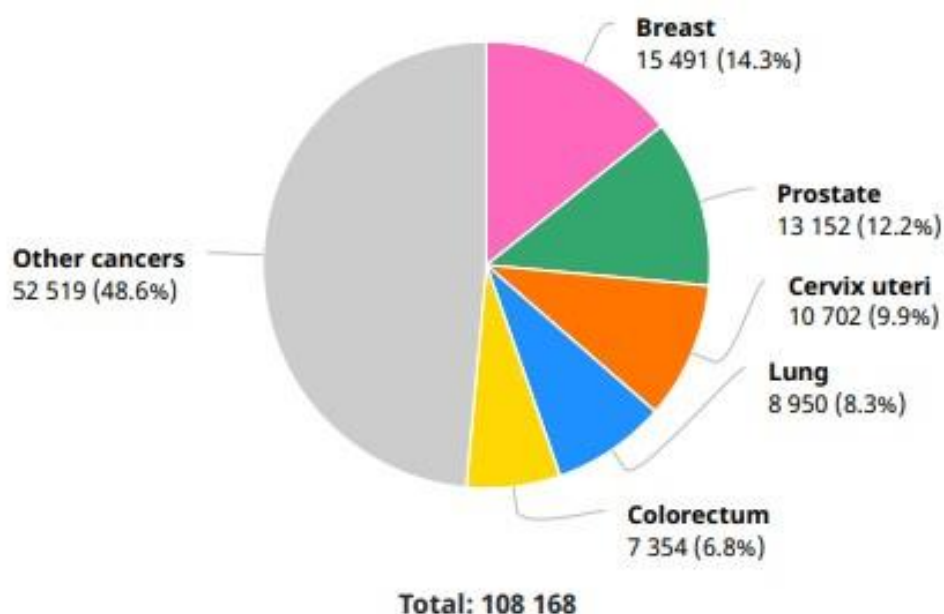


**Figure 1.1:** Number of new cases in 2020, both sexes and all ages (WHO, 2020)

## 1.2 Cancer-targeted therapy

The above figure (Figure 1.1) highlights the reason why most of the academic and pharmaceutical research efforts have been devoted to designing new anticancer compounds to combat the spread of cancer. Chemical design has become increasingly sophisticated over the years as a result of resistance to current cancer treatments and a lack of selectivity of the available drugs to tumor cells at the target site <sup>5,6</sup>. A possible solution to the aforementioned problem would be cancer-targeted therapy which selectively targets the cancer cells and reduces adverse effects experienced in the current treatment modalities. Cancer-targeted therapies are intended to increase efficacy and selectivity by interfering with specific molecular targets and in the process inhibiting the growth, progression, and invasion of cancer <sup>7</sup>. There are several targets or signaling pathways for cancer, wherein the targets have complex mechanisms of action. Hence, in this study, we focus on one target, the DNA topoisomerase I enzyme as our anticancer and antimicrobial target, which follows a less complicated mechanism of action.

### 1.3 DNA topoisomerase I enzyme

DNA topoisomerases are enzymes that unlink DNA and play an important part in a variety of biological processes involving DNA <sup>8-12</sup>. Targeting these enzymes is crucial in halting rapid cell proliferation where this process ends with the demise of the cancer cell via apoptotic cell death <sup>13</sup>. There are several inhibitors of this enzyme, further consideration of these inhibitors will be afforded in the literature review section to follow.

DNA topoisomerase inhibitors, while effective, have limitations such as drug resistance. Therefore, it is crucial to develop new compounds that can overcome the resistance faced by current inhibitors. In this study, we designed amide and ester derivatives of hydroxybenzoic acid, and the derivate compounds were then loaded onto pH-responsive polymeric nanomaterial as a drug delivery vehicle to deliver the derivate to the target site *in vitro* and to enhance the potency and selectivity of the designed compounds. The *in-silico* drug design and modelling were used to determine the potential activity of these compounds before undertaking the synthesis of compounds and subsequent *in vitro* studies.

### 1.4 Problem statement

The majority of chemotherapy drugs now in use are hydrophobic, which affects their bioavailability <sup>14</sup>. Nanomaterials have been used as drug carriers to improve their bioavailability and to reduce the drug's systemic toxicity but with minimal improvement. Hence, several attempts (on the synthesis of new drugs and corresponding nano-formulations) have been made over the years to overcome the challenges of available chemotherapeutic drugs <sup>15,16</sup>.

Hydroxybenzoic acid compounds were selected as precursors for the synthesis of bioactive compounds. The selection of these precursors was based on the hydrophilic nature and previously reported antimicrobial and anticancer activity amongst other applications. There is a need for a chemotherapeutic agent with dual anticancer and antimicrobial activity to avoid co-administration of multiple drugs for treating patients with cancer <sup>17</sup>. However, finding compounds that possess these abilities is very difficult and very costly due to multiple assays that would need to be performed to get lead compounds or candidate compounds. These circumstances make the computational approach very useful in designing lead compounds. Hence, in this study we implemented some computational methods to determine compounds that could show small levels of bioactivity in both anticancer and antimicrobial applications.

Moreover, the issues related to drug delivery may be addressed by the development of better drug delivery systems such as nanocarriers. The microenvironment of diseased areas necessitates the controlled release of drugs, drug stability, and site-specific delivery<sup>18,19</sup>. These nanocarriers must be biocompatible, biodegradable, and water-soluble to be effective in all aspects. Additionally, for the drug to be delivered to the tumor cells, it must be small enough to pass through the reticuloendothelial system (RES) barriers<sup>20,21</sup>. However, in most cases, nanoparticles have been widely associated with toxicity issues as a major disadvantage for their application. This is why in this work, derivatives of hydroxybenzoic acid were prepared and loaded onto polymeric nanomaterials that were designed to be biocompatible and biodegradable.

The polymer was selected and derivatized so that it has terminal groups that will enable the formed nanoparticles to respond to micro-environmental pH stimuli (as the endosomes and lysosomes have weak acid pH of 5.0-6.5) to assist in the controlled release of the drug (see Fig. 2.15 for illustration). In this study, N-isopropyl acrylamide was selected and derivatized with trans-cinnamic acid to introduce the ionizable pendant group to enable pH responsiveness.

### **1.5 Justification**

The prevalence of cancer and microbial infections in immunocompromised patients necessitates the development of more effective therapeutic agents. Existing treatment modalities for cancer often suffer from significant limitations such as toxicity and resistance, highlighting the urgent need for new chemotherapeutic agents. Hydroxybenzoic acid derivatives have shown promise due to their anticancer and antimicrobial properties. This research is justified by the potential to improve treatment outcomes through the synthesis of novel derivatives and their delivery via polymeric nanoparticles, which can enhance bioavailability and target-specific action.

### **1.6 Novelty of the study**

The research presented in this thesis focuses on the design, synthesis, and characterization of novel hydroxybenzoic acid amide and ester derivatives, and their incorporation into polymeric nanoparticles. The innovative aspect lies in the dual investigation of these compounds' antimicrobial and anticancer activities. This approach leverages the potential of hydroxybenzoic acid derivatives, previously known for their biological activities, and aims to enhance their efficacy and delivery through nanotechnology.

## **1.7 Hypothesis**

The central hypothesis of this study is that novel hydroxybenzoic acid amide and ester derivatives when synthesized and characterized, will exhibit significant antimicrobial and anticancer activities. Furthermore, loading these derivatives onto polymeric nanoparticles will enhance their biological efficacy due to improved delivery and reduced toxicity.

## Chapter 2: Literature Review

---

### 2.1 Cancer

Cancer is a term used to describe a collection of diseases characterized by abnormal cell proliferation with the ability to infiltrate or spread to other sections of the body <sup>22</sup>. Cancer occurs when the body's normal control mechanism stops working. When this happens the old cells do not die but instead grow out of control, forming new abnormal cells <sup>23</sup>. Then these extra cells may combine and aggregate together to form a mass of tissue known as a tumor <sup>23</sup>. However, not all cancers form tumors, others like leukemia grows in the bloodstream or bone marrow. Independence from growth signals, responsiveness to signals that stop cell division, uncontrolled replication, apoptosis evasion, sustained angiogenesis, and finally the ability to penetrate other tissues, known as metastasis, are all hallmarks of cancer <sup>22</sup>. In every country, cancer is a primary cause of death and a significant impediment to extending life expectancy <sup>24,25</sup>. Therefore, the global impact of this disease is of importance.

#### 2.1.1 Global prevalence of cancer

According to data from the World Health Organization (WHO) in 2019, cancer was reported as the primary or secondary reason for mortality in individuals under 70 years old in 112 out of 183 nations, while it ranks third or fourth in an additional 23 countries (Fig. 2). It was projected that there will be 609,820 cancer deaths and 1,958,310 new cancer cases in the United States in 2023 <sup>26</sup>. Except for the urinary bladder, the specified cancer estimates exclude basal cell and squamous cell skin cancers, which are not required to be reported to cancer registries, as well as *in situ* carcinomas (non-invasive cancer) <sup>27</sup>.

Overall, the global burden of cancer incidence and death is quickly increasing, reflecting both population aging and growth, as well as changes in the prevalence and distribution of the key cancer risk factors, many of which are linked to socioeconomic development <sup>25</sup>.

Health developments and the increasing burden of cancer have fuelled research in the oncology field to find new interventions to help unburden the global society in the fight against cancer. Treatment modalities for cancer have given patients a fighting chance because the right choice on treatment modalities can help patients live longer and increase their cancer survival chances.

There are varied cancer therapy modalities and the next section affords a summary of the available treatment modalities for cancer.

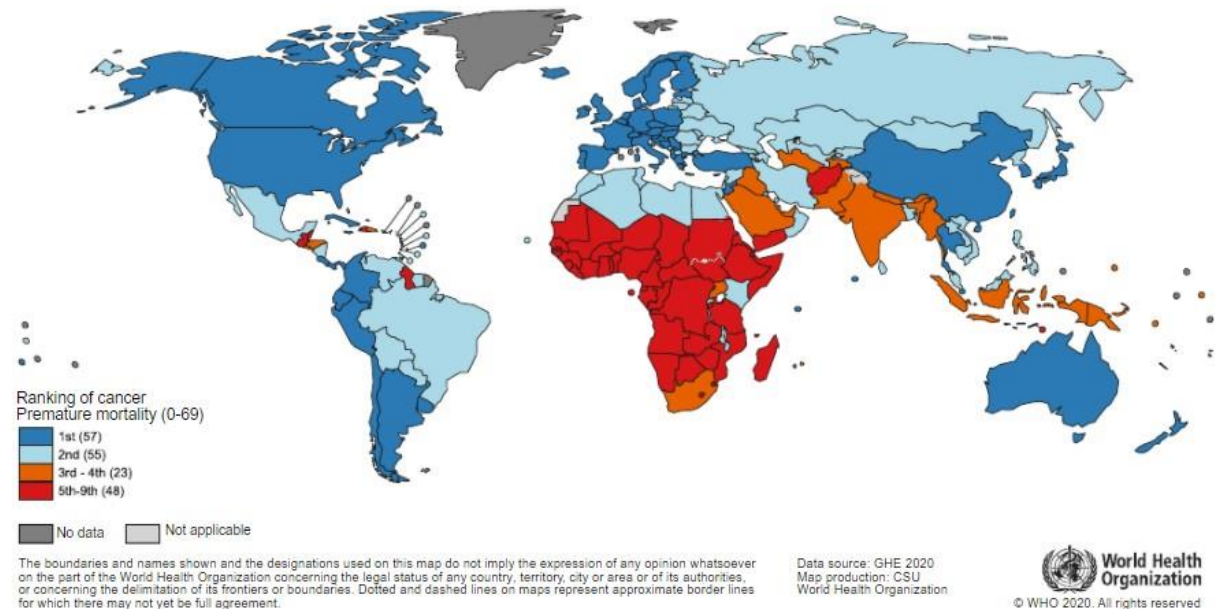


**Figure 2. 1:** The legend includes the national ranking of cancer as a cause of death among individuals under the age of 70 in 2019. It also provides the number of countries represented in each ranking group. Adopted from Sung et al.<sup>25</sup>

## 2.1.2 Treatment modalities of cancer

The goal of remarkable research has been to uncover new methods of high-quality cancer treatment approaches since the diagnosis of the disease<sup>28</sup>. Currently, cancer is the focus of about 60% of all ongoing medical quality therapy trials worldwide<sup>29</sup>. Treatment options and progress are determined by the type of cancer, its location, and its stage of progression<sup>28</sup>. Traditional and frequently utilized treatments include surgery, radiation-based surgical knives, chemotherapy, and radiotherapy<sup>28</sup>. Hormone-based therapy, anti-angiogenic modalities, stem cell therapies, immunotherapy, and dendritic cell-based immunotherapy are some of the recently used novel methods<sup>28,29</sup>. Different classifications are used to differentiate treatment modalities of cancer; surgery and radiation therapy are referred to as “local treatments” because of their treatment nature of treating a specific tumor or area of the body. While other treatment modalities such as chemotherapy, immunotherapy, or targeted therapy are referred to as “systemic treatment” since they have the potential to affect the entire body. The below sections will shed light on individual treatments.

### 2.1.2.1 Chemotherapy

When referring to treatments or drugs that cure cancer, the term "chemotherapy" ("chemo") is frequently used <sup>30</sup>. Drugs that are cytotoxic, or capable of killing tumor cells, are used in traditional or standard chemotherapy <sup>31</sup>. There are several ways that these drugs are used in cytotoxic chemotherapy and they are described in Table 2.1 below.

**Table 2. 1:** Types of chemotherapy used in the treatment of cancer and their descriptions <sup>32</sup>.

| Type of chemotherapy | Description                                                                                                                          |
|----------------------|--------------------------------------------------------------------------------------------------------------------------------------|
| Adjuvant therapy     | Is used to treat disease in conjunction with other treatments such as surgery or radiotherapy to lower the risk of cancer recurrence |
| Neo-adjuvant therapy | Is used to reduce tumors before they are surgically removed                                                                          |
| Curative therapy     | Is used as a cure for cancer                                                                                                         |
| Concurrent therapy   | Is used in conjunction with other treatments, such as radiotherapy, to increase the cancer cells sensitivity to radiation            |
| Palliative therapy   | While a complete cure may not be achievable, chemotherapy can be employed to reduce tumor growth and alleviate associated symptoms   |

The body's normal biological activity refreshes cells by eliminating excess or damaged cells and triggering the production of new cells <sup>31</sup>. Tumor cells, on the other hand, have a greater potential to divide and they have a characteristic of immortality since they are not subject to apoptosis <sup>33</sup>. Furthermore, cancer cells have damaged DNA, so they keep multiplying even when normal cells would cease <sup>34</sup>. The stages a cell goes through when it multiplies are described by the cell cycle <sup>33</sup>. Chemotherapy medications that are cytotoxic attack cells at different stages of the cell cycle <sup>35</sup>. Since they are not targeted therapies; they indiscriminately kill all rapidly dividing cells <sup>36</sup>. This then suggests that a substantial percentage of malignant cells are affected, but healthy cells are also affected. The chemotherapy's inability to target cancer cells only results in severe side effects depending on dosage, and these side effects include hair loss, nausea, fatigue, vomiting, etc. Patients become immunocompromised as a

result of aggressive chemotherapy treatment, which can lead to complicated infections and, ultimately, death <sup>37</sup>.

Cytotoxic drugs are classified based on their effect on cells as well as their chemical group <sup>38</sup>. When drugs are categorized based on their cell cycle activity, they are divided into two categories: cell cycle-specific and cell cycle non-specific <sup>39</sup>.

Drugs are classified chemically into the below groups based on their mechanism of action:

### **Antimetabolites**

Antimetabolites are drugs that are analogs for DNA and RNA units, and so they inhibit DNA and RNA growth when they are incorporated <sup>40</sup>. These drugs target the S phase of the cell and are used to treat leukemia, ovarian, breast, and intestinal cancers, among other cancers <sup>40</sup>. Antimetabolites are categorized based on the substances they interact with as shown in Table 2.2.

**Table 2. 2:** Antimetabolite targets and their commonly used inhibitors<sup>32</sup>.

| <b>Antimetabolite targets</b>  |  | <b>Commonly used drugs</b>                                            |
|--------------------------------|--|-----------------------------------------------------------------------|
| Folic acid antagonist          |  | Methotrexate                                                          |
| Pyrimidine antagonist          |  | 5-Fluorouracil, Foxuridine, Cytarabine, Capecitabine, and Gemcitabine |
| Purine antagonist              |  | 6-Mercaptopurine and 6-Thioguanine                                    |
| Adenosine deaminase inhibitors |  | Cladribine, Fludarabine, Nelarabine, and Pentostatin                  |

### **Alkylating agent**

Alkylating drugs cause direct DNA damage to cancer cells, stopping them from dividing and they are effective at all phases of the cell cycle <sup>41</sup>. Alkylating drugs are used to treat a variety of malignancies, including lymphoma, leukemia, multiple myeloma, Hodgkin's disease, and sarcomas <sup>41</sup>.

The alkylating drugs though, have the potential to cause damage to the bone marrow since they damage DNA <sup>42</sup>. Depending on the dosages used, long-term damage can lead to acute leukemia,



however, this is uncommon <sup>43</sup>. After 5 to 10 years of treatment with alkylating drugs, leukemia can arise.

The following Table 2.3 depicts drugs that are in the family of alkylating drugs.

**Table 2. 3:** Different classification of alkylating agents and their commonly used drugs <sup>44</sup>.

| Types of alkylating agents | Commonly used drugs                                                        |
|----------------------------|----------------------------------------------------------------------------|
| Mustard gas derivatives    | Mechlorethamine, Cyclophosphamide, Chlorambucil, Melphalan, and Ifosfamide |
| Ethylenimines              | Thiotepa and Hexamethylmelamine.                                           |
| Alkylsulfonates            | Busulfan                                                                   |
| Hydrazines and Triazines   | Altretamine, Procarbazine, Dacarbazine and Temozolomide                    |
| Nitrosoureas               | Carmustine, Lomustine, and Streptozocin                                    |
| Metal salts                | Carboplatin, Cisplatin, and Oxaliplatin                                    |

### Plant Alkaloids

Plant alkaloids are chemotherapeutic agents generated from particular plant species <sup>45</sup>. The periwinkle plant produces vinca alkaloids (*catharanthus rosea*) <sup>46</sup>. During metaphase, plant alkaloids attach to microtubule proteins, inducing mitotic arrest <sup>46</sup>. The cell dies because it is unable to divide. This group is mostly focused on the mitosis phase (M phase) of the cell cycle <sup>46</sup>. There are three subgroups in this group:

**Table 2. 4:** Subgroups of plant alkaloids and the commonly known and used alkaloids.

| Types of plant alkaloids | Commonly used alkaloids     |
|--------------------------|-----------------------------|
| Vinca alkaloids          | Vincristine and vinblastine |
| Epipodophyllotoxins      | Etoposide and teniposide    |
| Taxanes                  | Paclitaxel and docetaxel    |

## Topoisomerase Inhibitors

Topoisomerase inhibitors stop the enzymes (topoisomerases) from breaking and reconnecting DNA strands, which are required for cell division and growth <sup>47,48</sup>. The DNA topoisomerase enzymes work by cutting either one or two of the DNA strand(s), thereby relieving tension for the unwinding of the DNA <sup>49</sup>. Examples of compounds used as inhibitors of the topoisomerase I enzyme include, camptothecin, irinotecan and topotecan. In this study, we designed derivate compounds that could be used as possible inhibitors of the topoisomerase I enzyme and they were screened via *in-silico* studies.

### 2.1.2.2 Biological therapy (also known as immunotherapy)

The use of biological materials or biological response modifiers (BRMs) for therapeutic purposes is known as biotherapy. Biotherapies are a relatively new invention in the field of cancer, and they are increasingly being employed to treat childhood and adolescent cancers <sup>50</sup>. The BRMs differ from chemotherapy drugs in that they target specific cells rather than eliminating all rapidly dividing cells <sup>51</sup>. Like any other therapy, biotherapy also has major side effects such as fever, chills, nausea, appetite loss, and other flu-like symptoms that biological response modifiers can elicit <sup>52</sup>. Rashes or oedema at the injection site are possible side effects. Blood pressure (BP) may be altered as well, with most cases resulting in a decrease in BP. Another common side effect of biological response modifiers is fatigue. Bone pain and fatigue are other possible side effects of colony-stimulating factors.

There are several types of biotherapies and they are described in Table 2.5.

**Table 2. 5:** Different types of biotherapy inhibitors and their mechanism of action.

| Types of Biotherapy Inhibitors | Mechanism of action                                                                                              |
|--------------------------------|------------------------------------------------------------------------------------------------------------------|
| Angiogenesis inhibitors        | Stop the growth of blood vessels, preventing the tumor from getting the oxygen and nutrients it needs to survive |
| Cancer growth inhibitors       | Hinder cancer cells ability to grow and divide by interfering with their chemical signals                        |
| Cancer vaccines                | Boost the immune system to recognize cancer cells as aberrant and eliminate them                                 |

|                            |                                                                                                                                                                                 |
|----------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Gene therapy               | Is a relatively new concept that aims to insert genes into cancer cells to make them more susceptible to treatment                                                              |
| Interleukin and interferon | These are drugs that are given to help the body's immune system fight cancer. They encourage the immune system to kill cancer cells                                             |
| Monoclonal antibodies      | Work by recognizing and locking onto the protein on the surface of the cancer cell. The body's immune system responds by attacking the cancer cell, leading it to self-destruct |

However, with all these promising biotherapies, Immunotherapy has had mixed outcomes, with efficacy varying significantly between patients and cancer types.

### 2.1.2.3 Radiation therapy

Radiation therapy (also known as radiotherapy) is a type of cancer treatment in which high doses of radiation are used to kill cancer cells and shrink tumors <sup>53</sup>. Radiation therapy kills or slows cancer cells development by destroying their DNA at high dosages <sup>54</sup>. The major target of radiotherapy is DNA, which is damaged both directly and indirectly as a result of the interaction of radiation with water within the cell, which produces toxic free radicals <sup>54</sup>. Single-strand and double-strand breaks occur in DNA, and if they are not repaired, reproductive cells die when they try to proliferate <sup>55</sup>. In radiotherapy the radiation is transmitted in a beam to the area of the body that has to be treated. The radiations are characterized into two types:

- **Particle radiation:** comprises of low energy and has a penetrating limit of a few millimetres <sup>56</sup>. Depending on their energy and beam modulation, electrons, protons, and neutrons can penetrate to a depth of several centimetres <sup>56</sup>. Electrons are most typically utilized to treat superficial regions of the skin that are only a few centimetres deep. The examples of particle radiation are alpha and beta particles.
- **Ionizing electromagnetic radiation:** Examples are X-rays, also including gamma rays, which have a much higher energy level and can penetrate deeply into the skin or body <sup>57</sup>.

X-rays are currently the most widely used method of radiation. These X-rays are generated in a linear accelerator by bombarding tungsten metal targets with a stream of electrons, thereby

releasing energy <sup>58</sup>. Due to this high energy, radio-sensitivity occurs in the cells. Although malignant cells are the target of radiotherapy, healthy rapidly dividing cells in normal tissue (e.g., skin, gastrointestinal and urinary tract epithelium, gonads, and bone marrow) are equally vulnerable to radiation damage, resulting in radiotherapy side effects <sup>59</sup>.

#### **2.1.2.4 Surgery**

Surgery may be required for childhood and adolescent tumours, depending on the diagnosis. Moreover, surgery can serve as the initial step in treatment, which may involve conducting a biopsy. Alternatively, surgery could be performed following preliminary chemotherapy, aimed at reducing the size of the tumor <sup>60</sup>. For some types of tumors, surgery will be the only option. Purpose of surgery is to completely remove the tumor, ideally with disease-free margins <sup>60</sup>. Pathologists are the ones that confirm diagnosis of the malignancy via biopsy analysis. Assessing the proportion of necrosis (dead cells) is possible when chemotherapy is administered before surgery. Clinicians can use this to determine the appropriate treatment approach. The primary limitation of using surgery as the sole treatment modality is twofold. Firstly, it involves an invasive approach, which can carry inherent risks and potential complications for the patient. Additionally, the effectiveness of surgery relies heavily on the ability to accurately locate and access the tumor. However, in certain cases, particularly when malignancies have become invasive or have metastasized to other organs within the body, identifying and targeting the tumor site may prove challenging or even impossible, thus limiting the efficacy of surgical intervention. <sup>61</sup>.

#### **2.1.2.5 Hormone therapy**

Hormones are proteins or substances produced by the body that aid in the regulation of various cell types <sup>62</sup>. Some regions of the body, rely on sex hormones like estrogen, testosterone, and progesterone to function effectively <sup>63</sup>. Thyroid hormones, cortisol, adrenaline, and insulin are among the hormones found in human systems. Different organs or glands produce different types of hormones. The use of hormone therapy to treat cancer is derived from the fact that some tumor cells have receptors for specific hormones required for cell proliferation <sup>64</sup>. As a result, treatments that block or alter hormones can sometimes slow or stop the progression of these cancers <sup>64</sup>. Hormone treatment is most commonly used to treat breast and prostate cancers, since they rely on sex hormones to grow <sup>65</sup>. Hormone therapies are classified according to their function and/or the hormone that is affected as depicted in Table 2.6 below.

**Table 2. 6:** Hormone therapy target inhibitors and their functions <sup>66</sup>.

| Hormone therapies                                     | Function                                                                                                                                                                                                                                                                                                                                        |
|-------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Adrenal Steroid inhibitors                            | This type of hormone therapy interferes with the adrenal glands hormone production. Corticosteroids are hormones produced by the outer section of the adrenal gland (the adrenal cortex). Blocking the adrenal gland and corticosteroid hormones interferes with the activation of cancer growth in tumors that are impacted by these hormones. |
| Androgens inhibitors                                  | Antiandrogens are substances that prevent testosterone's effects. The male hormone testosterone is required for the growth of prostate cancer. It is possible to slow or shrink the cancer if the level of testosterone in the body is reduced.                                                                                                 |
| Antiestrogens                                         | Antiestrogens bind to the oestrogen receptor on cancer cells, preventing oestrogen from entering the cell. This disrupts cell development and ultimately results in cell death.                                                                                                                                                                 |
| Aromatase inhibitors                                  | Suppress the enzyme aromatase, which converts androgens (hormones produced by the adrenal glands) to oestrogen. Tumors that rely on oestrogen for growth will shrink in the absence of the oestrogen hormone.                                                                                                                                   |
| Luteinizing Hormone-Releasing Hormone (LHRH) Agonists | LHRH agonists act by instructing the pituitary gland in the brain to stop generating luteinizing hormone, which causes the testicles to release testosterone (in men) and the ovaries to release oestrogen (in women).                                                                                                                          |

On the downside hormone therapy affects men and women differently, therefore side effects vary from patient to patient. The possible side effects taken from previously treated patients

include; muscle strength and tone, sexual desire (lower testosterone levels or lower libido), production of bone marrow, prostate growth and function, changes in memory and concentration, and loss of body hair <sup>67</sup>.

#### **2.1.2.6 Target drug therapy**

Studies on molecular biology for site specific targets have drastically increased over the years because of the growing concern on the currently available and prescribed treatment modalities <sup>68</sup>. In the search for new treatment modalities and exploitation of tumor biology, target therapy which uses drugs or other substances to specifically attack certain type of cancer cells was established <sup>69</sup>. A targeted therapy can be used alone or in conjunction with other therapies like traditional or standard chemotherapy, surgery, or radiation therapy <sup>70</sup>. In targeted therapy, the drugs used are designed to specifically target only cancer cells without harming the normal cells, thereby reducing side effects experienced on other treatments <sup>70</sup>. Cancer cells have mutations in their genes that distinguish them from normal cells, hence it is easier to target them instead of normal cells <sup>41</sup>. The mentioned genes are instructions written in a cell's DNA that inform it what to do.

Unfortunately, even this “cream of the crop” approach has side effects and the severity depends on the drug used and what it targets, since there are several drugs that are used for targeted therapy <sup>32</sup>. Several side effects that have been previously shown in treated patients and this effects include skin problems, hand-foot syndrome, changes in hair growth, changes in and around the eye, changes in hair and skin colour, high blood pressure, bleeding or blood clotting problems, slow wound healing, autoimmune reactions, nausea and vomiting, increased risks of certain infections among others <sup>32</sup>.

#### **2.1.3 Shortfalls of the current treatment modalities**

Shortfalls of the different treatment modalities have been discussed previously (section 2.1.2). The section highlighted how the treatment modalities work and their side effects which patient are likely to encounter with the use of each treatment. However, cancer targeted therapy in conjunction with chemotherapy can be the hope going forward since targeted therapy is about targeting a specific cancer signaling pathway that is over or under expressed in the cancer cells.

#### **2.1.4 Cancer target therapy**

Molecular target therapy also referred to as “precision medicine” is a flexible approach allowing exploration of different chemotherapeutic drugs for different molecular targets <sup>71</sup>. The side effects of this approach can be minimized by choosing cancer targets that can be easily exploited as well as by the use of nanomedicine. Nanotechnology in drug delivery will be elaborated on further (refer to chapters 2 and 6), however, in this subsection, the focus is on the targeted cancer therapy and on possible cancer targets, ultimately zoning down to the cancer target for our study.

##### **2.1.4.1 Possible cancer targets**

Targeted therapy entails the development of pharmaceuticals that inhibit cancer cell proliferation, promote cell cycle regulation, or trigger apoptosis or autophagy, as well as the delivery of toxic substances directly to cancer cells to kill them <sup>72</sup>. Cancer has a “hallmark” of targets that can be exploited depending on the type of drug you want to design. The mechanism of action of some of the targets is very complex and requires knowledge in molecular biology to fully understand the concepts of the preferred target while some are easier to understand and explore with minimal knowledge in molecular biology. This study focused on the DNA topoisomerase I enzyme as a prime target for both anticancer and antibacterial activity.

##### **2.1.4.2 DNA topoisomerase enzymes**

Ever since 1971 when Jim Wang first discovered the DNA topoisomerase I (*in Escherichia Coli* on the omega ( $\omega$ ) protein) enzyme, molecular targets of both anticancer and antimicrobial drugs were revolutionized <sup>47,48,73</sup>. To perform the drug activity test, the supercoiled and relaxed DNA strands had to be separated. To accomplish this, the reaction products were run on a sucrose gradient, and the DNA contained in each fraction obtained from the centrifugation tubes was collected and analyzed <sup>73</sup>. Many topoisomerases have been identified and defined in all three domains of life (bacteria, eukaryotes, and archaea) over the last 40 years <sup>47</sup>.

DNA topoisomerases are a class of enzymes that regulate DNA topology <sup>47</sup>. These enzymes are involved in a variety of important biological activities in all cells such as DNA replication, transcription and recombination, or chromosome condensation <sup>74</sup>. These enzymes bind to the DNA phosphorus group covalently, splitting the DNA strand or strands, and then rejoining them again. There are two types of topoisomerases based on their mode of action: topoisomerases I (Top I) and topoisomerases II (Top II), which are further subdivided into five subfamilies (Type IA, IB, IC, IIA and IIB) <sup>74,75</sup>. The two main types of the topoisomerase

names (i.e. type I and II) are associated with the number of strand(s) they cut on the DNA helix. Type I cuts only one strand while type II cuts both strands of the DNA helix.

Topoisomerases are appealing targets for anticancer and antibacterial drugs due to their unique ability to maintain DNA topology <sup>76,77</sup>. Camptothecin and noncamptothecin anticancer drugs, in general, target topoisomerase I, whereas anthracyclines, etoposide, and mitoxantrone target eukaryotic topoisomerase II <sup>76,78</sup>. Furthermore, topoisomerase inhibitors have been demonstrated to be effective drug candidates in the treatment of malignancies such as sarcoma, lymphoma, leukemia, non-small cell lung, ovarian, and colorectal cancers that do not respond well to other drugs and treatments in clinical settings <sup>79-81</sup>.

#### **2.1.4.2.1 DNA topoisomerase I**

Topoisomerase I (TOP1) proteins were discovered in both eukaryotic and prokaryotic species <sup>82-85</sup>. Type I topoisomerases work by causing a temporary single-stranded break in the double helix, which is then followed by a single-stranded DNA passing event or regulated rotation of the broken strand and then reseals the broken strand <sup>47</sup>. All TOP1 enzymes employ the same chemistry to break the phosphodiester bond <sup>48</sup>. The enzyme's tyrosyl group attacks a phosphodiester bond on DNA, remaining covalently connected to one side of the break and releasing a free hydroxylated strand <sup>48</sup>. The hydroxyl end of the free strand attacks the phosphotyrosine bond, restoring a phosphodiester bond and releasing the enzyme for the next catalytic cycle <sup>48</sup>. As a result, these enzymes can resolve single-stranded hemicatenated recombination intermediates and relieve torsional stress (i.e., eliminate superhelical twists) in duplex DNA in the absence of the energy cofactor (meaning it does not require ATP to relax the supercoiled DNA) (see Fig. 2.2) <sup>80</sup>. The TOP1ccs (TOP1 cleavage complex step) is the most critical step and that is the most vulnerable step of topoisomerase I enzyme, since the inhibition of the normal TOP1 mechanism steps occur in that step preventing religation and causing cell death (see Fig. 2.2).



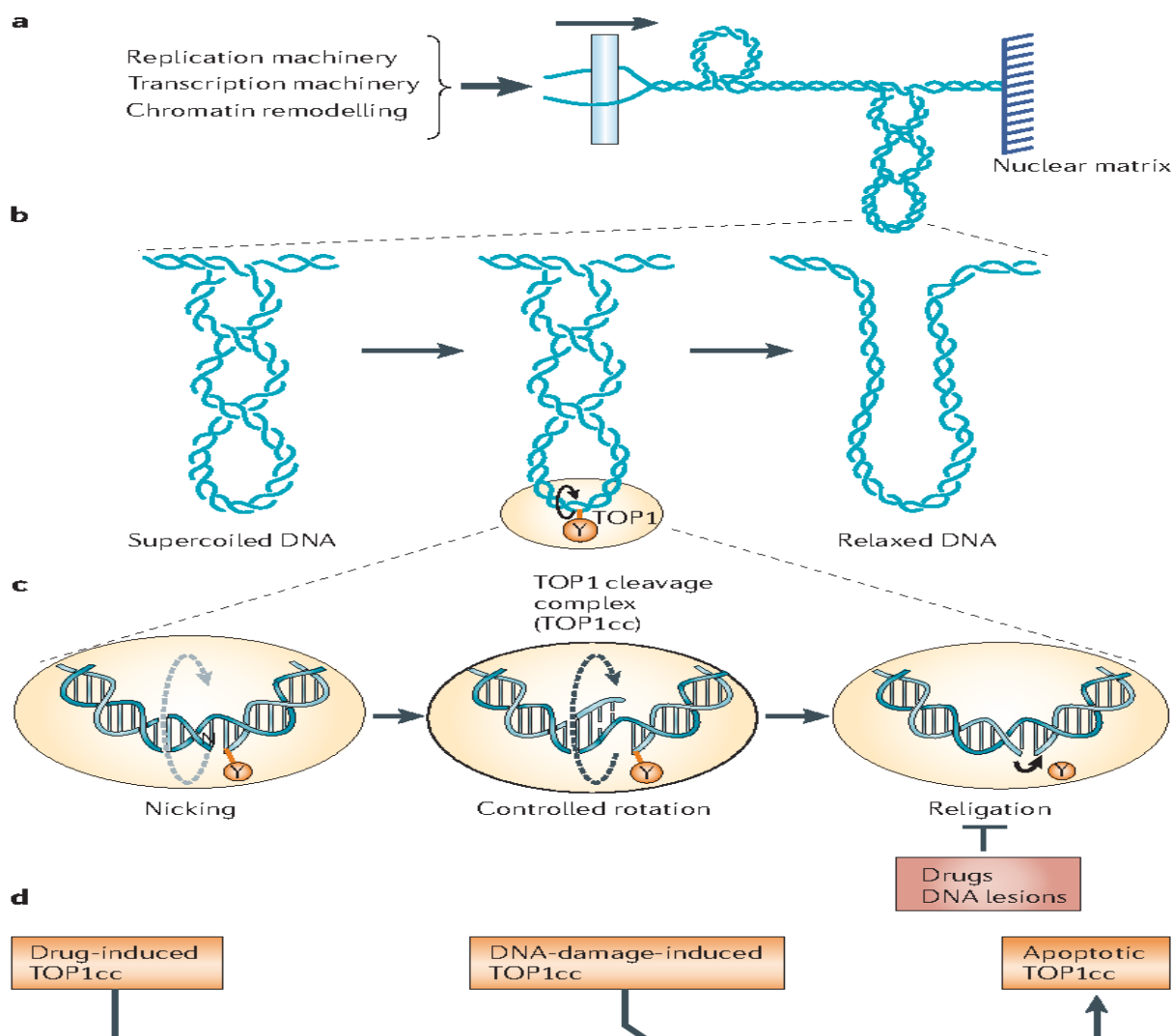


**Figure 2. 2:** Unwinding of the supercoiled DNA by TOP1-mediated DNA cleavage complex (adopted from Pommier <sup>86</sup>). (a) DNA supercoiling caused by DNA replication, transcription, and chromatin remodelling <sup>86</sup>. (b) Relaxation of the supercoiled DNA through an intermediate TOP1 cleavage complex step. (c) The intermediate TOP1-cleaved complex step were three processes takes place i.e. Nicking which is where the DNA single-strand breaks (nicks), controlled rotation when the TOP1 provides swivel points that enable the rotation of the intact DNA strand around the break and facilitate DNA relaxation, and the third step religation which is where the TOP1 realign and seals the broken strand. (d) By misaligning the ends of the broken DNA, drugs and DNA lesions prevent religation causing different cell death mechanisms.

#### 2.1.4.2.2 DNA topoisomerase I inhibitors

The most effective drugs currently in clinical trials are designed to target topoisomerases, in causing DNA damage <sup>87</sup>. The TOP1 inhibitors work insidiously, by converting topoisomerases from crucial normal enzymes to the most effective cellular poisons that fragment the genome <sup>88</sup>. Camptothecin (CPT) is a naturally occurring alkaloid and is a very powerful DNA TOP1 inhibitor, first discovered from the Chinese tree, *Camptotheca acuminata* <sup>89,90</sup>. The mechanism of action (MOA) of CPT and its analogues (structures shown in Fig. 2.3 and Fig 2.4) function

by integrating themselves into the TOP1/DNA cleavable complex, forming a ternary complex<sup>91</sup>. In order for CPT to bind, it requires the presence of both Top1 and DNA, as CPT does not bind substantially to one without the other (Leteurtre et al., 1993). By forming hydrogen bonds, CPT prevents both DNA religation and Top1 dissociation from the DNA<sup>91</sup>. Unfortunately, CPT presents numerous complications when used *in vivo*, as it has been noted to exhibit unfavourable pharmacokinetics and toxic properties<sup>92</sup>. For these reasons, novel semisynthetic exploration of the CPT analogues has been undertaken (Fig. 2.3 and Fig. 2.4). Approved analogues such as topotecan and irinotecan are the water-soluble FDA approved anticancer drugs that emanate from CPT. Topotecan has been approved as a second-line treatment for ovarian cancer and small cell lung cancer<sup>93</sup>. Irinotecan also licensed by the FDA, is used as a treatment agent for refractory colorectal carcinoma and as a primary therapy in conjunction with 5-fluorouracil (5-FU) for metastatic colorectal cancer<sup>92,94,95</sup>.

However, with all the activity shown for the approved water soluble analogues, Gounder and co-workers reported the shortfall of topotecan when the wild-type ABCG2 protein is expressed<sup>96</sup>. The expression of this protein rendered the resistance of the cell-lines under study, meaning the drug efflux proteins such as ABCG2 could significantly reduce the anti-proliferative activity of the widely relied on TOP1 inhibitors. Hence, a search of new and potent anticancer and antimicrobial drugs is on the rise.

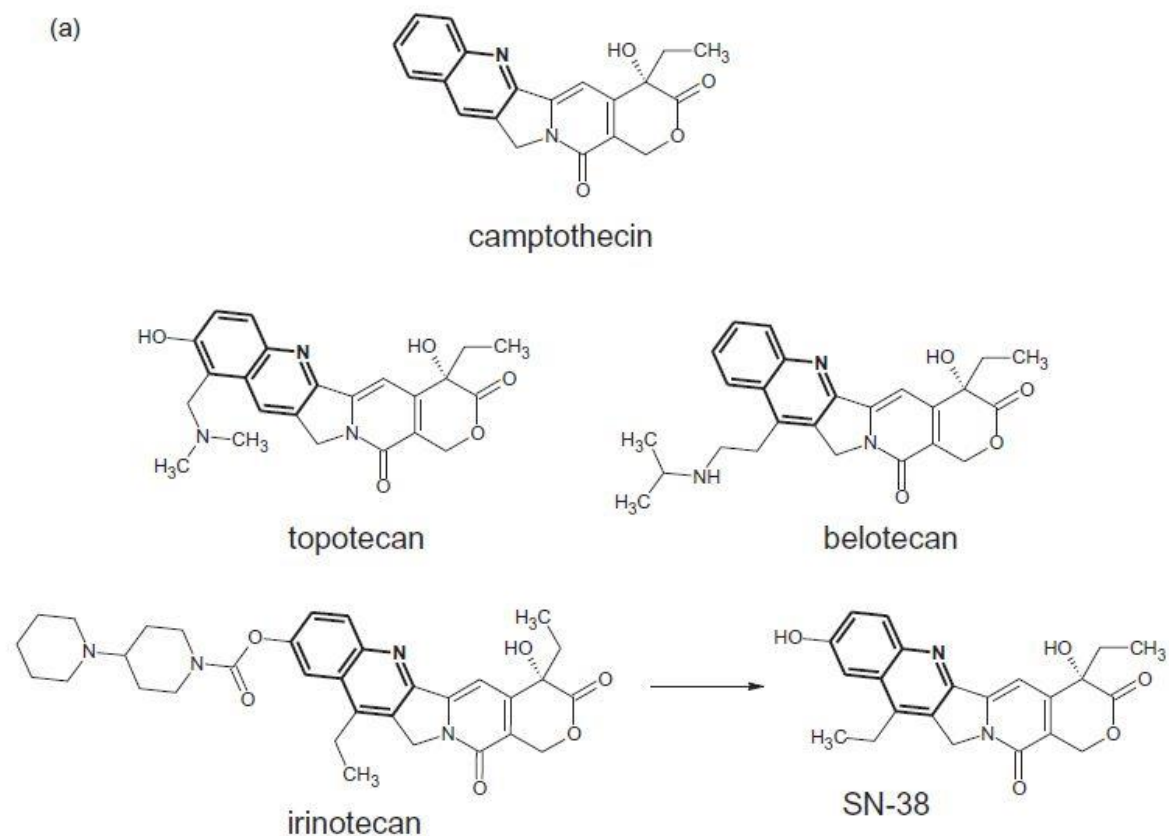


**Figure 2. 3:** The FDA approved CPT analogues adopted from Musiol <sup>92</sup>.

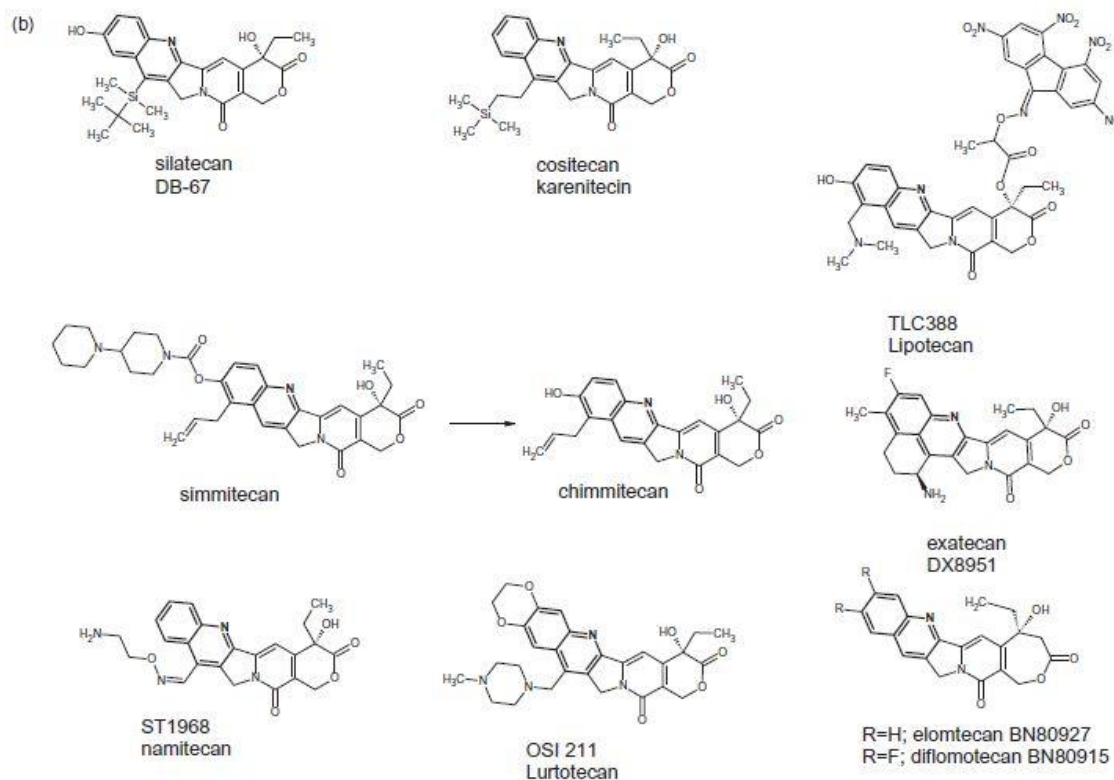


**Figure 2. 4:** The other camptothecin analogues adopted from Musiol <sup>92</sup>.

### 2.1.5 The search for new chemotherapeutic agents

Treatment modalities such as surgery and radiation therapy are capable of killing and damaging cancer cells but only in certain areas of the body. Unlike the aforementioned therapies, chemotherapy is a cancer treatment that employs medications or treatments to kill cancer cells that have migrated far from the original tumor and can function throughout the body, eliminating cancer cells that have spread far from the original tumor <sup>32</sup>. Therefore, finding potent novel therapeutic drugs that can efficiently destroy cancer cells or stop tumors from growing is one strategy to increase patient survival rates. Many methods have been employed towards finding new effective compounds like the use of supercomputers and other sophisticated computer programs to engage in parallel screening of thousands of compounds at the same time without physical lab presence. The new computer (*in-silico*) approach has become a prerequisite in pharmaceutical industries for drug discovery and development. This *in-silico* approach was instrumental in the design of hydroxybenzoic acid derivate compounds in this study and will be further elaborated on in a later section (refer to section 2.4).

### 2.1.6 Hydroxybenzoic acid (HBA)

Hydroxybenzoic acids are derived directly from benzoic acid and contain a C6–C1 structure and only the aromatic cycle's hydroxylations and methoxylations give them structural variations <sup>97</sup>. Hydroxybenzoic acids, contain up to four hydroxyl groups encircling a single benzene ring and are frequent metabolites of flavonoids and several hydroxycinnamic acids (e.g., chlorogenic acid). The family of these compounds is dominated by four acids: vanillic, syringic, and protocatechuic acids, as well as p-hydroxybenzoic acid <sup>98</sup>. Another well-known plant acid that contributes to the synthesis of hydrolyzable gallotannins is gallic acid <sup>98</sup>. All these compounds are widely distributed in the plant kingdom and the section below highlights functionalized hydroxybenzoic acids and their bioactivity.

#### 2.1.6.1 Functionalized HBA

Hydroxybenzoic acids are also classified as phenolic acids from non-flavonoid groups. There are many hydroxybenzoic acid compounds, but in this study, focus was directed to 3-hydroxybenzoic acid, 3,5-dihydroxybenzoic acid, and gallic acid (3,4,5-trihydroxybenzoic acid) as precursors for derivatization. The structures of these hydroxybenzoic acid compounds are displayed in Fig. 2.5 below.

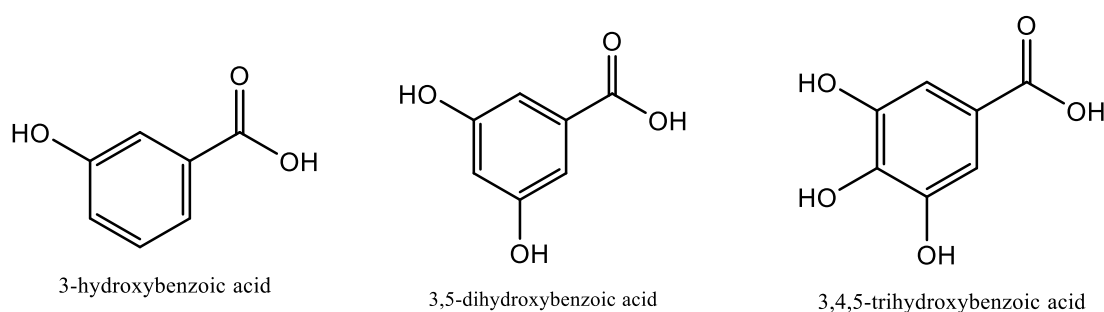


**Figure 2. 5:** Selected hydroxybenzoic acids for this study.

### *3-hydroxybenzoic acid (3-HBA) and derivative biological applications*

Grapefruit (*Citrus paradisi*), olive oil (*Olea europaea*), and medlar fruit (*Mespilus germanica*) are the main sources of 3-hydroxybenzoic acid (3-HBA)<sup>99</sup>. 3-HBA has shown diverse biological activity with and without modification and is also one of the constituents in plants.

### *3-hydroxybenzoic acid antimicrobial activity*

Kumar and co-workers studied the antimicrobial and antioxidant activity of *Araucaria cunninghamii* sweet bark which had the main constituent as 3-HBA (55.39%), this extract showed great antimicrobial activity against a series of pathogenic plant strains (0-500 µg/mL) and also had a good antioxidant activity with the IC<sub>50</sub> values ranging from 53.52-65.2 µg/mL<sup>100</sup>. Ohlan et al.<sup>101</sup> synthesized and evaluated the antimicrobial activity of urea inclusion complexes that involved 3-HBA as one of the precursors. In their study, they undertook a study on gram-positive strains: *Staphylococcus aureus* (*S. Aureus*), *Bacillus subtilis*, and gram-negative strain: *Escherichia coli* as well as on the fungal strains: *Candida albicans* and *Aspergillus niger*. The results obtained showed that in all the synthesized complexes, the 3-HBA complex showed higher antimicrobial activity against *S. Aureus* (3.1 µg/mL) and moderately higher activity on other strains<sup>101</sup>.

### *3-hydroxybenzoic acid other biological properties*

Potent inhibitory effects of some phenolic acids on Lactoperoxidase were studied by Koksall et al.<sup>102</sup>, where 3-HBA showed competitive inhibition on Lactoperoxidase with an inhibition constant (K<sub>i</sub>) of 0.405 mM. Aslan & Beydemir<sup>103</sup>, studied the effect of phenolic compounds on the inhibition of the polyol pathway enzyme (which is responsible for the pathogenesis of various diabetic complications in individuals with diabetes mellitus). In their results, 3-Hydroxybenzoic acid inhibited the aldose reductase (AR) (49.0 µM) and sorbitol

dehydrogenase (SDH) (6730  $\mu$ M) better than both 3,5-dihydroxybenzoic acid (AR: 92.0  $\mu$ M, SDH: 8350  $\mu$ M) and gallic acid (AR: 103.0  $\mu$ M, SDH: 26.0  $\mu$ M). However, gallic acid performed way better in terms of SDH inhibition <sup>103</sup>.

#### *3,5-dihydroxybenzoic acid (3,5-DHBA) and derivatives biological applications*

Peanuts (*Arachis hypogaea*), chickpeas (*Cicer arietinum*), red sandalwood (*Pterocarpus santalinus*), and hill raspberry (*Rubus niveus*) contain  $\alpha$ -resorcylic acid (3,5-dihydroxybenzoic acid) as one of their constituents <sup>99</sup>.

#### *3,5-dihydroxybenzoic acid antimicrobial and anticancer activity*

Kalinowska et al.<sup>104</sup> did a comprehensive study of structural, anti-/pro-oxidant, lipophilic, antimicrobial, and cytotoxic activity in MDA-MB-231 and MCF-7 cell lines of plant-derived and dietary hydroxybenzoic acid. The hydroxybenzoic acids of interest in their study were 3,5-DHBA and gallic acid (GA) <sup>104</sup>. Their results showed that GA acid is a more potent antioxidant than 3,5-DHBA since GA showed activity in all the antioxidant assays undertaken and where the activity was attributed to the *para* and *ortho* hydroxyl groups combination in GA, while 3,5-DHBA has hydroxyl groups in the *meta* positions. For the pro-oxidant assay, 3,5-DHBA outperformed GA since it showed the highest pro-oxidant activity after 120 minutes. Similarly for the antimicrobial assay, 3,5-DHBA showed good results (3 mg/mL) but only against *P. Aeruginosa* while GA also showed similar results against *E. coli*, *B. subtilis* and *S. enteritidis* at the same concentration (3 mg/mL). Regarding cytotoxicity studies in MCF-7 and MDA-MB-231 cell lines, GA was the most potent compound showing cytotoxicity concentrations (IC<sub>50</sub>) of 0.44 mM against MCF-7 and 0.36 mM against MDA-MB-231 cell line while other studied hydroxybenzoic acids did not show any toxicity against these cell lines <sup>104</sup>.

#### *3,5-dihydroxybenzoic acid other biological properties*

A study by Liu and co-workers showed that 3,5-DHBA is a specific agonist of hydroxycarboxylic acid 1 (HCA1), wherein activation of HCA1 in adipocytes resulted in the inhibition of lipolysis in adipocytes <sup>105</sup>. While another study by Sultana and co-workers <sup>106</sup> showed the nematocidal activity of 3,5-DHBA. In the aforementioned study, plant-parasite nematodes were inhibited by 3,5-DHBA and gallic acid as the main phenolic acids amongst other plant isolates. The findings suggested a strong inhibition capability of 3,5-DHBA, gallic and ethyl galactoside and they were regarded to be the most potent isolated extracts.

#### *3,4,5-trihydroxybenzoic acid (gallic acid) and derivatives biological application*

Amongst the aforementioned hydroxybenzoic acid compounds, gallic acid (GA) has been the most studied and it has an extensive literature compared to 3-HBA and 3,5-DHBA.

Gallic acid is one of the most abundant phenolic acids found in plants and is widely used in the food and pharmaceutical industries <sup>107</sup>. Gallnuts, sumac, witch hazel (*Hamamelis virginiana*), clove (*Syzygium aromaticum*), tea leaves, oak bark, sundew, and other plants (Fig. 2.6) contain this natural chemical. GA is also found in foods such as blackberry, hot chocolate, common walnut, Indian gooseberry, vinegar, wine, and white tea <sup>108</sup>. The structures of the naturally occurring derivatives of gallic acid are illustrated in Fig. 2.7 below.

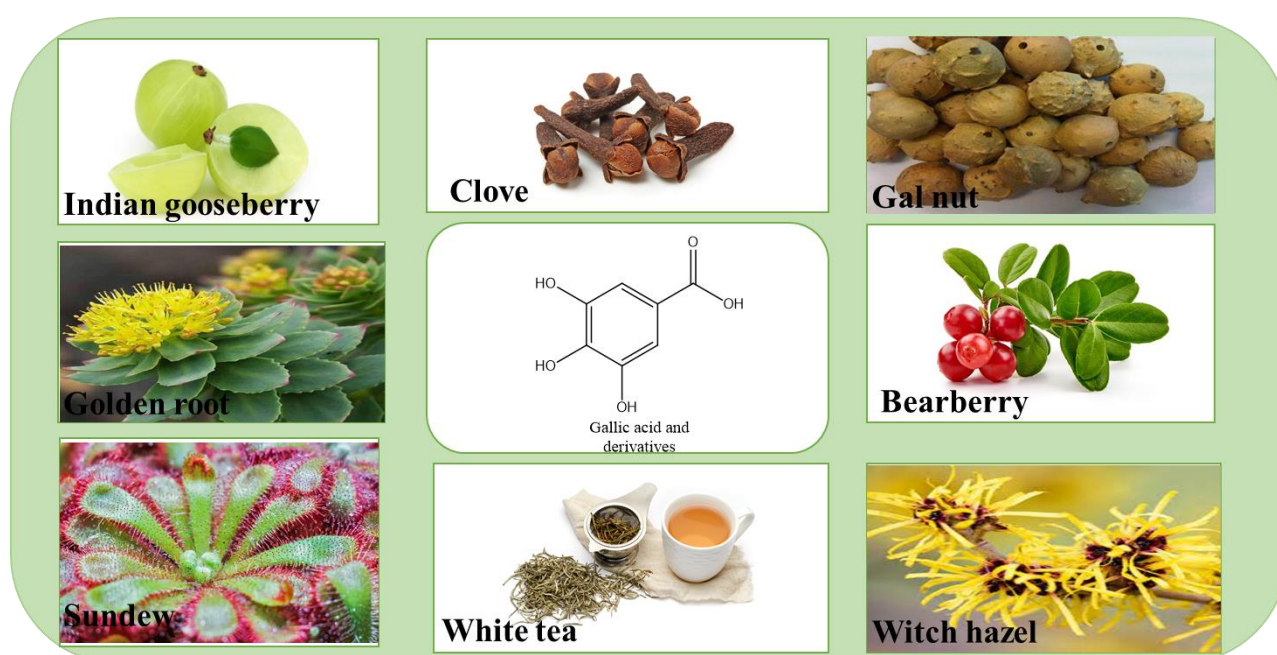


**Figure 2. 6:** Natural sources of gallic acid and its derivatives.

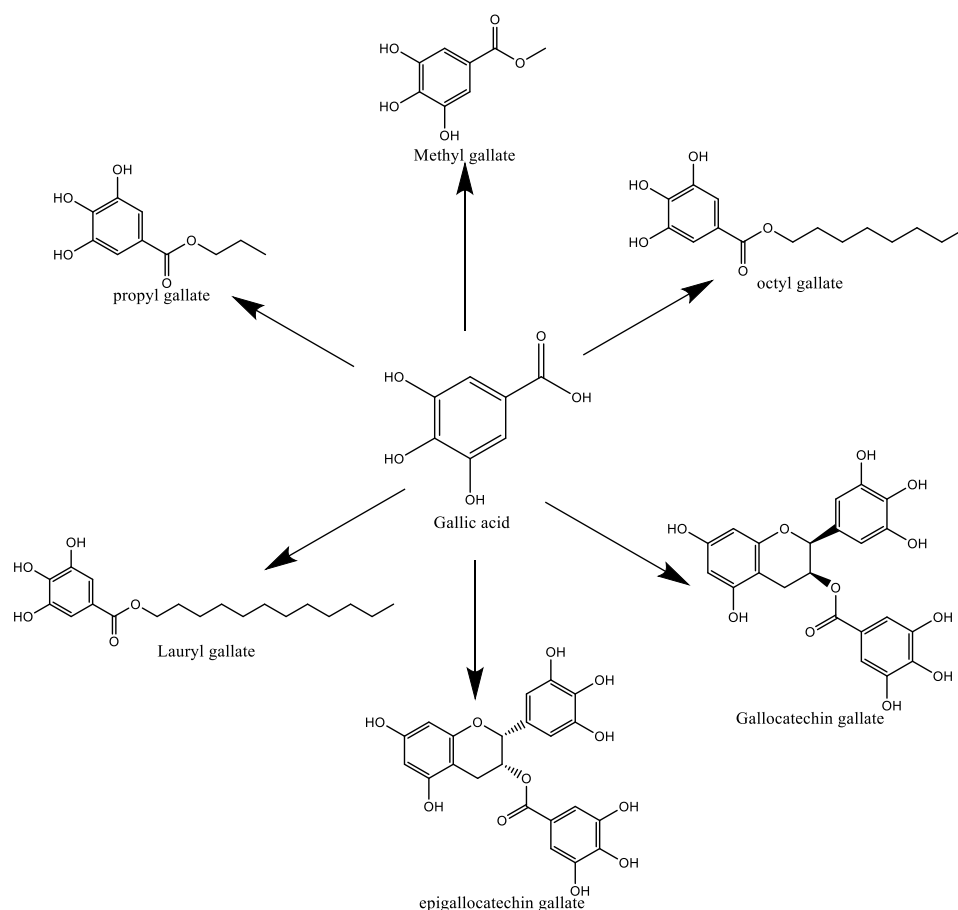


**Figure 2. 7:** Naturally occurring gallic acid and derivatives.

### *Gallic acid antimicrobial activity*

Borges and co-workers reported the antibacterial activity and mode of action of gallic acid against pathogenic bacteria. Their findings suggested that GA has good antibacterial activity with the MIC of 500  $\mu\text{g/mL}$  for *P. aeruginosa*, 1500  $\mu\text{g/mL}$  for *E. coli*, 1750  $\mu\text{g/mL}$  for *S. aureus*, and 2000  $\mu\text{g/mL}$  for *L. monocytogenes*. The mode of action indicated that through hydrophobicity changes, decrease of negative surface charge, and occurrence of local rupture or pore formation in cell membranes, GA caused irreversible changes in membrane properties (charge, intracellular and extracellular permeability, and physicochemical properties), resulting in leakage of essential intracellular constituents<sup>109</sup>. A study by Sorrentino et al<sup>110</sup> showed the antimicrobial activity of gallic acid against food-related *Pseudomonas* strains and its use as biocontrol tool to improve the shelf life of fresh black truffles. The results obtained showed MIC value of 2.5 mg/mL for all tested strains with only *P. fluorescens* DSMZ 50090<sup>T</sup> proving resistant against GA with<sup>110</sup> MIC value of 5 mg/mL. Furthermore, Sarjit and co-workers



showed the versatility of GA in their study titled antimicrobial activity of gallic acid against thermophilic *Campylobacter* as a strain specific and associated with a loss of calcium ions. The results obtained against the eight studied strains showed the MIC values in the range of 15.56 – 250 µg/mL<sup>111</sup>. In another study by Li et al.<sup>112</sup> titled Antibacterial activity and mechanism of a laccase-catalyzed chitosan–gallic acid derivative against *Escherichia coli* and *Staphylococcus aureus*, the findings showed that the derivative was more potent than the parent chitosan polymer and also that *S. aureus* was more sensitive to the derivative than *E. coli*. The mechanism of action of the derivative was via entry into the bacterial cells through damaged cell membranes and by inhibiting DNA synthesis<sup>112</sup>.

From a mechanistic perspective, gallic acid can inhibit the motility, adhesion, and biofilm formation of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus mutans*, *Chromobacterium violaceum*, and *Listeria monocytogenes*<sup>113–115</sup>.

#### *Gallic acid anticancer activity*

Gallic acid (GA) has previously been shown to inhibit a variety of cancer cell lines through a variety of mechanisms. Small cell carcinoma (SBC-3), squamous cell carcinoma (EBC-1), adenocarcinoma (A549), and cisplatin-resistant sub-clone of SBC-3 were used to study the apoptosis-inducing effect of GA in four human lung cancer cell lines by Ohno et al.<sup>116</sup>. Results showed a dose dependent activity of GA towards the studied cell lines. In addition, after GA treatment, cell morphology changed, DNA fragmentation occurred, and cell viability was lost<sup>116</sup>. Devi et al.<sup>117</sup> studied the anticancer activity of gallic acid on cancer cell lines, HCT15 (human colon cancer) and MDA-MB 231 (human breast cancer). Findings suggested potency of gallic acid since the IC<sub>50</sub> values were found to be 96 µg/mL and 80 µg/mL on HCT15 and MDA-MB 231 respectively. Furthermore, the photomicrograph morphological examination revealed morphological alterations in the treated cells, such as cell shrinkage, rounding, and detaching from the substratum indicating cell death via apoptosis<sup>118</sup>. GA stimulates caspase-3 and -8 expression, a change in the Bcl-2/Bax ratio, suppression of tyrosine phosphorylation of BCR/ABL kinase, and a downregulation of COX-2 levels in K562 and HL-60 leukemia cell lines, according to Chandramohan Reddy et al.<sup>119</sup> and Yeh et al.<sup>119</sup>. Liu and co-workers showed that GA is a cancer-selective agent that induces apoptosis in human pancreatic cancer cell lines (CFPAC-1 and MiaPaCa-2), since it showed dose and time dependant activity with the IC<sub>50</sub> values of 102.3 and 135.2 µM at 48 h for CFPAC-1 and MiaPaCa-2 cells respectively. The activity was attributed to GA activating caspase-3, caspase-9, and reactive oxygen species

(ROS), with elevation in the Bax expression and  $[Ca^{2+}]_i$  and reduced mitochondrial membrane potential in MiaPaCa-2 cells <sup>120</sup>.

#### *Gallic acid in other biological properties*

In food industries, gallic acid and its derivatives including, lauryl gallate, propyl gallate, octyl gallate, tetradecyl gallate, and hexadecyl gallate, have been used to prevent oils and fats from oxidizing and becoming rancid due to their free radical scavenging and antioxidant properties <sup>107</sup>. Variya and co-workers reported the antidiabetic potential of gallic acid from *Emblica officinalis* (Indian gooseberry) since the results revealed that GA improved glucose transporters and insulin sensitivity through PPAR- $\gamma$  and Akt signaling pathways <sup>121</sup>. The anti-inflammatory effect of the feiyangchangweiyan (FYC) capsule and its main components on pelvic inflammatory disease in rats was suggested to proceed via the regulation of the NF- $\kappa$ B and BAX/BCL-2 <sup>122</sup>. Results showed that GA as one of the components of FYC successfully inhibited the BAX pathway and significantly increased the expression of Bcl-2. The mechanism and pathway for action were determined from analyses involving the histological examination and enzyme-linked immunosorbent assay (ELISA) as well as the western blot analysis to detect the expression of NF- $\kappa$ B, BAX, BCL-2, and phosphorylation of the c-Jun N-terminal kinase (JNK) <sup>122</sup>.

There are other several biological applications that have shown the biological effect of GA. However, this study aims to highlight the involvement of GA in the selected applications and to show the molecular involvement and the signalling pathways that GA alters. 3-HBA and 3,5-DHBA have interesting activities in the biological applications highlighted above but their involvement in other biological applications is very limited. Therefore, this study will probe the potential activity of 3-HBA, 3,5-DHBA and GA amide and ester derivatives in antimicrobial and anticancer application.

#### **2.1.6.2 Amide derivatives of HBA**

The amide bond is one of the most abundant functional groups in organic, pharmaceutical, and material chemistry, as well as an essential component of all natural peptides and proteins <sup>123,124</sup>. It accounts for over 25% of the currently existing pharmaceutical drugs that are on the market <sup>123,125</sup>.

In this study we focus on the hydroxybenzoic acid amide derivatives and their biological or industrial applications. Studies that involve the biological activity of 3-HBA and 3,5-DHBA

amide derivatives were not obtained during the literature search, therefore the focus will only be on GA amide derivatives since many reports were sourced on these.

Chen et al.<sup>126</sup> demonstrated the impact of di-amide bonding activity in GA derivatives compared to mono-amide linkage. In their study, the authors synthesized a series of GA amide derivatives and evaluated their activity against  $\alpha$ -synuclein aggregation known to be involved in all Parkinson's disease (PD) cases. The di-amide compounds were the most active compounds with IC<sub>50</sub> values ranging from 0.98  $\mu$ M – 1.95  $\mu$ M. The most active compound with an IC<sub>50</sub> value of 0.98  $\mu$ M had a backbone with hydroxyl groups on the periphery<sup>127</sup>. Mahdavi et al.<sup>128</sup> studied the dual activity (antimicrobial and anticancer) of the gallic acid amide derivatives synthesized using 2-aminothiophene. The results suggested the activity of the synthesized compounds against a series of Gram-negative and Gram-positive strains as well as anticancer activity against HeLa and HCT116 cell lines<sup>128</sup>.

### **2.1.6.3 Ester derivatives of HBA**

The ester derivatives of hydroxybenzoic acid are the most prominent in terms of the available literature. Earlier reports have even suggested that hydroxybenzoic acid esters are more stable than hydroxybenzoic acids, i.e. propyl gallate was found to be more stable than gallic acid, especially in the human body's weak alkaline condition (pH 7.4) or the intestine alkaline condition (pH 8.6)<sup>129,130</sup>. Moreover, hydroxybenzoic acids esters are quite important in our everyday lives since they are used widely in food industries which in turn sustain us. In addition, gallic acid esters such as propyl (E310), octyl (E311), and dodecyl (E312) esters are employed as food additives having antioxidant properties. Furthermore, parabens are p-hydroxybenzoic acid esters that operate as food preservatives, such as methyl or ethyl parabens, E214 and E218, respectively<sup>131,132</sup>. In many cases, esterification of phenolic acids improves their properties; this improves many functional and antioxidative properties, and in the case of phenolic lipids, the oxidative stability and nutritional value of edible oils are increased, and these compounds by virtue of their ultraviolet adsorbing activity are also valuable in the cosmetics industry<sup>133,134</sup>.

## **2.2 Microbial infections**

Microbial infections are increasingly emerging as a significant and challenging issue for human health globally<sup>135</sup>. When microorganisms infiltrate a person's body (host) and causes harm, the resultant effect is called an infection. The microorganisms can survive, reproduce, and colonize different regions in the host body. Patients with decreased immunity, neoplastic illnesses, and

those receiving organ transplants are the most common victims of microbial infections <sup>135</sup>. Microorganisms can either be benign or pathogenic. The benign microorganisms are harmless but when untreated they can be pathogenic <sup>136</sup>. Pathogens are microscopic organisms that are infectious and can multiply rapidly and can also be fatal <sup>136</sup>. There are three examples of pathogenic microorganisms (i) Bacteria, (ii) Fungi and (iii) Viruses. In this study, we focused only on bacteria and fungi since they are opportunistic type of organisms that can prove fatal to immunocompromised patients. Therefore, we paid attention only to clinical strains of selected bacteria and fungi.

### 2.2.1 Clinical strains

Patients that are admitted at the intensive care unit (ICU) are at higher risk of contracting secondary microbial infections (leading causes of healthcare-associated infection) which can either affect the respiratory, urinary, and circulatory systems more frequently <sup>137</sup>. Bacterial and fungal co-infection complications can be associated with decreased immunity of the patients in the ICU due to several diseases that individual patients might be suffering from at the time of admission <sup>138</sup>. Cancer is one of the many diseases that could weaken the patient's immunity severely. Therefore clinical manifestation of microbial infections from *Candida* microbial strains (that includes *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. parapsilosis*) and bacterial infections from *Staphylococcus Aureus* (*S. Aureus*), *Escherichia coli* (*E. coli*), *Pseudomonas Aeruginosa* (*P. aeruginosa*), and *Streptococcus Pyogenes* (*S. Pyogenes*) amongst other strains possess a serious threat to ICU patients. Each strain is worthy of consideration, to understand the prevalence and virulence as highlighted below:

*Candida* spp.:

*Candida spp.* are commensal oval budding yeasts (forms hyphae and pseudohyphae) that are found in the typical human skin and gut microbiota and can be detected in up to 60% of healthy people <sup>139–143</sup>. *Candida* lives normally on the skin and inside the body, in areas including the mouth, throat, gut, and vagina, without creating any problems. However, in certain predisposing conditions there are at least 15 different *Candida* species that could cause human disease, but the five most prevalent pathogens causing more than 95% of invasive disease include: *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* <sup>140,143</sup>. Invasive candidiasis is the term used for serious infections caused by these organisms <sup>143</sup>.

## *Staphylococcus Aureus*

*Staphylococcus aureus* are Gram-positive bacteria (stain purple by Gram stain) that are cocci-shaped and tend to cluster in "grape-like" clusters as shown in Figure 2.7 <sup>144</sup>. *Staphylococcus aureus* is a human pathogen as well as a commensal bacteria <sup>145</sup>. *S. aureus* is the causative agent of a variety of human infections, including bacteremia, infective endocarditis, impetigo, folliculitis, furuncles, carbuncles, cellulitis, scalded skin syndrome, and others), osteomyelitis, septic arthritis, prosthetic device infections, pulmonary infections (e.g., pneumonia and empyema), gastroenteritis, meningitis, toxic shock <sup>145</sup>. These bacteria can induce invasive infections and/or toxin-mediated diseases, depending on the strains involved and the site of infection <sup>146</sup>.



**Figure 2.8:** Scanning electron microgram (SEM) of *S. aureus* showing spherically shaped bacterium and clinical manifestations of *S. aureus*. Images adopted from Tong et al. <sup>145</sup>.

## *Escherichia Coli*

*E. coli* is a Gram-negative, facultatively anaerobic, rod-shaped coliform bacterium (as shown in Figure 2.9) found in the lower intestine of warm-blooded species <sup>147,148</sup>. Except in immunocompromised hosts or when the normal gastrointestinal barriers are breached, as in peritonitis, these commensal *E. coli* strains rarely cause disease <sup>147</sup>. However, Enteric/diarrheal

disease, urinary tract infections (UTIs), and sepsis/meningitis can all be caused by the *E. coli* pathogenic type <sup>147</sup>.



**Figure 2.9:** The images show a gram-stained facultative anaerobic, rod-shaped coliform bacterium (*Escherichia coli*) viewed under a light microscope and symptoms of *E. coli*. Images were obtained from Islam et al.<sup>149</sup>.

### *Pseudomonas Aeruginosa*

*Pseudomonas aeruginosa* is a Gram-negative, encapsulated, Rod-shaped aerobic bacteria (as shown in Fig. 2.10) that can cause disease in plants and animals, including humans <sup>150,151</sup>. *P. aeruginosa* emergence as a major opportunistic human disease over the last century may be due to its resistance to antibiotics and disinfectants that can kill other bacteria <sup>152</sup>. *P. aeruginosa* is currently a common cause of bacteremia in burn victims, urinary tract infections in catheterized patients, and hospital-acquired pneumonia in ventilator-dependent patients <sup>153</sup>. These infections are difficult to treat, partly because of *P. aeruginosa*'s natural resistance to antibiotics, and thus can result in lung failure and death <sup>152</sup>.



**Fig. 2.10:** The images show encapsulated rod-shaped aerobic bacteria and skin-related skin manifestations of *P. aeruginosa*. The images are adapted from Wu et al.<sup>154</sup>.



### *Streptococcus pyogenes*

*Streptococcus pyogenes* is a Gram-positive, aerotolerant bacteria (see Fig. 2.11) that belongs to the *Streptococcus* genus. It is an extracellular bacteria made up of non-motile and non-spore-forming cocci (round cells) that tend to form chains. *S. pyogenes* is a common human-specific bacterial pathogen that can produce a range of disorders, from mild localized infections to life-threatening invasive infections<sup>155,156</sup>. Pharyngitis, scarlet fever, impetigo, cellulitis, type II necrotizing fasciitis, streptococcal toxic shock syndrome, acute rheumatic fever, and post-streptococcal glomerulonephritis are some of the diseases caused by *S. pyogenes*<sup>155,156</sup>.

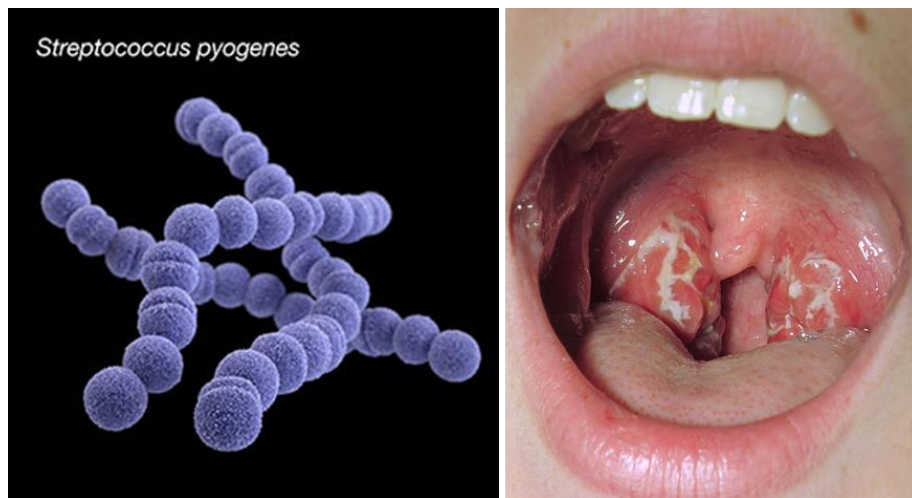


**Figure 2.11:** The image on the left shows an extracellular bacteria made up of non-motile and non-spore-forming cocci (round cells) that tend to form chains. The image on the right shows a patient with a sore throat caused by *P. aeruginosa*. The images were adopted from centre for disease control (CDC) retrieved from (<https://www.cdc.gov/streplab/groupa-strep/index.html>).

The commonality about the selected fungal and bacterial strains above is that they are all pathogenic and are causes of opportunistic diseases found on the healthcare setting. They can cause invasive infections that immensely contribute to the mortality of immunocompromised hosts. Therefore, cancer patients being immunocompromised are at risk of contracting some of these infections on the hospital bed-side.

## **2.2.2 Microbial strains that affect cancer patients**

### **2.2.2.1 Candida species**

Cancer treatments such as chemotherapy and radiation therapy are very important to cancer patients since they bring “hope” of recovery from this illness. However, their potential toxic effects on normal cells are a worrying factor. These treatments suppress salivation while also increasing mucosal fragility and associated mucositis <sup>157</sup>. The patients become more susceptible to *candidal* infection and they subsequently develop oral candidiasis due to the overgrowth of *Candida* species <sup>157</sup>. *Candida* species are commonly found in oral flora under normal conditions. The yeast can convert from a commensal to a pathogen under specific physiological and pathological conditions, especially in people with cancer because of their immunocompromised state <sup>158</sup>.

### **2.2.2.2 Gram-positive bacteria**

It is reported that at least half of all microbiologically documented infections in cancer patients are caused by Gram-positive bacteria <sup>159</sup>. *Staphylococci*, *streptococci*, and *enterococci* are the most common gram-positive bacteria that cause significant infections in cancer patients <sup>160</sup>. In comparison to gram-negative bacteria, Gram-positive bacteria have a thicker peptidoglycan cell wall. The peptidoglycan layer of the gram-negative cell wall is 2 to 3 nm thick and covered by an outer lipid bilayer membrane, whereas the polymer layer of the gram-positive cell wall is 20 to 80 nm thick. In this study, only two Gram-positive bacteria namely, *S. aureus* and *S. pyogenes* were included in the study. In cancer patients *S. aureus* has been previously linked to skin and soft tissue infections and accounts for the major part of the Gram-positive bacteria found on cancer patients <sup>161</sup>, while *S. pyogenes* has been reported to cause streptococcal toxic shock syndrome (STSS), which is a severe infectious disease that involves rapidly progressive septic shock <sup>162</sup>. Soft tissue necrosis, acute renal failure, adult respiratory distress syndrome, disseminated intravascular coagulation (DIC), and multiple organ failure develop tens of hours after the onset of STSS in patients, resulting in a shock and death <sup>162</sup>.

### **2.2.2.3 Gram-negative bacteria**

Although the overall rate of Gram-negative infection has decreased over the last decade, data from several large surveillance studies conducted at major cancer centres in the United States and Europe show that *Enterobacteriaceae* are responsible for 65 to 80 % of documented Gram-negative infections in their patients <sup>163</sup>. In immunocompromised hosts, however, *Pseudomonas aeruginosa* is linked to significant morbidity and mortality <sup>163</sup>. Hence, the focus of this study



for Gram-negative bacteria was on *P. aeruginosa* and *E. coli* only. *P. aeruginosa* has been reported to cause bacteremic pneumonia, a deadly infection that generally occurs in neutropenia patients on chemotherapy <sup>164</sup>. Whilst, *E. coli* has been reported to be the most common Gram-negative bacteria that causes bloodstream infections (BSI) <sup>165</sup>.

Therefore, microbial infections are the major cause of death in cancer patients since they contribute to the onset of other diseases in addition to cancer and for this reason, antimicrobial agents have gained increased interest, especially in the oncology field.

## **2.2.3 Antimicrobial agents used to treat cancer patients and their shortfalls**

### **2.2.3.1 Antifungal agents**

Immunosuppression, a typical adverse effect of treatment, predisposes patients to invasive fungal infections, which have a rising prevalence in many chronic diseases <sup>166</sup>. Different *candida* strains have been treated with different antifungal agents shown below in Figure 2.12. Amongst the antifungal agents, fluconazole has been used to treat a broad spectrum of candida strains. *Candida albicans* and *Candida glabrata* have been treated with Fluconazole or echinofungin (caspofungin, anidulafungin and micafungin) as their primary treatment <sup>143,166</sup>. The echinocandins target the  $\beta$ -D-glucan synthase enzyme, which is required for cell wall formation <sup>140</sup>. Fluconazole shows fungistatic activity by interfering with cell wall synthesis, growth, and cell adhesion <sup>167</sup>. Amphotericin B or echinofungins have been used as a first-line antifungal agent in the treatment of *Candida tropicalis*-related infections <sup>168</sup>. Amphotericin B's target site of action is ergosterol, the primary sterol in the fungus cytoplasmic membrane <sup>169</sup>. *Candida parapsilosis* has a treatment plan that involves fluconazole, amphotericin B, nystatin, and voriconazole as some of the treatment options <sup>170,171</sup>.

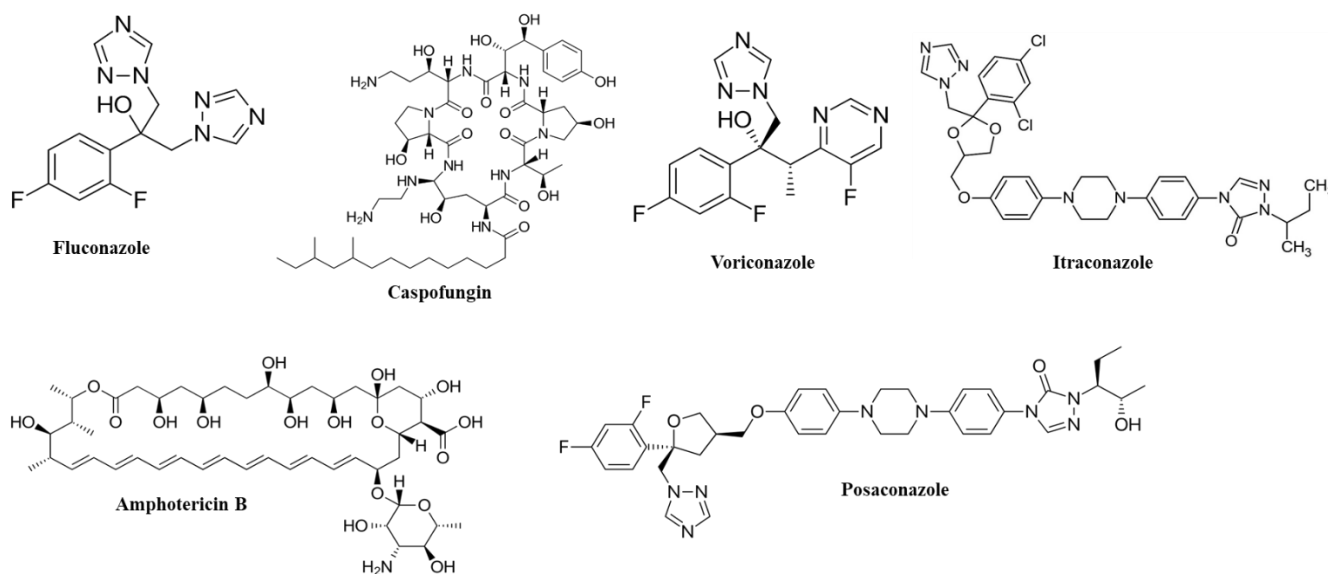


**Figure 2.12:** Some of the antifungal agents used to treat infections in cancer patients.

### 2.2.3.2 Antibacterial agents

Bacterial species are characterized according to their cell wall and are then divided into Gram-negative and Gram-positive depending on the type of cell wall or peptidoglycan layer they represent. Figure 2.13 and Figure 2.14 show the type of drugs used to treat Gram-negative and Gram-positive bacteria respectively. The figures below show the currently used antibiotics for the treatment of Gram-positive and Gram-negative bacteria and they follow different mechanisms of action. Antibiotics have lowered patient mortality, but they have also increased the threat of multidrug-resistant microorganisms<sup>161</sup>. Among immunocompromised patients, multidrug-resistant bacteria are prevalent<sup>161</sup>.

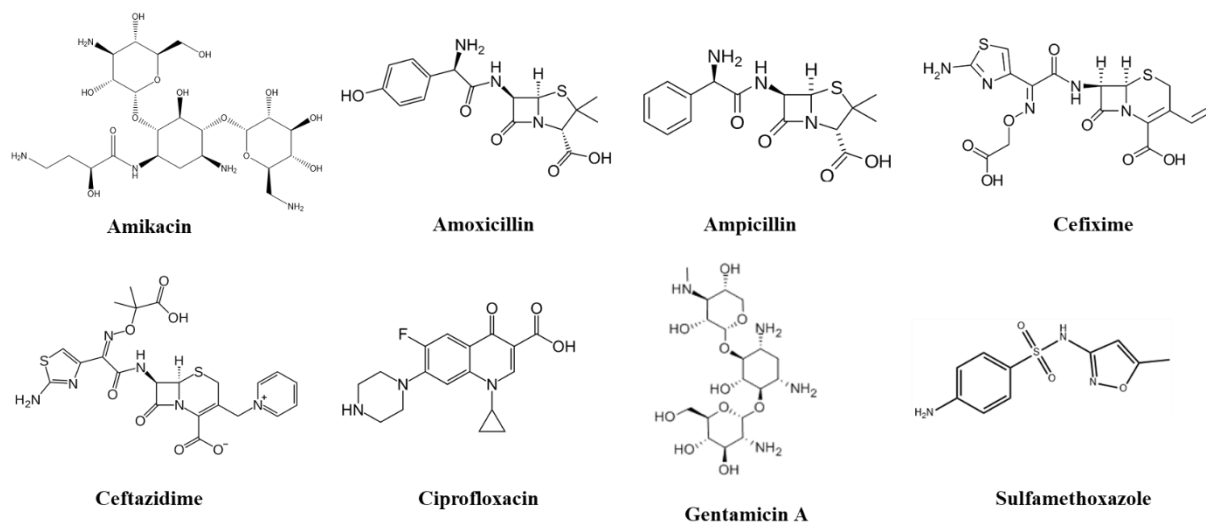


**Figure 2.13:** Some of the Gram-negative antibacterial drugs used to treat infections in cancer patients.

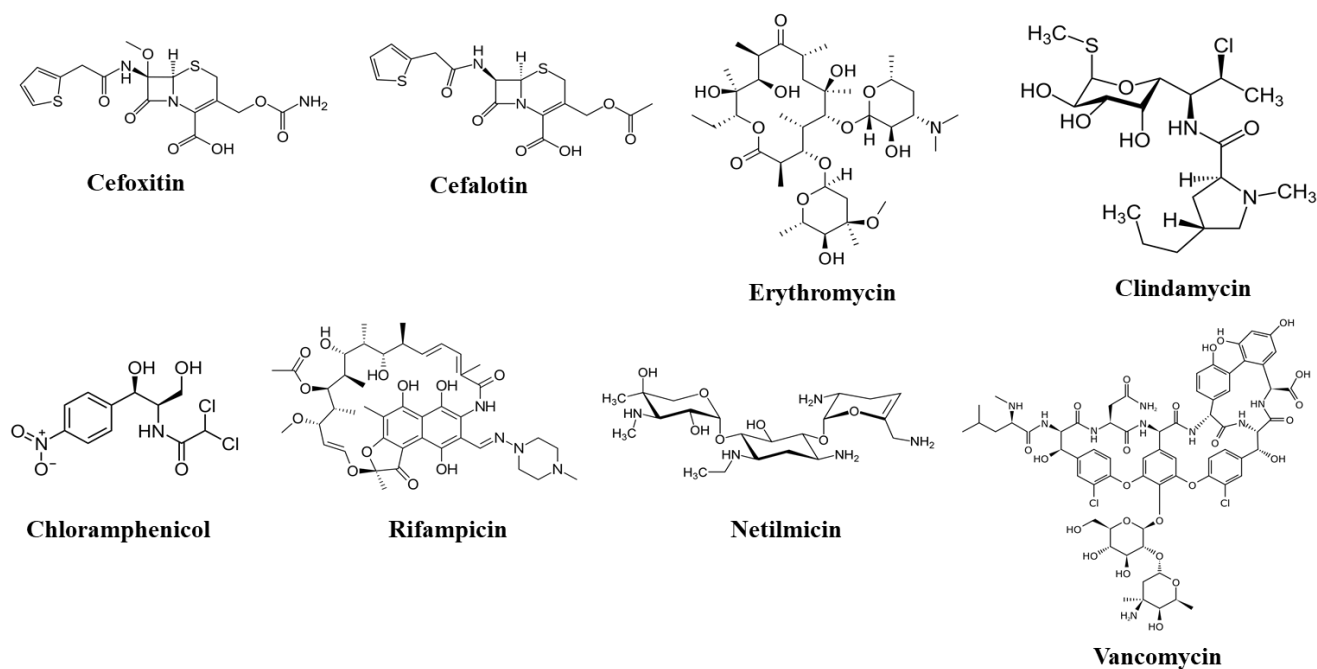


**Figure 2.14:** Some of the Gram-positive antibacterial drugs used to treat infections in cancer patients.

The mechanism of action for the drugs highlighted in Figure 2.13 and Figure 2.14 is subdivided into four major mechanisms namely; inhibition of bacterial cell wall synthesis, enhancement of bacterial cell membrane permeability, interference with bacterial protein synthesis and inhibition of nucleic acid replication and transcription<sup>172</sup>. Figure 2.14 below shows the class of antibiotics and their mechanism of action (see Fig. 2.15). Drugs that affect

the cell wall synthesis are shown to be mostly similar to penicillin in structure, meaning drugs that contain the  $\beta$ -lactam moiety are most likely going to inhibit the cell wall synthesis. Moreover, drugs with an aminoglycolic group (such as amikacin) are most likely to enhance the bacterial cell membrane permeability and also polypeptide-based drugs are likely to have the same effect as the aminoglycolic-based drugs. While compounds that contain several hydroxyl groups such as gentamicin, erythromycin, clindamycin, chloramphenicol and netilmicin are shown to have an effect on the bacterial protein synthesis. Furthermore, compounds with a quinolone group are most likely to affect bacterial nucleic acid replication and transcription.

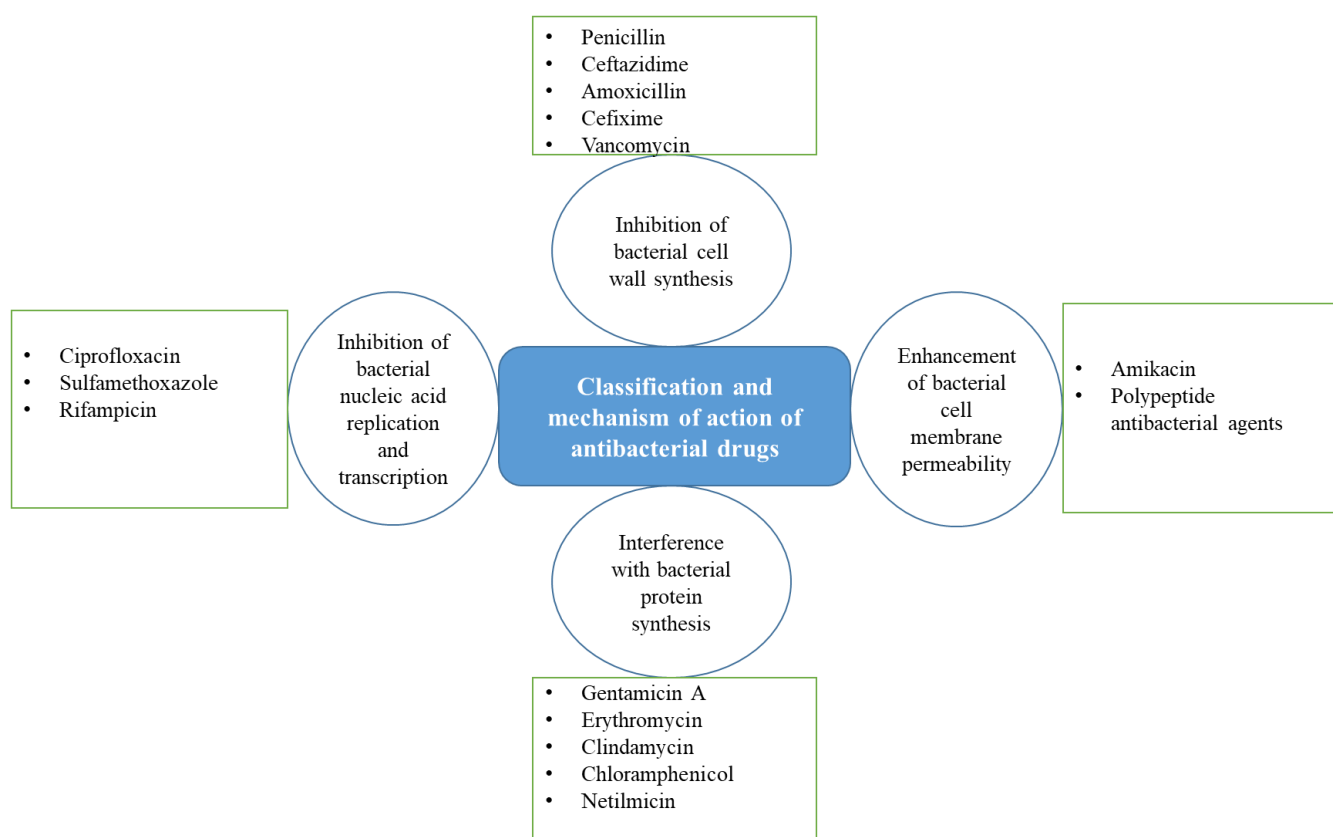


**Figure 2.15:** Classification and mechanism of action of antibacterial drugs. Inhibition of bacterial cell wall synthesis, enhanced permeability of bacterial cell membranes, interference with bacterial protein synthesis, and inhibition of bacterial nucleic acid replication and transcription are the four major mechanisms. Adopted with modification from Gao and co-workers <sup>173</sup>.

Antibiotics are regarded as "miracle medications" in the fight against microorganisms. Antibiotics have been used for decades in a variety of industries, including agriculture and veterinary, not only for therapeutic purposes but also as a preventative measure <sup>174</sup>. However,

uncertainty has arisen as a result of microorganisms developing resistance to common antibiotics while the host is oblivious to the problem. Therefore, the synthesis of new antimicrobial compounds is crucial to combat the ever-evolving microorganism resistance and improving the survival of cancer patients affected by secondary infections.

#### **2.2.4 Antimicrobial agents that bear hydroxyl moieties or hydroxybenzoic acid**

The figures (Figures 2.13 and 2.14) above show that most of the antibacterial agents currently administered bear the hydroxyl moiety. This justifies the growing interest in medications that are derived from phenolic compounds. Drugs shown in Figure 2.12 and Figure 2.13 also show interesting bonding patterns that contribute to the antimicrobial activity. Apart from the hydroxyl pendant groups available, the ester and amide bonding are also highly dominant in those administered drugs. Insight into the groups present in the administered drugs shapes the design of novel drugs that could have potential as antibacterial agents as these are designed to bear hydroxyl groups and to have either ester bonding, amide bonding, or the hybrid system of both the ester and the amide bonding for improved efficacy. Gallic acid and its related and other related phenolic acids such as 3-HBA and 3,5-DHBA have shown the importance of having the hydroxybenzoic acid motif in the design of new compounds.

#### **2.2.5 Important HBA compounds for antimicrobial activity**

Previous studies show that amongst the HBA compounds, gallic acid has been the most promising compound and has attracted extensive interest in a broad biological spectrum. The study by Khatkar and fellow researchers (2017) synthesized a series of gallic acid amide, anilides, and ester derivatives and evaluated their antimicrobial activity and quantitative structural activity relationship (QSAR). The QSAR and the *in vitro* antimicrobial activity showed that the amides and the esters were more potent than the anilides<sup>175</sup>. Another study by Sherin and co-workers, delved into the synthesis of a series of gallic acid derivatives having different moieties such as halogen groups and other forms of electron withdrawing and electron donating groups<sup>176</sup>. Their results from structural activity relationship studies showed that, groups such as halogens had a significant impact on the antimicrobial activity and that aryl derivatives had a more significant impact towards enhancing the potency of the synthesized compounds against Gram-negative, Gram-positive, and fungal strains that were studied<sup>176</sup>.

In summary, the antimicrobial activity of compounds is governed by the type of substituents on the periphery of the molecule of interest. Gallic acid potency is enhanced by the type of

bonding, with aryl derivatization increasing potency more than alkyl derivatization. Furthermore, amidation and esterification generally show improved potency. However, testing *in vitro* potency as a source of identifying lead compounds to design potent compounds is time consuming and costly. Research has ventured into the new *in-silico* approach shown to be effective in determining bioactive and potent compounds without undertaking *in vitro* experiments. Results of *in-silico* studies are accurate and reliable for determining *in vitro* and sometimes *in vivo* activity.

### **2.3 *In silico* drug design**

In addition to experimental procedures, computational approaches have since become an essential component of drug discovery<sup>177</sup>. Virtual screening, pose prediction, and binding energy calculation are commonly thought of as three different stages in the lead discovery process<sup>177</sup>. Virtual screening, also known as *in silico* screening, is a computational method for identifying drug-like molecules for a specified target<sup>178</sup>. Molecular docking simulations, pharmacophore-based screening, similarity searches, and more complex artificial intelligence techniques are examples of these computational methodologies<sup>177</sup>.

It is commonly acknowledged that the process of discovering and developing novel drugs is time-consuming, risky, and costly<sup>179</sup>. From concept to market, the typical drug discovery and development cycle lasts about 14 years and costs between \$800 million and \$1 billion USD<sup>180</sup>. Rapid advancements in combinatorial chemistry and high-throughput screening technologies have created an environment that has sped up the drug discovery process by making it possible to screen and synthesize vast libraries of compounds rapidly<sup>181,182</sup>. However, because of the low efficiency and high failure rate in drug discovery, even though the amount invested in the creation of new drugs has increased dramatically over the past few decades, the output is not positively proportional to the investment<sup>183</sup>. As a result, numerous strategies have been developed to shorten the research cycle, lower costs, and eliminate failure risks in the quest for new drugs. One of the most efficient ways to accomplish these goals is through computer-aided drug design (CADD)<sup>179</sup>.

The development of digital repositories for studying chemical interactions, computer programs for generating compounds, and tools for systematically evaluating possible lead candidates are only a few of the facets of drug discovery that CADD encompasses<sup>184</sup>. Computational tools can be used for the majority of the drug discovery and development processes, from target identification and validation to lead discovery and optimization, and the tools can even be used

for preclinical trials in the post-genomic era, benefiting from the dramatic increase in biomacromolecule and small molecule information<sup>183,185–187</sup>. Figure 2.16 illustrates a variety of computational drug discovery methods that have been used at different stages of the drug discovery and development pipeline, including target identification and validation, lead discovery and optimization, and preclinical tests. The use of computational techniques could cut the price of developing new drugs by up to 50%<sup>179,186</sup>.

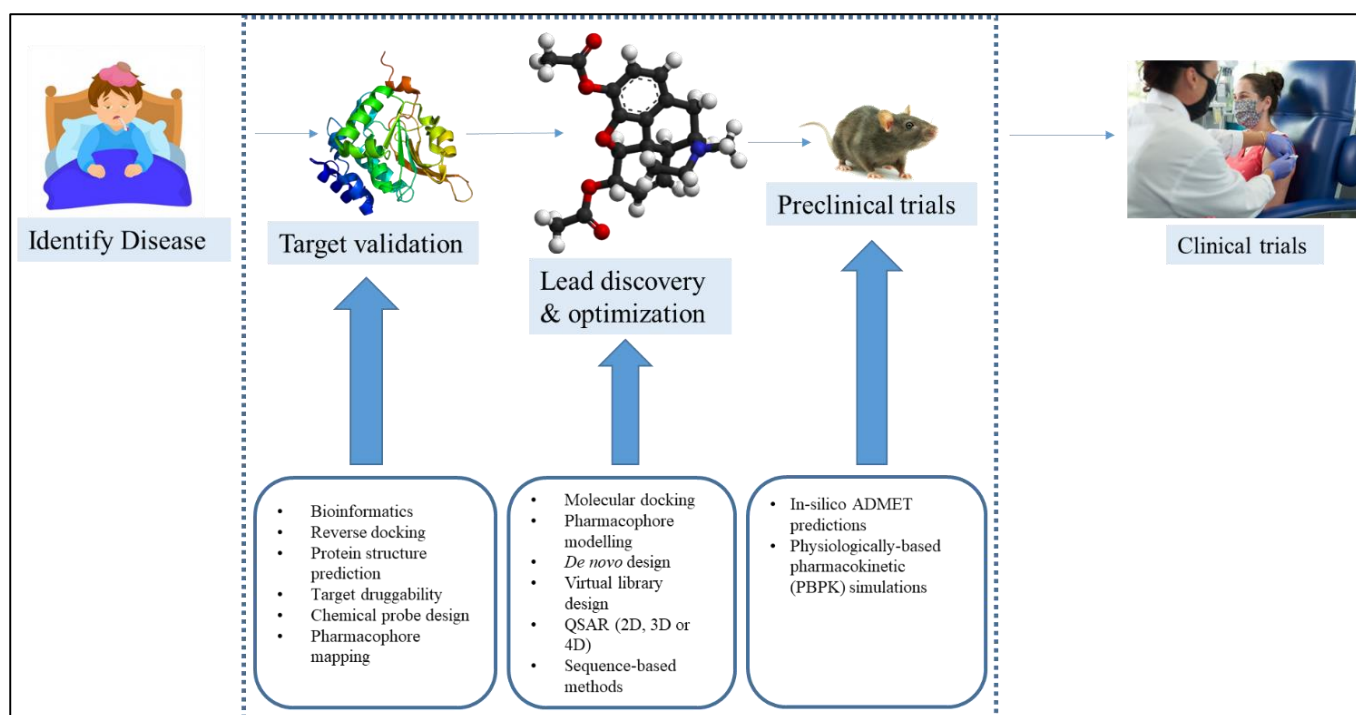


**Figure 2.16:** Drug discovery and development pipeline and stages<sup>188</sup>. The demarcated section shows areas or stages where the computer-aided-drug design (CADD) approach is used as an alternative to reduce the time and cost of producing a single-market drug, adopted with modifications from Tang et al.<sup>188</sup> and Ou-Yang et al.<sup>179</sup>.

The computer-aided drug design commonly uses three approaches namely the structure-based drug design (SBDD), ligand-based drug design (LBDD) and sequence-based approach. *De novo* drug design and molecular docking are two SBDD techniques that rely on homology models, crystal structures, and NMR data to determine the structure of the target macromolecule<sup>189</sup>. LBDD tools, such as qualitative structural activity relationship (QSAR), pharmacophore modelling, molecular field analysis, and 2D or 3D similarity assessment, can

offer vital insights into the nature of the interactions between drug targets and ligands in the absence of three-dimensional (3D) structures of potential targets, allowing predictive models suitable for lead discovery and optimization to be constructed <sup>190</sup>. The SBDD and LBDD traditional workflow is shown in Figure 2.17 demonstrating every detail of CADD using these two approaches from inception. Recently, sequence-based approaches that use bioinformatics tools to examine and compare different sequences have been created to find prospective targets from scratch and to undertake lead discovery in scenarios where neither the target structure nor the ligand information is known <sup>191</sup>.

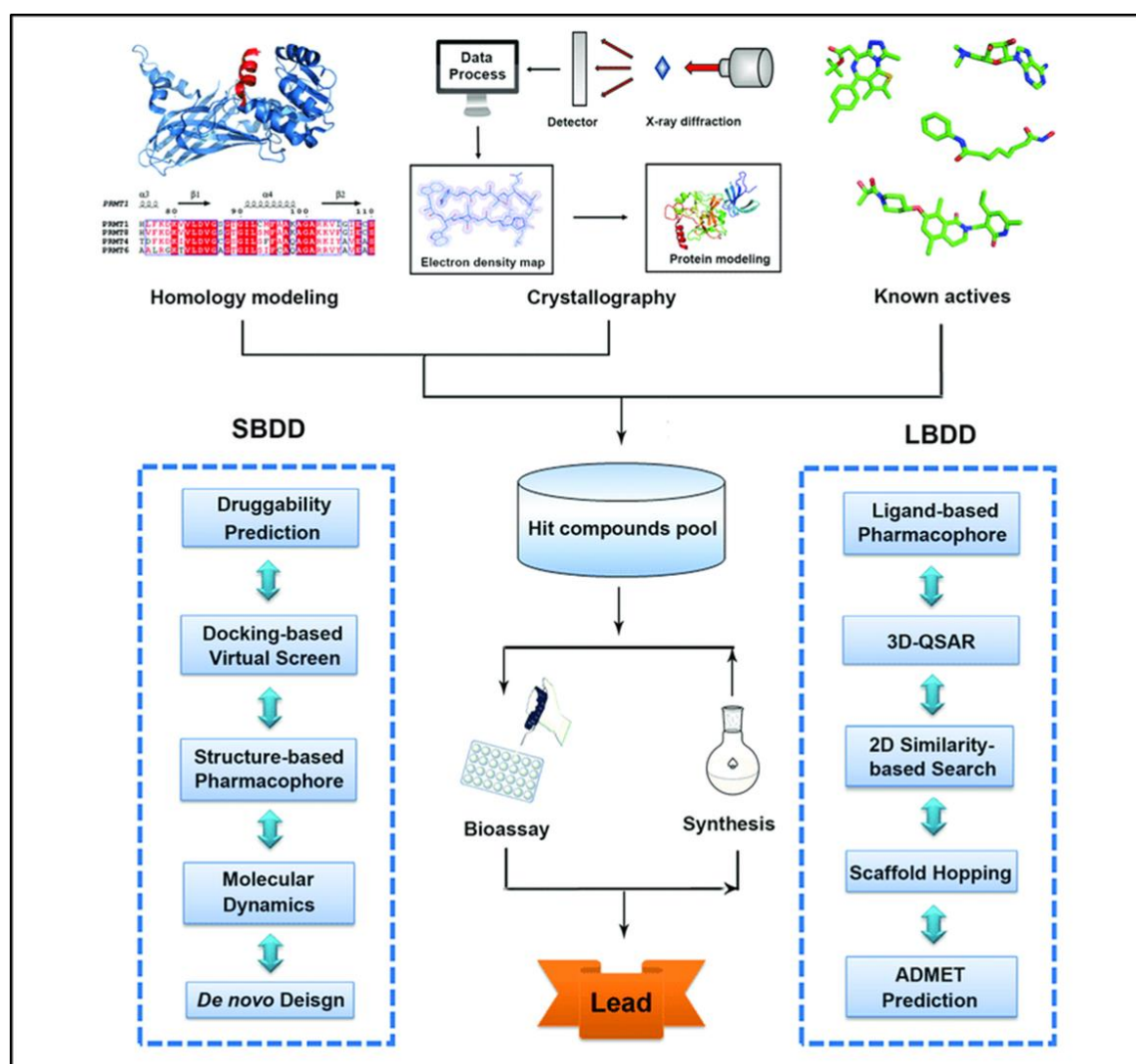


**Figure 2. 17:** Structure-based drug design (SBDD) and ligand-based drug design (LBDD) traditional workflow reprinted from Lu et al.<sup>192</sup> via license: Creative Commons Attribution 4.0 International.



Proteins (primarily enzymes, receptors, and transport proteins) and nucleic acids (DNA and RNA) are the primary targets for drugs at the molecular level. The high-throughput miniaturization of chemical synthesis and biological evaluation of multiple compounds on gene/protein expression and function, in conjunction with the sequencing of over 180 different genomes, pave the way for global drug-discovery approaches that are no longer narrowly focused on a single target but instead on an entire family of related proteins or on a full metabolic pathway <sup>193</sup>. Understanding protein function, elucidating signal transduction networks, and drug design research are significantly impacted by the identification of protein interaction sites <sup>194</sup>. Predictive techniques using sequence information exclusively for protein interaction site prediction have received increasing interest due to the exponential growth of protein sequence data <sup>194</sup>. In general, computational techniques for sequence-based approach can be divided into two categories: molecular docking of two proteins with known structures and the discovery of potential interaction sites on an isolated protein without knowledge of the structure of its partner or complex <sup>195</sup>. Therefore, it is helpful to predict protein interaction sites from sequences to direct experiments like site-specific mutagenesis, two-hybrid systems, or the synthesis of inhibitors <sup>195</sup>.

The above mentioned computational methods are crucial in the design and discovery of novel drugs. In all three of the aforementioned techniques, the method that suits simple and known compounds to predict their activity in certain proteins is the SBDD approach. SBDD does not only rely on the mining of chemical structures (which can give complex structures that may be almost impossible to synthesize) like the sequence-based and LBDD. Prior knowledge of the backbone structure is supported by the SBDD which gives freedom to modify the backbone structure with any compound or groups of interest. However, with all this in mind, side effects and the half-life of drugs in the physiological environment are still lingering issues. A need arises to supplement this knowledge of designed compounds with drug delivery systems as these can significantly dampen the aforesaid challenges. With this in mind, the section below considers in detail the different kinds of drug delivery systems available for use.

## **2.4 Drug delivery systems (DDS)**

The desired pharmacological effect of drugs has been limited over the years, due to the lack of specificity and inability of the drug to reach the target site. Due to these challenges, new ways to deliver drugs to the disease site have been explored. It has been determined that the bioavailability of the drug is compromised by the inability of the drug to reach the target site with the administered dosage <sup>196</sup>. Below is the mechanism of drug delivery systems (DDS).

### 2.4.1 Mechanism of DDS

Delivery techniques have significantly aided in the transformation of potential drugs into effective therapies<sup>197–199</sup>. Drug delivery strategies and technology quickly evolved to accommodate changing requirements for drug delivery as the therapeutic landscape evolved<sup>200</sup>. Drug delivery systems (DDS), which include particulate carriers mostly made of lipids and/or polymers and their associated treatments, can improve many of the pharmacological properties of conventional ("free") drugs<sup>197</sup>. Drug delivery systems (DDS) are formulated to modify the pharmacokinetics (PK) and biodistribution (BD) of the drugs they carry, either by acting as drug reservoirs (for sustained release) or by affecting both parameters simultaneously<sup>197</sup>.

Delivery efforts first focused on enhancing the drug's solubility, regulating their release, increasing their activity, and modifying their pharmacokinetics (PK) because the delivery of small molecules is mostly determined by their physicochemical properties, substantially influencing the bioavailability of the drugs<sup>201,202</sup>. However, major challenges with target-specific delivery, tonic effectiveness, *in vivo* instability, poor bioavailability, poor solubility, poor absorption in the body, and adverse drug effects arise when large-sized materials are used in drug delivery<sup>203</sup>. Therefore, adopting innovative drug delivery systems to direct drugs into specific body areas may be an alternative solution to address these serious challenges<sup>204,205</sup>. As a result, nanotechnology has a big part to play in modern drug formulations that successfully target specific areas of the body and release regulated amounts of drugs into those areas<sup>203</sup>.

According to reports, nanostructures can prevent drugs from degrading in the gastrointestinal tract, and they aid drugs to bypass the liver, thereby preventing the first-pass metabolism, and enabling targeted drug delivery to different parts of the body<sup>15,197,203,206–208</sup>. Because of their unique absorption mechanisms including absorptive endocytosis and the ability to stay in the blood circulation for a long period, nanotechnology boosts the oral bioavailability of drugs<sup>203</sup>. This results in less drug interaction with the mucus barriers and thereby decrease adverse side effects<sup>209</sup>. According to Emeje and co-workers, the uptake of nanostructures is 15–250 times greater than that of microparticles in the 1–10  $\mu\text{m}$  range<sup>210</sup>.

Therefore due to the aforesaid reasons, there is an emerging rise in nanocarriers research in the biomedical field. Section 2.4.2 below gives an overview of the types and functions of nanocarriers for drug delivery purposes.

## 2.4.2 Nanocarriers

One thing remains consistent among the many ways that nanoscience has been defined in books, journals, and on the internet in that, nanoscience entails the study of the control of matter at the atomic and molecular level <sup>211</sup>. Molecular and nano level research is often done at a range of less than 100 nm in at least one dimension <sup>212</sup>. Although the first uses of nanomaterial were in the fields of biology, chemistry, mechanics, electrical engineering, and magnetism, current focus has been on their use in pharmacy, particularly in the field of drug delivery <sup>213</sup>. Applying nanostructures and nano-phases to various scientific fields has demonstrated how nanotechnology can overcome the divisions between biological and physical sciences <sup>206</sup>; especially in nanomedicine and nano-based drug delivery systems, where such particles are considered extremely important and are of great research interest <sup>206,207</sup>. The currently used drug delivery systems include molecular targeted therapy, liposomes, micelles, polymeric nanoparticles, nanoparticles, etc. Amongst the studied drug delivery systems, nanocarriers have been the subject of interest because of the interesting properties they exhibit in the nano-size regime. Nanocarrier properties of interest include a high surface-to-volume ratio, easy modification for site-specific purposes, and the ability to penetrate through the leaky vasculatures of tumors <sup>18,19,214</sup>.

However, adverse side effects of metallic-based, carbon-based, protein-based, and liposomal-based nanoparticles in drug delivery applications have also been reported. A review of the properties and toxicity traits possessed by these different nanocarriers is worthy of consideration and it is attempted in the following subsections.

### 2.4.2.1 Carbon-based nanoparticles (CNPs)

Nanomaterials made of carbon are incredible scientific advancements with distinct characteristics (high mechanical strength, high conductivity, attractive optical properties, chemical versatility, etc.) <sup>215</sup>. The most often employed materials in chemical analysis among them are graphene and carbon nanotubes <sup>210</sup>. Due to their widespread use in cancer therapy, carbon nanotubes (CNTs) have attracted a lot of attention among the many nanocarriers <sup>216</sup>. The CNTs are rolled-up, hollow tubes made of seamless cylindrical graphene nanostructures that are sealed at both ends by hemispherical fullerene caps <sup>217</sup>. CNTs are classified in two types; the single-walled carbon nanotubes (SWCNT) and the multi-walled carbon nanotubes (MWCNT) (Fig. 2.18) <sup>216,218,219</sup>. Researchers are concerned about the utilization of CNT's advanced physical, mechanical, and chemical properties in drug delivery systems <sup>220</sup>. This is

due to CNTs ability to traverse cell membranes while commuting the carrier biomolecule <sup>220</sup>. As a result of the toxicity concerns, a plethora of studies looking into CNTs toxicity traits were conducted. According to these studies, CNTs can have harmful effects to the nervous system, the lungs, the immunological system, the embryo, the liver, the heart, and the blood vessels <sup>217,221–226</sup>.

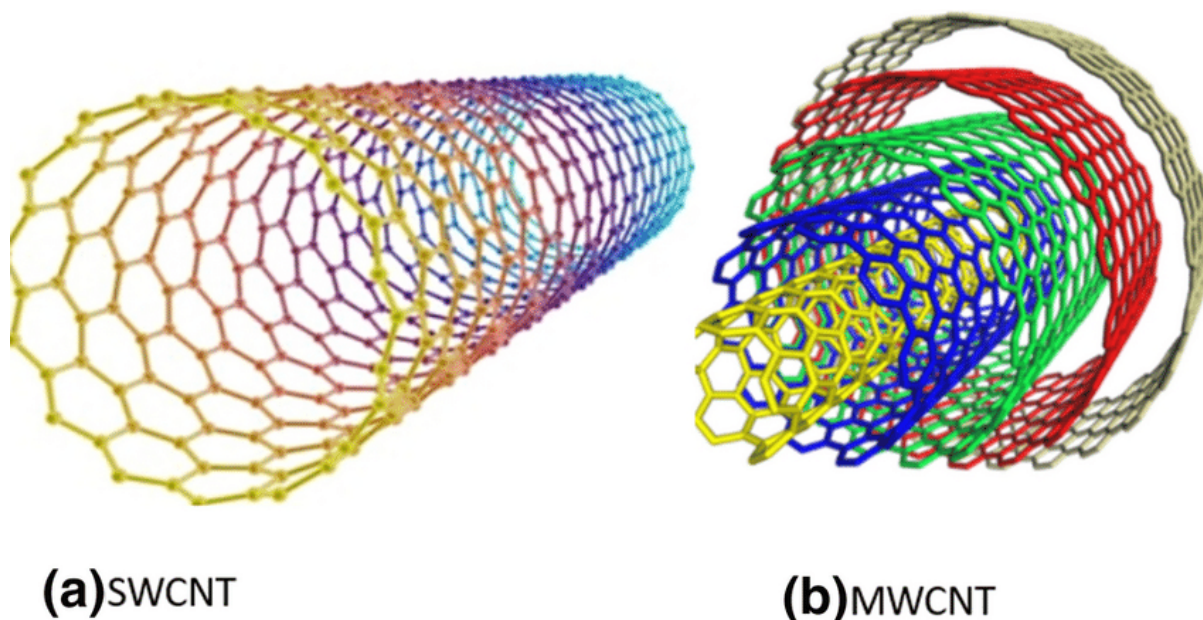


**Figure 2.18:** The structures illustrating (a) single-walled carbon nanotubes and (b) multi-walled carbon nanotubes. Adopted from Devi et al.<sup>117</sup>.

#### 2.4.2.2 Metallic-based nanoparticles

Nanoparticles in their different form and makeups generally present with different physicochemical properties (shapes, sizes, charge, and aggregation) as illustrated in Fig. 2.19. Metallic-based nanomaterials have been extensively explored in several fields because of their inherent small and modifiable sizes. However, the pros and cons of small-sized materials are significant in biological applications. Due to their diminutive size, NPs can enter the human body, pass through numerous biological barriers, and possibly even reach the most sensitive organs <sup>227,228</sup>. There are four main processes in the synthesis of metallic nanoparticles: gas phase, vapor deposition, colloidal formation, and attrition <sup>227</sup>. Each of these steps carries the risk of toxicity when the skin, lungs, or gastrointestinal tract are exposed to these materials <sup>227</sup>. These metallic-based or rather inorganic-based nanomaterials include but are not limited to metal oxides (iron oxide, cerium oxide, titanium dioxide, silicon dioxide), metals (gold and silver), and semiconductor NPs known as quantum dots (size range of 1-20 nm) <sup>229</sup>. Therefore,

alternative to these nanomaterials could be achieved through the use of polymeric nanomaterials.

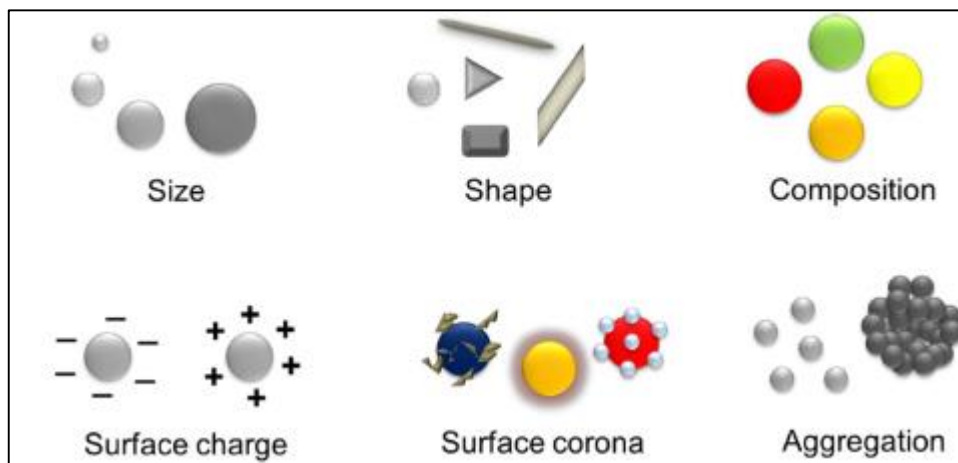


**Figure 2. 19:** Schematic illustration of key physicochemical characteristics of nanomaterials that affect their potential for use in biomedicine. Adopted from Navya and co-workers <sup>229</sup>.

#### 2.4.2.3 Polymeric nanomaterials

Polymers are one of the most studied large molecules composed of repeating structural units called monomers, which are covalently bonded together to form long chains or networks <sup>230</sup>. These monomers can have any structure as long as they have at least two functional groups that can interact with another monomer <sup>230</sup>. Ideally, a polymer may be prepared to have particular properties by choosing the appropriate monomer or monomers. Polymers became important molecules as NP precursors for drug delivery systems because they are versatile macromolecules that could be easily modified to meet specific properties, and some of their molecular degradation produces biocompatible metabolites <sup>231</sup>. However, unlike the metallic-based and carbon-based nanoparticles, the polymeric colloidal nanoparticle has a size range of 10-1000 nm, having either a spherical, branched, or core-shell morphology <sup>232</sup>. Furthermore, polymeric NPs are usually fabricated from biodegradable monomers which render them non-toxic in comparison to the aforementioned nanoparticles.

Polymeric nanoparticles offer advantages such as biocompatibility, biodegradability, and stimuli-responsive abilities <sup>232</sup>. The stimuli-responsiveness is attained by incorporating certain functionalities to modulate the responsiveness of these nanostructures to specific stimuli through the use of remote activation <sup>233</sup>. Stimuli-sensitive polymers are widely studied because

they enhance the movement of the therapeutic drug in the body, thereby assisting in the targeted and sustained release of the drug <sup>234</sup>. Examples of biodegradable polymers include synthetic polymers such as poly (lactide-co-glycolide) copolymers (PLGA), poly (lactide) (PLA), poly ( $\epsilon$ -caprolactone) (PCL), poly (amino acids) and poly N-isopropyl acrylamide (PNIPAM) in addition to natural polymers such as gelatin, albumin, chitosan, and alginate <sup>233</sup>.

In drug delivery systems, the size, shape, and surface properties of the polymer play a key role in the *in vivo* cellular absorption and biodistribution of the polymer nanoparticles <sup>235</sup>. This is in addition to the polymer's biodegradability. Size and surface functionality of the particle are both highly correlated with the opsonization and subsequent macrophage recognition <sup>236</sup>. Elsabahy and Wooley<sup>233</sup> deliberate on the traits of NPs involved in the design and development of nanodrugs and this is demonstrated in Figure 2.20.

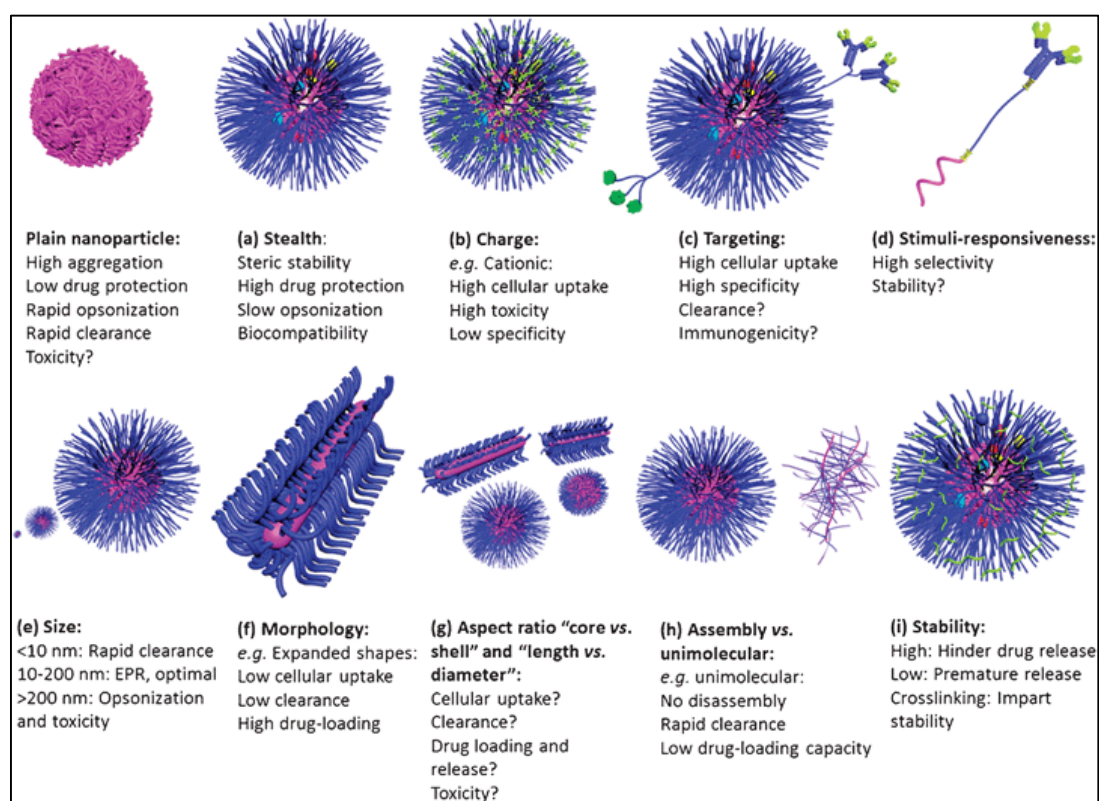


**Figure 2.20:** Physicochemical properties of ideal PNPs and their characteristics based on size, surface and morphology. Adopted from Elsabahy and Wooley <sup>233</sup>.

Based on the traits (of PNPs) shown in Figure 2.20, an ideal polymeric nanoparticle would have stealth, small size, and stimuli-responsiveness, the latter of which will serve as the

foundation for this work. Smaller size polymeric nanoparticles (< 200 nm) have been shown to have escaped recognition from the reticuloendothelial system (RES) and thus prolonged the half-life of the NPs in the bloodstream<sup>235</sup>. However, particles less than 100 nm were found to be ineffective at times since they are easily cleared by the reticuloendothelial system (RES), thus reducing the uptake efficiency because of the reduced circulation time<sup>237</sup>.

Several reports have shown the importance of smart/responsive polymeric nanoparticles. Srividya and co-workers<sup>238</sup> copolymerized polyacrylic acid (Carbopol® 940) and hydroxypropylmethylcellulose (Methocel E50LV) to form a pH-triggered *in-situ* gelling system. The system formulation was therapeutically efficacious since it provided a sustained release of the drug over an 8 h period<sup>238</sup>. In contrast to this study was a study done by Yang and co-workers, who designed novel pH-responsive paclitaxel (PTX)-loaded poly (ethylene glycol)-phenylhydrazone-dilaurate (PEG-BHyd-dC12) micelles for *in vitro* anticancer activity. Their results showed that as the pH decreased from 7.4 to 5.5, there was a fast or premature release of the bound paclitaxel and this was not an ideal situation since a nanocarrier has to sustainably release a drug over time not instantly to improve the bioavailability of the drug<sup>239</sup>. However, a study by Jin and co-workers on the dual temperature- and pH-responsive ibuprofen delivery from poly(N-isopropylacrylamide-co-acrylic acid) nanoparticles and their fractal features. The release behaviors of IBU-loaded copolymers exhibited high responsiveness to temperature and pH values over 24 hours at 37 °C using pH of 7.4<sup>240</sup>.

Amongst the stimuli responses studied, either physical (temperature, electric field, magnetic field, or ultrasound) or chemical (redox, enzyme, ionic and pH) responsive properties, chemical or more specifically pH-responsive nanomaterials have gained popularity. pH-response ability is achieved mostly by using an ionizable pendant group<sup>240</sup>. The process/mechanism of pH-responsive nanomaterials on the cancer cells is illustrated in Fig. 2.21. The below scheme shows that, by mediating both passive and active targeting, pH-responsive nanoparticles can accumulate in tumor tissue due to enhanced permeability and retention effects (EPR)<sup>241</sup>. The tumor microenvironment (TME), which has an acidic pH between 5.5 and 6.8, causes physicochemical changes in the nanoparticles (swelling, solubility changes, charge changes, and cleavage of covalent bonds), to effectively release drugs into the TME<sup>241</sup>.



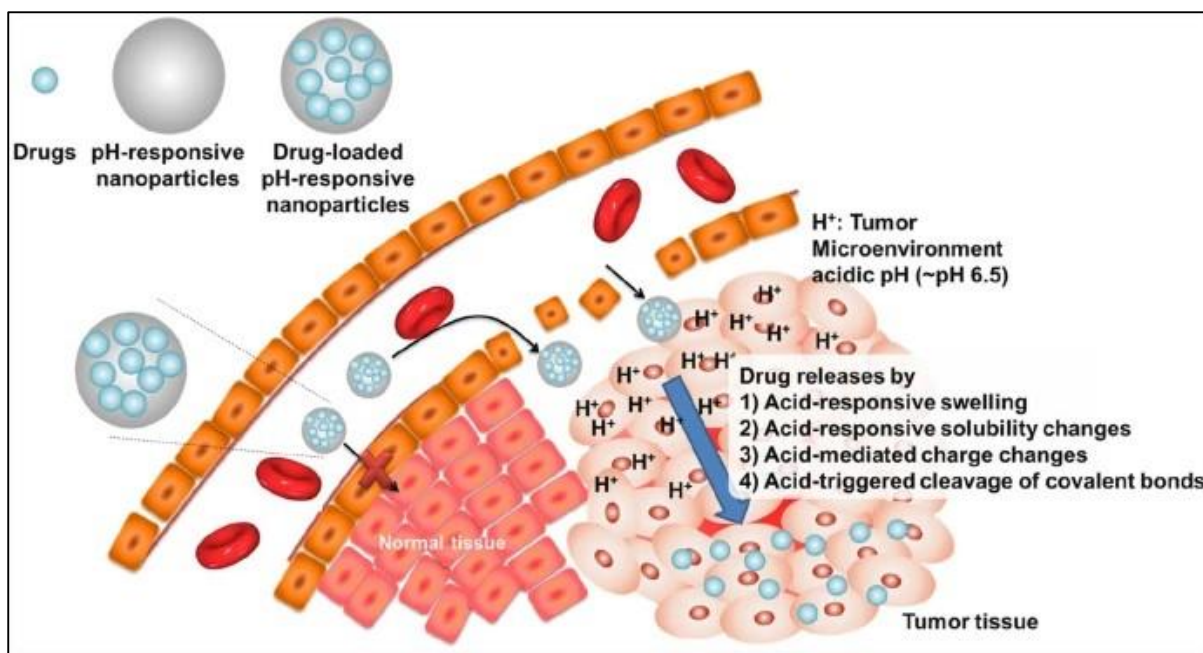


**Figure 2.21:** Schematic diagram of nanoparticles that are responsive to acidic pH of the tumor tissue <sup>241</sup>.

#### 2.4.2.3.1 Poly(*N*-Isopropylacrylamide) (PNIPAM)

The selected polymer or monomer for this study was *N*-isopropyl acrylamide (NIPAM). Poly(NIPAM) (PNIPAM) is one of the most studied thermo-reversible polymers with gelation properties in aqueous solutions, it has gelling temperatures ranging between 32-35 °C and reverts into a solution upon cooling <sup>240,242–247</sup>. Changing the temperature below or above a critical value, sometimes referred to as the lower critical solution temperature (LCST), causes the hydrophilic/hydrophobic condition to be reversible <sup>247</sup>. PNIPAM undergoes a phase transition when heated above its LCST and it changes from a soluble hydrated state to an insoluble dehydrated state <sup>247</sup>. Although this phase transition is often thought to occur at 32 °C, the actual temperatures can vary by 5 to 10 degrees Celsius (or even more) depending on the polymer concentration, molar mass of the polymer chains, polymer dispersity, and terminal moieties <sup>248</sup>.

Many researchers have looked into PNIPAM copolymers for potential uses in tissue engineering and controlled drug delivery, since PNIPAM expels its liquid contents at a temperature close to that of the human body <sup>249,250</sup>. In addition, PNIPAM can be further tuned or modified due to its readily functionalized structure. Functionalization with ionizable functional groups would render PNIPAM copolymers with pH-responsive abilities.



## 2.5 Conclusion and present work

The problem faced by patients in hospitals is serious because in hospitals patients are admitted with one disease and leave with other ailments or more severe secondary diseases. This is possible for patients receiving chemotherapy or other cancer-related treatment modalities because of their immunocompromised status. However, the problem can be said to start from the drugs used to treat these diseases, because they may cause more harm to the patient subsequently leading to an immunocompromised state and susceptibility to secondary opportunistic infections (contracted at the hospital settings). Therefore, the search for drugs or medications that can ease one or more of the aforementioned challenges would go a long way in addressing the survival of cancer patients. In this study, we designed compounds that are derivatives of beneficial compounds from plant materials to ensure the safety of the drugs themselves, and also we used computational methods to determine the predicted bioavailability and toxicity of these materials before any *in vitro* work to ensure that the foundation is laid on toxicity free materials. Furthermore, we employed polymeric nanoparticles that respond to the acidic microenvironment of the cancer cells for the selective release of these designed compounds.

.

## **Chapter 3: Aims and objectives, thesis outline and research design**

---

### **3.1 Aims and Objectives**

#### **3.1.1 Aims**

- i. To design, synthesize, and characterize hydroxybenzoic acid amide and ester derivatives as well as the corresponding polymer nanocomposites.
- ii. To determine the antimicrobial and anticancer activity of prepared compounds, and compound-polymer composites using in vitro studies.

#### **3.1.2 Objectives**

- To synthesize novel amide and ester derivatives of hydroxybenzoic acid.
- To characterize these derivatives by Fourier-Transform Infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR), and Mass spectrometry (MS).
- To synthesize pH-sensitive stimuli polymeric nanoparticles (PNPs).
- To characterize these PNPs by transmission electron microscopy (TEM) and dynamic light scattering (DLS).
- To load the synthesized hydroxybenzoic acid derivatives onto the prepared PNPs and characterize the resulting nanocomposites by TEM and DLS.
- To carry out cytotoxicity studies on the prepared derivatives, polymer-based nanoparticles, and corresponding nano-composites on pancreatic cancer cell line (Mia Paca-2) and Human Embryonic Kidney cells (HEK 293T).
- To conduct antimicrobial activity in vitro studies on the derivatives, polymer-based nanoparticles, and corresponding nano-composites.

### **3.2 Thesis outline**

**Chapter 1** provides an overview of cancer and microbial infections that often occur in immunocompromised cancer patients. It outlines the prevalence of cancer in South Africa and briefly introduces cancer-targeted therapy. Additionally, it introduces one of the main targets of the study, the DNA topoisomerase enzyme.

**Chapter 2** delves into the intricacies of cancer formation, discussing its global prevalence and the shortcomings of existing treatment modalities. It explores the concept of cancer-targeted therapy, particularly focusing on DNA topoisomerase I, as a primary target, along with the current inhibitor's mechanism of action and limitations. Furthermore, it highlights the potential of hydroxybenzoic acid derivatives as candidate compounds due to their known anticancer and antimicrobial properties, among other biological applications. The chapter also addresses microbial infections originating from hospital settings, reviewing identified pathogens, their inhibitors, and associated challenges with drug agents. In addition, it discusses the role of in-silico drug design in lead compound identification, emphasizing its efficiency in reducing time, effort, and costs. To mitigate compound toxicity, the chapter examines the use of drug delivery systems, particularly polymeric nanoparticles, which enhance drug bioavailability and target-specific delivery.

**Chapter 3** provides the project's framework and design, effectively outlining its scope through the aims and objectives.

**Chapter 4** presents the methodologies, findings, and discussions of the in-silico study, organized by each protein target. Moreover, it effectively demonstrates the potential inhibition of all the designed compounds.

**Chapter 5** details the synthesis of newly designed ester and amide derivatives of hydroxybenzoic acid, followed by their characterization using NMR, FTIR, and MS techniques. These analyses provide insight into the structure of the designed compounds.

**Chapter 6** delves into the synthesis, characterization, and loading of the designed compounds onto the synthesized polymeric nanomaterials. The polymer nanomaterial and its conjugates underwent characterization using TEM and DLS. It's important to note that SEM analysis and drug entrapment efficiency studies were not feasible for this study due to the unique properties of the novel-designed polymeric nanomaterial. The resulting polymer nanoparticles exhibited a gel-like consistent texture that was resistant to drying even with lyophilization. Despite this, TEM and DLS analyses confirmed the formation of small nanoparticles within the nano range, validating the creation of the polymeric nanoparticles.

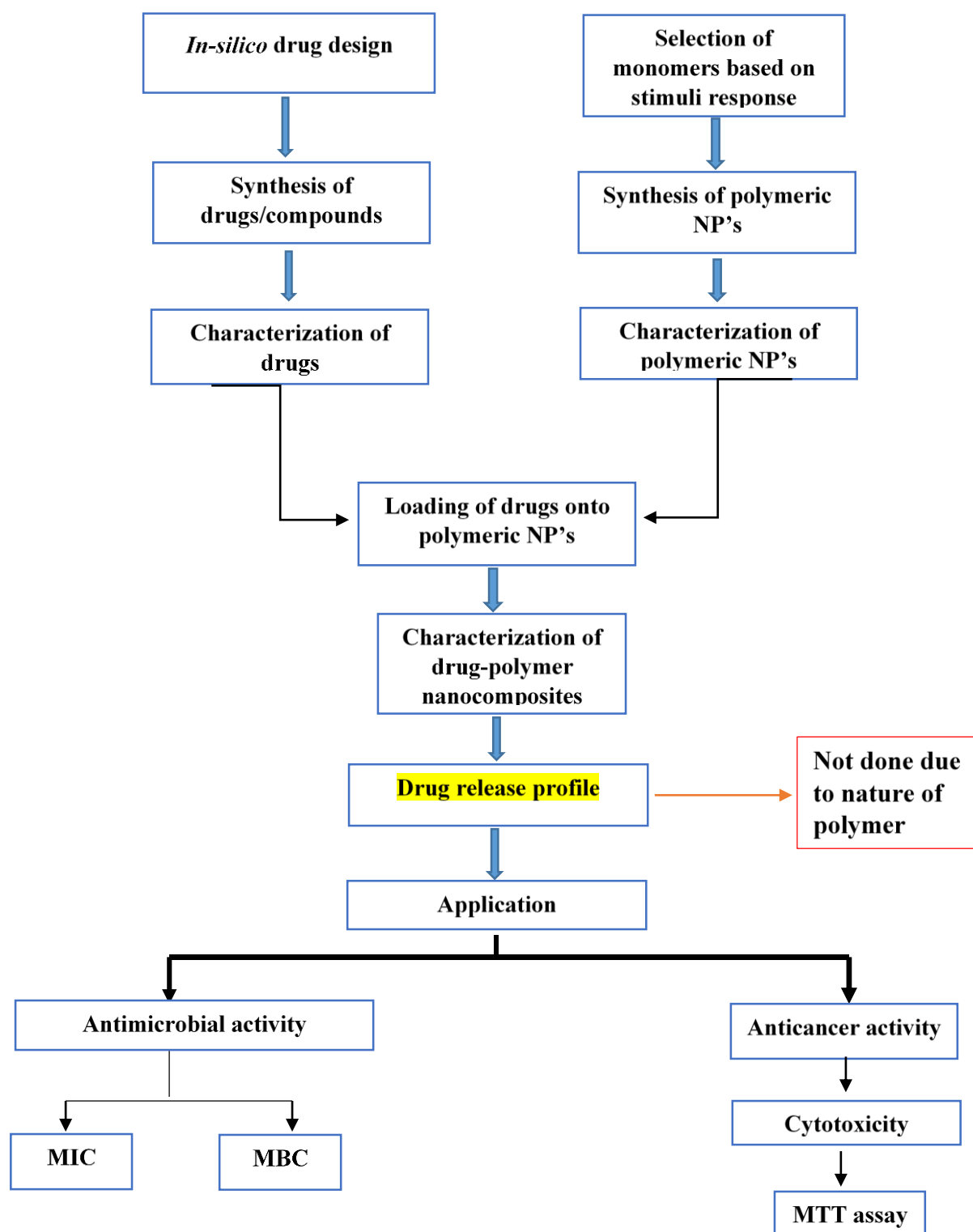
**In Chapter 7**, the antimicrobial activity of the designed compounds is investigated both with and without loading onto the polymeric nanomaterials. Loading onto the nanomaterials was exclusively performed for compounds demonstrating notable antimicrobial or anticancer activity.

**Chapter 8** presents the results of the in-vitro cytotoxicity evaluation of the designed compounds, both with and without loading onto the polymeric nanomaterials. It was observed that the loaded compounds exhibited superior activity compared to their unloaded counterparts.

**Chapter 9** gives a detailed general discussion of the study.

**Chapter 10** This chapter concludes the study, outlines the limitations, and give future recommendations.

### 3.3 Study design



## Chapter 4: *In-silico* drug design

---

### 4.1 Introduction

Drug development is increasingly relying on computer-aided drug design (CADD) techniques, which are essential for efficiently identifying viable drug candidates <sup>251</sup>. These computational techniques are important for reducing the use of animal models in pharmacological research, assisting in the rational design of novel and safe drug candidates, and repurposing already-marketed medications, thus supporting medicinal chemists and pharmacologists throughout the drug discovery process <sup>251</sup>. Due to their potential to speed up the process of drug discovery in terms of time, labour, and cost, *in-silico* techniques have recently attracted a lot of attention <sup>252</sup>. CADD has addressed the attrition rate of the drug candidates which was reported to be as high as 96% <sup>253</sup>. Poor drug effectiveness, insufficient drug distribution, metabolism, and excretion, as well as toxicity (ADME-Tox), are the causes of this high attrition rate <sup>254</sup>. The CADD affords the virtual testing of ADME-Tox properties using sophisticated computer programs, which drastically reduces the attrition rate and also saves time and cost by reducing the amount of *in-vitro* and *in-vivo* tests to be undertaken to obtain safe and bioavailable lead compounds.

However, with all these interesting developments, conditions to perform these tasks still play an important role in the success of the CADD approach, in this case, the structure-based drug design that was previously discussed plays a pivotal role. Identification of target protein is one of the important factors that contribute immensely to the identification of lead compounds. In this study, the proteins of interest were DNA topoisomerase I and ATP-binding cassette transporter G2 (ABCG2) protein. DNA topoisomerases are important enzymes and comprise the therapeutic target in the treatment of cancer <sup>255</sup>. This is because they influence the *in vivo* DNA topological state by cleaving and removing DNA strands in a controlled manner <sup>256</sup>. To solve topological issues caused by DNA metabolism and chromosome decatenation during cell division, topoisomerase enzymes temporarily break the DNA molecule's single or double strands <sup>257–259</sup>. The above mentioned enzyme is crucial for cellular functions such as DNA transcription, replication, recombination, and DNA repair <sup>260</sup>. The focus of this study is on DNA topoisomerase I (DNA Top 1) enzyme, which temporarily break a single strand during DNA metabolic processes. DNA Top 1 is a valuable pharmacological target for the production of potent anticancer and antibacterial drugs due to its crucial function in DNA metabolic

functions<sup>261,262</sup>. With encouraging results, camptothecin (CPT) and its derivatives (topotecan and irinotecan) have been utilized as DNA topoisomerase I inhibitors, and their mechanism of action (MOA) has been thoroughly investigated<sup>91,263</sup>. However, due to cancer cells' acquired resistance to camptothecin and its analogues, their use as DNA Top 1 inhibitors is mostly limited<sup>75,264</sup>.

The ATP-binding cassette transporters (ABC transporters) superfamily's efflux pumps are mainly responsible for the multidrug resistance of cancer cells<sup>265–267</sup>. The plasma membrane protein ATP-binding cassette transporter G2 (ABCG2), also known as breast cancer resistance protein (BCRP), is regarded as one of the most prominent drug resistance factors<sup>267,268</sup>. Moreover, ADMET (absorption, distribution, metabolism, excretion, and toxicity) features are thought to be caused by ABC transporters<sup>268</sup>. Additionally, ABCG2 is directly responsible for detoxifying and protecting cells from cytotoxic agents by effluxing xenobiotics<sup>265,269</sup>. The overexpression of ABCG2 renders cancer cells resistant to several chemotherapy treatments<sup>269</sup>. The most recent information indicates that no approved drugs could counter ABCG2-mediated multidrug resistance<sup>269</sup>. Therefore, inhibiting the ABCG2 transporter is essential for treating cancer in humans. Hence, to combat the multidrug resistance that is present with the market's prescribed drugs, it is necessary to design new compounds with increased potency. Figures 4.1 and 4.2 show the designed amide and ester compounds that are used in this study.

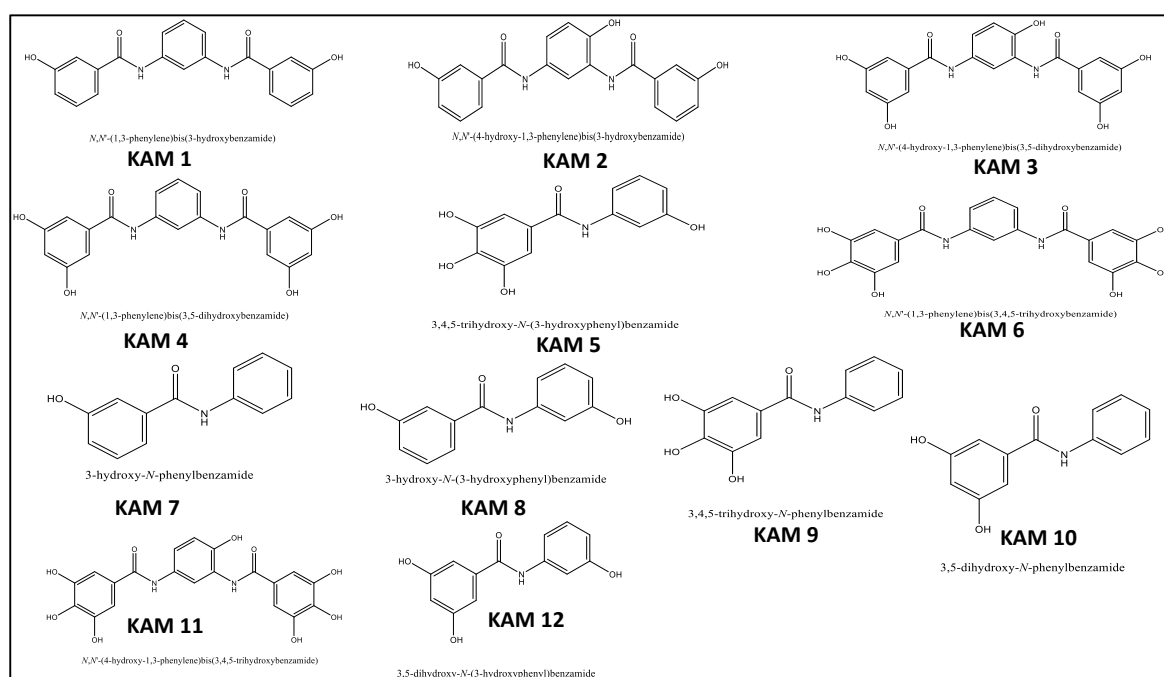


**Figure 4. 1:** 2D structures of the designed hydroxybenzoic acid amide derivative compounds.

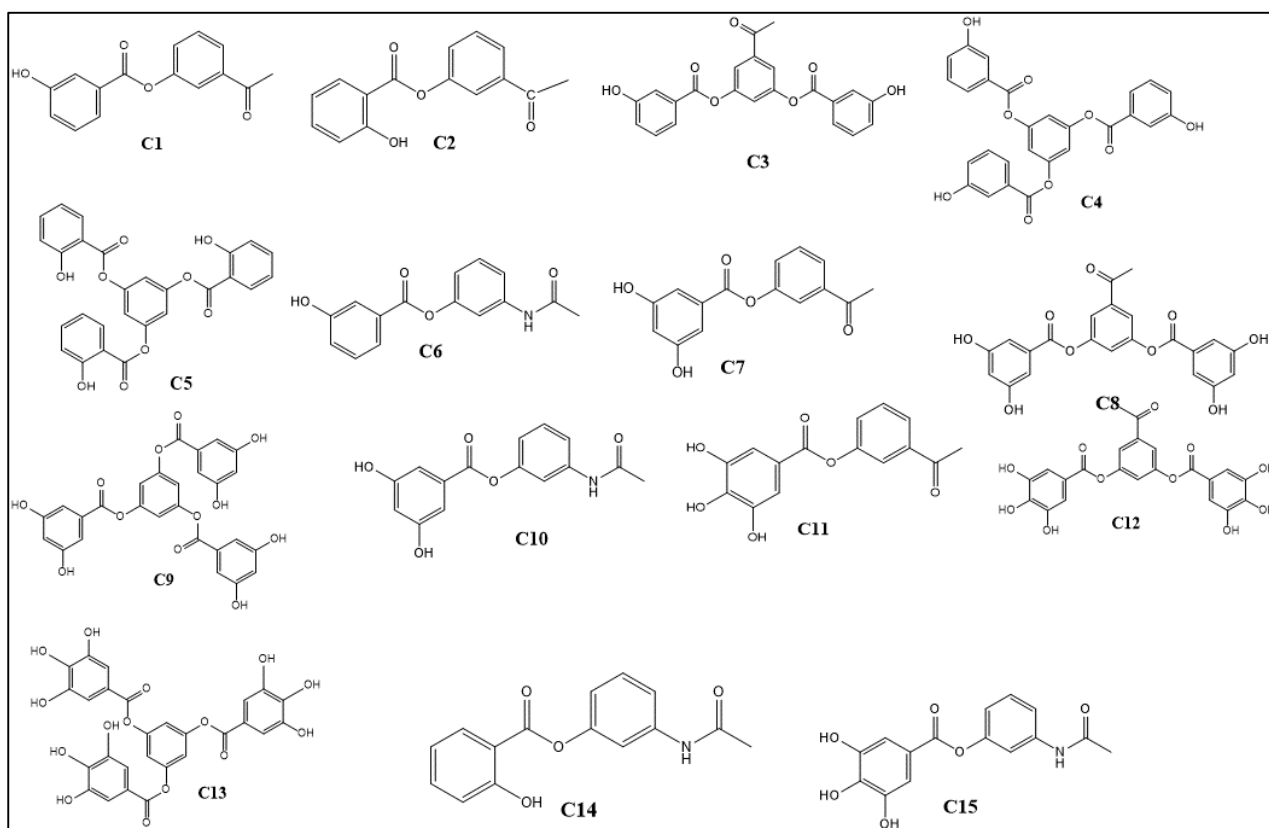


**Figure 4. 2:** 2D structures of the designed hydroxybenzoic acid ester derivative compounds.

## 4.2 Computational methods

### 4.2.1 Tools used

Bioinformatics tools: ChemDraw Professional 16.0 was used for drawing the designed compounds, while the proteins used in this study were obtained from RCSB PDB (Research Collaboratory for Structural Bioinformatics Protein Data Bank). Molecular Operating Environment (MOE v2015) software was used to prepare both the ligand and protein and for docking purposes. MOE was also used to obtain the 2D protein-ligand interactions. Molecular dynamics simulations were performed by CHARMM36 force field and NAMD package version 2.13. OSIRIS property explorer was used for toxicity and drug score determination, while molinspiration was used to obtain information related to drug-likeness. SwissADME was used to predict the physicochemical properties of the designed compounds. Gaussian 9.0 was used to calculate the density functional theory (DFT) parameter values. GaussView 6.0 was used to visualize the frontier molecular orbitals.



#### **4.2.2 Preparation of ligands and proteins**

Structure optimization of the compounds that were used as anticancer agents was performed by Gaussian 09 program package using the density functional theory with B3LYP and the 6-311++G(d,p) basis sets. The compounds were protonated, and energy was minimized using the default parameters of the Molecular Operating Environment (MOE v2015) software (gradient: 0.05, Force Field: MMFF94X). Protonation of the compounds was performed to ensure that they are in their most stable ionic forms, which is essential for accurate energy minimization. In molecular modeling, protonation states can significantly affect the structure and interactions of molecules. By protonating the compounds, the simulation can accurately reflect the physiological conditions under which these compounds would exist, leading to more reliable results in the subsequent energy minimization process.

The structural coordinates for Multidrug Transporter ABCG2 and DNA Topoisomerase I bound to topotecan were retrieved from the protein databank (PDB code; 7NEZ and 1K4T respectively). Hydrogens and Gasteiger charges were added, and all the hetero-atoms and water molecules were removed from the protein structure. The protonated protein was initially optimized to remove all the unwanted steric clashes using the AMBER99 force field.

#### **Protein preparation and membrane environment consideration**

The protein, Multidrug Transporter ABCG2 (PDB ID: 7NEZ), was selected for the computational studies. ABCG2 is a membrane protein, and its interactions with the lipid bilayer are essential for its structural integrity and function. To accurately model these interactions, the membrane environment was explicitly considered during the docking and molecular dynamics (MD) simulations.

#### **4.2.3 Molecular docking**

Refined structures were used for the docking study using the default parameters of MOE; Placement: Triangle Matcher, rescoring 1: London dG, Refinement: Forcefield, Rescoring 2: GBVI/WSA. Before running the docking protocol, a total of ten conformations for the ligand were selected. The top-ranked conformations based on docking score were selected for protein-ligand interaction (PLI) analysis. Protein-ligand binding free energy using Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) was calculated as the difference between the energy of the bound complex and the energy of the unbound protein and inhibitor compound. The method allows for free energy decomposition into contributions originating

from different types of physico-chemical interactions. Specifically, the energy is calculated for the protein–ligand complex, the ligand, and the protein, and their energies were computed using the CHARMM force field with the generalized Born implicit solvent model, in order to calculate the averaged binding free energy ( $\Delta G$ ) according to the following equation:

$$\Delta G_{binding} = \Delta E_{MM} + \Delta G_{solv} - T\Delta S$$

Where  $\Delta E_{MM}$  includes  $\Delta E_{internal}$  (bond, angle, and dihedral energies),  $\Delta E_{elect}$  (electrostatic), and  $\Delta E_{vdw}$  (van der Waals) energies;  $\Delta G_{solv}$  is the electrostatic solvation energy (polar and non-polar contributions).

#### 4.2.4 Molecular dynamic simulations

Simulations were conducted in a periodic water box for 100 ns using the CHARMM36 force field and NAMD package version 2.13. The force field for ligands was generated from the CHARMM-GUI server. The water box (including 150 mM NaCl) was created by adding water for 20 Å in the positive and negative x, y, and z directions around the protein, yielding a cuboidal box. LJ cutoff was defined at a distance of 12 Å, with a switching distance of 10 Å. Long-range electrostatic interactions were handled using the particle mesh Ewald (PME) method. Before the production run, the systems were minimized for 5000 steps using a conjugate gradient algorithm. The simulations were performed in an NPT ensemble; the temperature and the pressure of the system were fixed at 300 K and 1 bar, respectively, using a Langevin thermostat and barostat. Root mean square deviation (RMSD), root mean square fluctuation (RMSF), and number of intermolecular hydrogen bonds (H-bonds) were chosen as parameters to analyze MD trajectories between the protein and compound.

#### 4.2.5 Absorption, distribution, metabolism, excretion (ADME)/Toxicity properties

*In silico* toxicity risk assessments on the designed compounds were conducted to determine mutagenic, tumorigenic, irritant, and reproductive effects along with fragment-based drug-likeness and the overall drug score of hydroxybenzoic acid derivatives by using the OSIRIS property explorer program ([www.organicchemistry.org/prog/peo/](http://www.organicchemistry.org/prog/peo/)). The bioactivity properties of all the designed 27 compounds were predicted using the Molinspiration online server tool ([www.molinspiration.com](http://www.molinspiration.com)). Furthermore, the physicochemical properties were predicted by using a robust SwissADME server. Compounds were then docked against DNA topoisomerase I and ABCG2 proteins.

#### 4.2.6 Density Functional Theory (DFT) calculations

The molecular structures of the compounds that showed consistently high binding affinities, KAM 8, KAM 12, KAM 5, KAM 1, KAM 4, and KAM 6 for amide compounds and C8, C12, C4, C9 and C13 for ester compounds were optimized using DFT calculations. The level of theory used was B3LYP (Becke's, 3-parameter, Lee–Yang–Parr) correlation with a standard 6-31++G(d,p) basis set, these levels were implemented on Gaussian 09 software on default criteria. The data obtained was visualized on GausView 6.0 program under the DFT-B3LYP/6-31++G (d,p) methods.

#### 4.3 Results

A web-based system called OSIRIS Property Explorer (OPE) was used to determine drug attributes such as toxicity risk assessment, predictions of fragment-based drug-likeness, and overall drug-score<sup>270</sup>. The molinspiration predictions on the other hand, show possible bioactivity against the outline targets. Table 4.1 shows results of amide compounds (mono and di-substituted amides). OSIRIS predictions, showed that all the amide compounds were non-toxic since there were no traits of any toxicity from the designed compounds. On the other hand, Fig. 4.2 shows results for ester compounds that were also similar to amide compounds in terms of the non-toxic predictions. Compounds KAM 1, KAM 2, KAM 5, KAM 6, KAM 8, KAM 9, and KAM 11 showed a positive drug-likeness (DL) scores, indicative of fragments belonging to known moieties of known drugs or Fluka's database recognised compounds. Furthermore, compound KAM 5 and KAM 6 showed a high DL score and drug score (DS) suggestive of possible bioavailability. However, KAM 5 showed a negative values on druggable targets which is in contrast to the high drug-likeness value. While KAM 6 showed positive value for predicted kinase inhibition (KI), which also showed positive values for compounds KAM 1-KAM4. Hinting, on possible activity against one of the cancer signalling pathways.

**Table 4. 1:** Toxicity and bioavailability properties of the designed HBA amide derivatives.

| Compound | OSIRIS Prediction |    |    |    |             |      | Molinspiration Prediction |       |             |       |       |       |
|----------|-------------------|----|----|----|-------------|------|---------------------------|-------|-------------|-------|-------|-------|
| Name     | ME                | TE | IE | RE | DL          | DS   | GL                        | ICM   | KI          | NRL   | PI    | EI    |
| KAM 1    | -                 | -  | -  | -  | <b>0.03</b> | 0.56 | -0.05                     | -0.15 | <b>0.05</b> | -0.08 | -0.05 | -0.06 |
| KAM 2    | -                 | -  | -  | -  | <b>0.33</b> | 0.61 | -0.07                     | -0.28 | <b>0.03</b> | -0.13 | -0.13 | -0.9  |
| KAM 3    | -                 | -  | -  | -  | -3.52       | 0.41 | -0.07                     | -0.28 | <b>0.04</b> | -0.10 | -0.14 | -0.08 |

|                              |   |   |   |   |             |             |              |       |             |       |       |             |
|------------------------------|---|---|---|---|-------------|-------------|--------------|-------|-------------|-------|-------|-------------|
| KAM 4                        | - | - | - | - | -3.94       | 0.4         | -0.05        | -0.14 | <b>0.06</b> | -0.07 | -0.06 | -0.05       |
| KAM 5                        | - | - | - | - | <b>3.07</b> | <b>0.92</b> | -0.21        | -0.17 | -0.03       | -0.26 | -0.31 | -0.04       |
| KAM 6                        | - | - | - | - | <b>2.95</b> | 0.79        | <b>-0.06</b> | -0.13 | <b>0.06</b> | -0.13 | -0.06 | -0.02       |
| KAM 7                        | - | - | - | - | -1.47       | 0.53        | -0.41        | -0.23 | -0.31       | 0.47  | -0.53 | -0.20       |
| KAM 8                        | - | - | - | - | <b>0.03</b> | 0.69        | -0.35        | -0.21 | -0.21       | -0.40 | -0.46 | -0.17       |
| KAM 9                        | - | - | - | - | <b>1.53</b> | 0.85        | -0.28        | -0.18 | -0.12       | -0.38 | -0.36 | -0.06       |
| KAM 10                       | - | - | - | - | -5.41       | 0.46        | -0.33        | -0.20 | -0.20       | -0.35 | -0.45 | -0.14       |
| KAM 11                       | - | - | - | - | <b>3.1</b>  | 0.79        | -0.08        | -0.26 | <b>0.04</b> | -0.16 | -0.13 | -0.05       |
| KAM12                        | - | - | - | - | -3.79       | 0.48        | -0.28        | -0.21 | -0.12       | -0.29 | -0.39 | -0.13       |
| <b>Topotecan</b><br><b>*</b> | - | - | - | - | <b>6.64</b> | 0.84        | 0.41         | -0.16 | <b>0.22</b> | -0.04 | -0.11 | <b>0.96</b> |

\*\*ME: Mutagenic effect, TE: Tumorigenic effect, IE: Irritant effect, RE: Reproductive effect, DL: Drug likeness, DS: Drug score, GL: GPCR ligand, ICM: Ion channel modulator, KI: Kinase inhibitor, NRL: Nuclear receptor ligand, PI: Protease inhibitor, EI: Enzyme inhibitor, Potential toxicants: - Non-toxic.

The ester compounds on the other hand, also showed non-toxic traits as previously stated. However, drug-likeness and drug scores were mostly showing lower negative values that signify the novelty of the designed compounds since they are no fragments that corresponds to the drugs in Fluka's company database and known drugs of this class. Moreover, the anonymity of these designed compounds could also pose a threat since there are no comparable known structures related to these compounds. The gallic acid and other hydroxybenzoic acid motifs are extensively studied, therefore the results somewhat contrary to well-known phytochemicals like gallic acid. Nevertheless, compounds C3, C4, C8, and C9 showed a positive value for nuclear receptor ligand (NRL) as shown in Fig. 4.2, another possible target for several diseases that includes cancer among others. These compounds showed a positive value in targets that topotecan shows negative values, suggesting versatility for use in other druggable targets.

After *in silico* toxicity screening it was important to screen the compounds for potential to be used as an oral drug. Therefore, Lipinski's rule of five was necessitated to determine the potential oral bioactivity.

**Table 4. 2:** Toxicity and bioavailability properties of the designed HBA ester derivatives.

| Compound                | OSIRIS Prediction |    |    |    |             |             | Molinspiration Prediction |       |             |             |       |             |
|-------------------------|-------------------|----|----|----|-------------|-------------|---------------------------|-------|-------------|-------------|-------|-------------|
| Name                    | ME                | TE | IE | RE | DL          | DS          | GL                        | ICM   | KI          | NRL         | PI    | EI          |
| C 1                     | -                 | -  | -  | -  | -3.59       | 0.44        | -0.38                     | -0.17 | -0.51       | -0.11       | -0.49 | -0.18       |
| C2                      | -                 | -  | -  | -  | -4.15       | 0.45        | -0.21                     | -0.11 | -0.42       | -0.23       | -0.51 | -0.42       |
| C3                      | -                 | -  | -  | -  | -9.67       | 0.32        | -0.16                     | -0.13 | -0.24       | <b>0.05</b> | -0.19 | -0.10       |
| C4                      | -                 | -  | -  | -  | -7.09       | 0.22        | -0.07                     | -0.08 | -0.09       | <b>0.10</b> | -0.09 | -0.04       |
| C5                      | -                 | -  | -  | -  | -3.22       | 0.34        | -0.13                     | -0.03 | -0.07       | -0.12       | -0.10 | -0.01       |
| C6                      | -                 | -  | -  | -  | <b>0.04</b> | <b>0.66</b> | -0.35                     | -0.24 | -0.29       | -0.21       | -0.40 | -0.23       |
| C7                      | -                 | -  | -  | -  | -8.88       | 0.44        | -0.33                     | -0.18 | -0.43       | -0.03       | -0.43 | -0.16       |
| C8                      | -                 | -  | -  | -  | -8.94       | 0.35        | -0.15                     | -0.11 | -0.21       | <b>0.04</b> | -0.17 | -0.09       |
| C9                      | -                 | -  | -  | -  | -9.02       | 0.26        | -0.07                     | -0.20 | -0.10       | <b>0.06</b> | -0.08 | -0.06       |
| C10                     | -                 | -  | -  | -  | -3.94       | 0.45        | -0.30                     | -0.24 | -0.23       | -0.14       | -0.30 | -0.21       |
| C11                     | -                 | -  | -  | -  | -1.93       | 0.50        | -0.30                     | -0.17 | -0.38       | -0.08       | -0.36 | -0.11       |
| C12                     | -                 | -  | -  | -  | -6.79       | 0.36        | -0.16                     | -0.11 | -0.20       | -0.00       | -0.17 | -0.06       |
| C13                     | -                 | -  | -  | -  | -4.14       | 0.28        | -0.13                     | -0.43 | -0.22       | -0.12       | -0.08 | -0.18       |
| C14                     | -                 | -  | -  | -  | -4.32       | 0.16        | -0.22                     | 0.31  | -0.34       | -0.12       | -0.07 | -0.02       |
| C15                     | -                 | -  | -  | -  | <b>2.59</b> | <b>0.87</b> | -0.28                     | -0.23 | -0.19       | -0.19       | -0.31 | -0.17       |
| <b>Topoteca<br/>n *</b> | -                 | -  | -  | -  | <b>6.64</b> | 0.84        | <b>0.41</b>               | -0.16 | <b>0.22</b> | -0.04       | -0.11 | <b>0.96</b> |

Table 4.3 shows results obtained from the web-based SwissADME predictions that states that not more than one of Lipinski's parameters should be violated for oral active compounds. From the obtained results for amide compounds, KAM 4, KAM 6 and KAM 11 violated either one or more of the Lipinski's parameters. KAM 11 (a di-amide compound derived from gallic acid and 2,4-diaminophenol) violated two of the parameters namely; surface area and H-bond donors, while KAM 4 and KAM 6 only violated one (H-bond donors).

Table 4.4 shows results for ester compounds, the ester compounds violated over 1 of the parameters. Specifically compounds C9, 12 and C13 violated more than 3 of the parameters. Compounds C9 and C13 are tri-substituted compounds which are bulky and due to their size, they are prone to violate more of parameters because of possible varying conformational structures and rotatable bonds. These two aforementioned compounds violated 4 of the similar parameters (molar mass, surface area, H-bond donors and acceptors). However, this does not necessarily mean that these compounds are toxic, the Lipinski's rule of five only predicts possible oral bioactivity. Therefore, due to the ruled out toxicity traits from the OSIRIS predictions, the compounds were deemed safe and they were further tested for other *in silico* studies.

**Table 4. 3:** SwissADME predicted physicochemical properties of the designed amide compounds (Lipinski's RO5).

| Compound Name | Molar Mass (g/mol) | XLog P3 | Surface Area (Å <sup>2</sup> ) | H-Bond donors | H-Bond Acceptors | nViolation |
|---------------|--------------------|---------|--------------------------------|---------------|------------------|------------|
|               | <500               | <5      | <180                           | <5            | <10              |            |
| KAM 1         | 348.35             | 3.01    | 98.66                          | 4             | 4                | 0          |
| KAM 2         | 365.35             | 2.53    | 118.89                         | 5             | 5                | 0          |
| KAM 3         | 396.35             | 1.83    | 159.35                         | 7             | 7                | 1          |
| KAM 4         | 380.35             | 2.17    | 139.12                         | 6             | 6                | 1          |
| KAM 5         | 261.23             | 1.34    | 110.02                         | 5             | 5                | 0          |
| KAM 6         | 412.35             | 1.46    | 179.58                         | 8             | 8                | 1          |
| KAM 7         | 213.23             | 2.26    | 49.33                          | 2             | 2                | 0          |
| KAM 8         | 229.23             | 1.91    | 69.56                          | 3             | 3                | 0          |
| KAM 9         | 245.23             | 1.49    | 89.79                          | 4             | 4                | 0          |

|               |        |      |               |          |   |   |
|---------------|--------|------|---------------|----------|---|---|
| <b>KAM 10</b> | 229.23 | 1.91 | 69.56         | 3        | 3 | 0 |
| <b>KAM 11</b> | 428.35 | 1.11 | <b>199.81</b> | <b>9</b> | 9 | 2 |
| <b>KAM 12</b> | 245.23 | 1.68 | 89.79         | 4        | 4 | 0 |

**Table 4. 4:** Swiss predicted physicochemical properties of the designed ester compounds (Lipinski's RO5).

| <b>Compound Name</b> | <b>Molar Mass (g/mol)</b> | <b>XLog P3</b> | <b>Surface Area (Å<sup>2</sup>)</b> | <b>H-Bond donors</b> | <b>H-Bond Acceptors</b> | <b>nViolation</b> |
|----------------------|---------------------------|----------------|-------------------------------------|----------------------|-------------------------|-------------------|
|                      | <b>&lt;500</b>            | <b>&lt;5</b>   | <b>&lt;180</b>                      | <b>&lt;5</b>         | <b>&lt;10</b>           |                   |
| <b>C1</b>            | 256.25                    | 2.66           | 63.60                               | 1                    | 4                       | 0                 |
| <b>C2</b>            | 256.25                    | 2.71           | 65.40                               | 1                    | 4                       | 0                 |
| <b>C3</b>            | 392.36                    | 3.72           | 110.13                              | 2                    | 7                       | 0                 |
| <b>C4</b>            | 486.43                    | <b>5.09</b>    | 139.59                              | 3                    | 9                       | 1                 |
| <b>C5</b>            | 486.43                    | <b>5.15</b>    | 142.05                              | 3                    | 9                       | 1                 |
| <b>C6</b>            | 217.27                    | 2.33           | 75.63                               | 2                    | 4                       | 0                 |
| <b>C7</b>            | 272.25                    | 2.12           | 83.83                               | 2                    | 5                       | 0                 |
| <b>C8</b>            | 424.36                    | 3.01           | 150.59                              | 4                    | 9                       | 0                 |
| <b>C9</b>            | <b>534.43</b>             | 4.02           | <b>200.28</b>                       | <b>6</b>             | <b>12</b>               | 4                 |
| <b>C10</b>           | 287.27                    | 1.95           | 95.86                               | 3                    | 5                       | 0                 |
| <b>C11</b>           | 288.25                    | 1.95           | 104.06                              | 3                    | 6                       | 0                 |
| <b>C12</b>           | 456.36                    | 2.30           | <b>191.05</b>                       | <b>6</b>             | <b>11</b>               | 3                 |
| <b>C13</b>           | <b>582.43</b>             | 2.96           | <b>260.97</b>                       | <b>9</b>             | <b>15</b>               | 4                 |
| <b>C14</b>           | 217.27                    | 2.50           | 77.10                               | 3                    | 4                       | 0                 |
| <b>C15</b>           | 303.27                    | 1.62           | 116.09                              | 4                    | 6                       | 0                 |

#### 4.3.1 Docking Scores Analysis

The extent of inhibition for all the designed compounds was assessed by docking the compounds to the selected proteins shown in Figure 4.3. Docking results obtained were compared to inhibition results for the commercially used standard drug topotecan. The docking procedure was conducted using two X-ray crystal structures of the DNA topoisomerase I and

ABCG2 protein (PDB codes: 1K4T and 7NEZ respectively) which were docked with twenty-seven hydroxybenzoic acid derivatives.

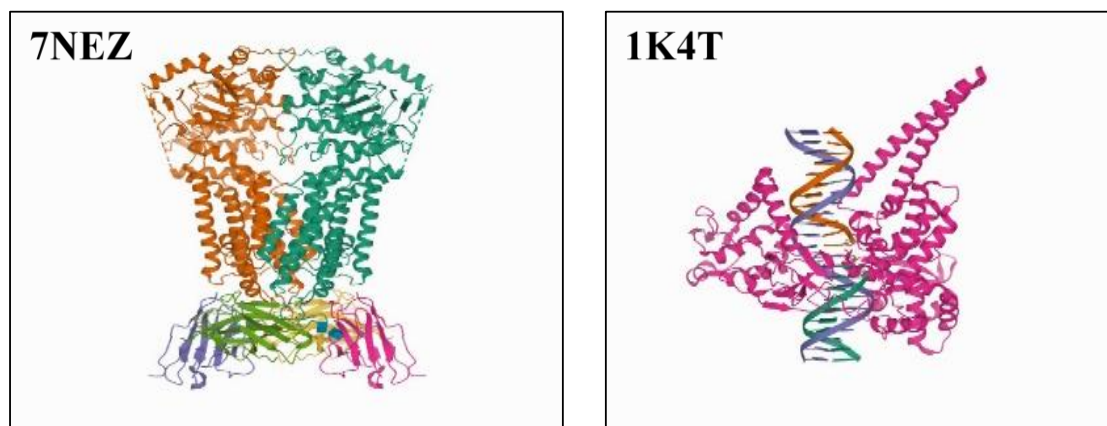


**Figure 4. 3:** Three-dimensional X-ray crystal structures of the selected DNA topoisomerase I and ABCG2 proteins obtained from the protein data bank (PDB) with their PDB IDs (1K4T and 7NEZ).

After docking, eight different binding poses were obtained. The lowest binding energy pose with the lowest RMSD (root-mean-square deviation) value was selected as the best pose. The docking studies indicate that the esters were better than the amides with C13 (tri-substituted ester compound) having the best overall binding energy of -12.56 kcal/mol against 1K4T and -8.72 kcal/mol against 7NEZ protein (see Table 4.5). Amongst the amides, KAM 6 had the best binding energy of -11.30 kcal/mol compared to the reference compound topotecan (TTC) with -9.14 kcal/mol against 1K4T. Against 7NEZ, TTC was observed to be the best against amides with a binding energy of -8.25 kcal/mol. The esters revealed C4 to have the best binding energy of -12.68 kcal/mol, followed by C9 and C13 both with -12.56 kcal/mol binding energy. Against 7NEZ, C13 was the best ester compound in comparison to the TTC binding energy of -8.25 kcal/mol. The binding strength of CE12 against 1K4T was confirmed by MM-GBSA analysis which showed an energy of -83.42 kcal/mol whilst against 7NEZ was observed to be -77.79 kcal/mol.



**Table 4. 5:** Docking results for the designed compounds against DNA-topoisomerase I and ABCG2 proteins.

| 1K4T       |                     | 7NEZ         |               | 1K4T       |                      | 7NEZ         |               |
|------------|---------------------|--------------|---------------|------------|----------------------|--------------|---------------|
|            | $\Delta G$          |              | $\Delta G$    |            | $\Delta G$           |              | $\Delta G$    |
| KAM 1      | -9.34 -43.77        | -7.17        | -72.64        | C1         | -7.61 -32.86         | -6.15        | -49.34        |
| KAM 2      | -9.92 -46.99        | -7.23        | -71.19        | C2         | -7.62 -33.74         | -6.72        | -63.45        |
| KAM 3      | -10.63 -58.07       | -7.54        | -73.33        | C3         | -10.40 -56.54        | -7.66        | -71.94        |
| KAM 4      | -10.25 -37.91       | -7.46        | -68.62        | C4         | -12.68 -57.55        | -7.88        | -73.61        |
| KAM 5      | -7.64 -43.30        | -6.01        | -55.57        | C5         | -9.50 -55.76         | -7.45        | -69.83        |
| KAM 6      | -11.30 -56.32       | -7.40        | -73.68        | C6         | -7.97 -45.58         | -6.48        | -57.86        |
| KAM 7      | -6.82 -38.48        | -5.46        | -44.59        | C7         | -7.74 -36.54         | -6.45        | -54.46        |
| KAM 8      | -7.13 -44.09        | -5.59        | -54.75        | C8         | -11.08 -63.13        | -7.71        | -78.19        |
| KAM 9      | -7.18 -33.97        | -5.79        | -49.12        | C9         | -12.56 -37.91        | -7.96        | -66.98        |
| KAM 10     | -7.02 -24.61        | -5.64        | -44.73        | C10        | -8.19 -50.28         | -6.44        | -52.61        |
| KAM 11     | -10.99 -51.87       | -7.51        | -74.67        | C11        | -7.77 -51.56         | -6.48        | -58.80        |
| KAM 12     | -7.36 -37.15        | -5.77        | -46.66        | C12        | -11.58 -66.08        | -8.15        | -79.02        |
| -          | - -                 | -            | -             | <b>C13</b> | <b>-12.56 -83.42</b> | <b>-8.72</b> | <b>-77.79</b> |
| -          | - -                 | -            | -             | C14        | -7.55 -31.45         | -6.50        | -60.43        |
| -          | - -                 | -            | -             | C15        | -8.12 -35.32         | -6.59        | -61.47        |
| <b>TTC</b> | <b>-9.14 -43.43</b> | <b>-8.25</b> | <b>-43.43</b> | <b>TTC</b> | <b>-9.14 -35.01</b>  | <b>-8.25</b> | <b>-43.43</b> |

TTC – Topotecan

#### 4.3.2 Protein-ligand interactions:

In *in-silico* protein-ligand interactions, the aim of ligand–protein docking is to discover a ligand's most common binding mode(s) from its interaction with a protein with a known three-dimensional structure<sup>271</sup>. Protein-ligand interactions are crucial for understanding biological regulation mechanisms and providing a theoretical foundation for the design and development of new drug targets<sup>272</sup>. The interpretation of the top 10 compounds against 1K4T found showed that compound C4 forms H-donor interactions with Asp440 (-9.7 kcal/mol;2.37 Å), C9 forms H-donor interactions with Lys425 (-0.9 kcal/mol;3.09 Å) as well as with Asp440 (-0.7

kcal/mol;3.08Å), C13 forms H-donor bonds with Asp440 (-1.0 kcal/mol;3.28 Å), Asn722 (-1.1 kcal/mol;2.74 Å) and H-acceptor interactions with Lys436 (-1.0 kcal/mol;3.25 Å) as shown in Fig. 4.4 (b). C12, C11 and KAM 3 formed no hydrogen interaction, KAM 6 forms H-donor bonds with DA113 (-3.1 kcal/mol;2.72 Å), CE05 forms H-donor bonds with Lys425 (-0.7 kcal/mol;2.86 Å), KAM 11 forms H-donor interactions with Lys436 (-0.4 kcal/mol;2.85 Å) and the reference compound TTC formed H-acceptor bonds with DA113 (-2.0 kcal/mol;2.98 Å), two ionic interactions with Lys425 (-7.6 kcal/mol;2.62 Å) and Lys425 (-1.0 kcal/mol;3.80 Å). Against 7NEZ, the top 10 interpretation shows C13 (see Fig. 4.4 (d)), C9, C4, C8, C3 form no hydrogen interaction, C12 forms H-acceptor bond with Asn436 (-2.7 kcal/mol, 3.24 Å) and  $\pi$ - $\pi$  interaction with Phe439 (-0.0 kcal/mol;3.94Å), KAM 3 forms H-donor with Gln398 (-0.7 kcal/mol;3.47Å), KAM 6 forms H-donor with Gln398 (-2.3 kcal/mol;2.88 Å) and the reference compound TTC forms H-donor with Met549 (-0.9 kcal/mol;3.65 Å), Met549 (-0.8 kcal/mol;3.75 Å) and Phe439 (-0.6 kcal/mol;3.43 Å). The summarized results of the lead compounds H-bond residues are shown in Table 4.6 below.

**Table 4. 6:** Selected lead compounds protein-ligand interaction H-bond residues.

| Compound | H-bond residues                     |                    |
|----------|-------------------------------------|--------------------|
| Name     | 1K4T                                | 7NEZ               |
| KAM 1    | Tyr A426, DT C112                   | -                  |
| KAM 2    | DT C110, Ile A424                   | Asn A436           |
| KAM 3    | DT C110, Leu A429                   | Asn A436, Gln A398 |
| KAM 4    | DT C110                             | Asn A436, Gln A398 |
| KAM 6    | DC C111, DC C111, DA C113, Met A428 | -<br>Gln A398      |
| KAM 11   | DC C111, DT C110, Met A428          | Thr A402, Thr A402 |
| C4       | DT C110, Asp A440                   | -                  |
| C8       | DC C111, DT C110                    | Asn A436           |
| C9       | DT B9, DT C110, Met A428            | -                  |
| C12      | DC C111, DC C111, Met A428          | Asn A436, Asn A436 |
| C13      | DT B10, DT C110, Lys A751           | -                  |

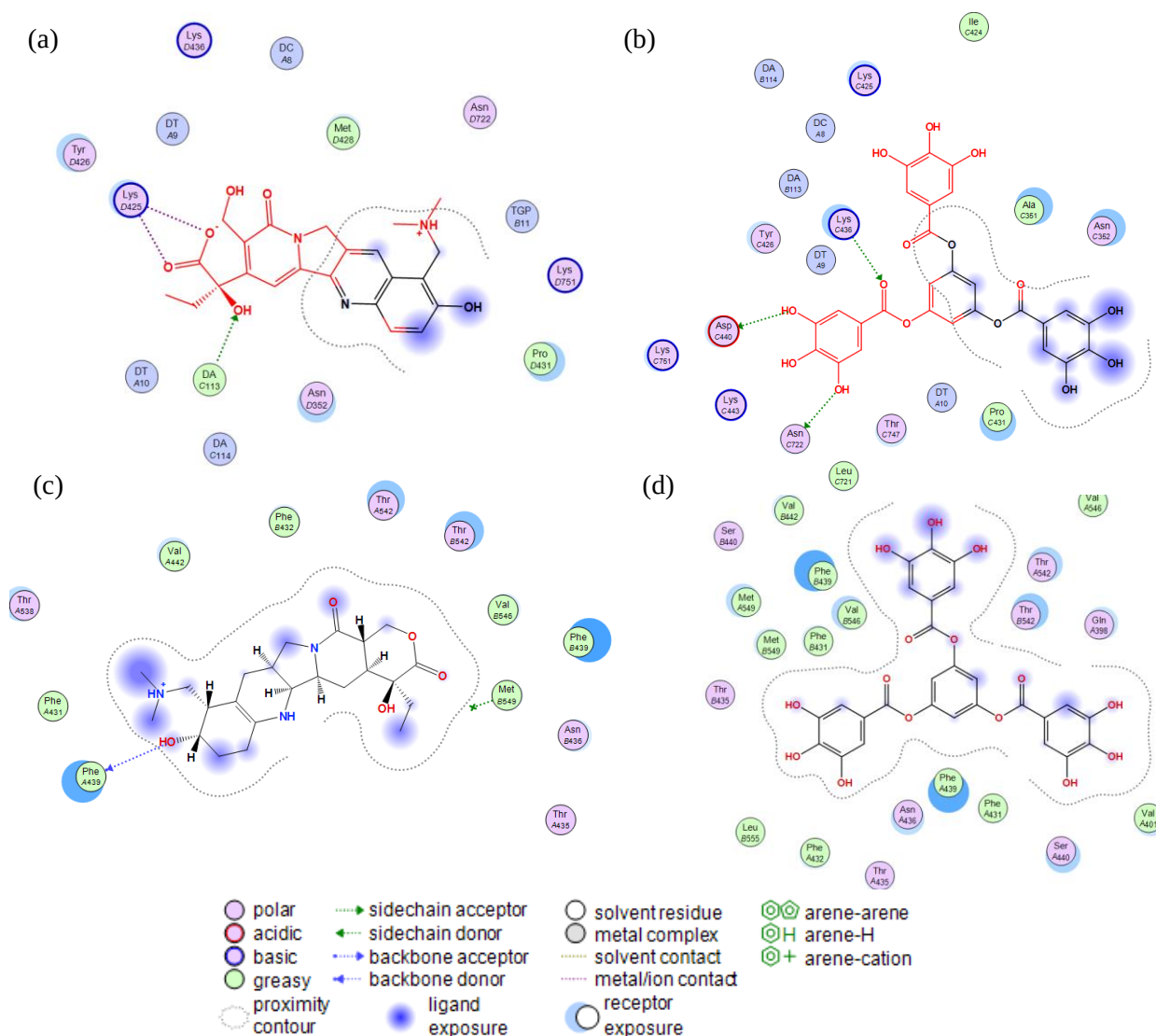


**Figure 4. 4:** 2D docking protein-ligand interactions of C13 (b & d) and reference compound TTC (a & c) against 1K4T and 7NEZ.

#### 4.3.3 Density Functional Theory (DFT) calculations - Frontier molecular orbitals (FMOs):

The highest occupied molecular orbitals (HOMO) and the lowest unoccupied molecular orbitals (LUMO) of the compounds that showed high binding affinities (KAM 1, KAM 2, KAM 3, KAM 4, KAM 6, and KAM 11 for amide compounds and C4, C8, C9, C12, and C13 for ester compounds) consistently were calculated using the DFT method at the hybrid system of B3LYP/6-31G(d,p) level of theory. The results shown in Tables 4.7 and 4.8 represent the obtained results for the HOMO, LUMO, and their frontier molecular energy gap difference

( $\Delta E$ ) descriptors of selected amides and esters respectively, the selection was based on their consistent high binding affinity scores.

These molecular descriptors are very important in defining a molecule's chemical stability and reactivity as well as biological activity<sup>273</sup>. The HOMO eigenvalue is used to determine the molecule's electron-donating capacity, while the LUMO eigenvalue is used to determine the molecule's electron-accepting capacity<sup>273–275</sup>. The frontier molecular gap on the other hand is used to determine the reactivity and the stability of a molecule. A high  $\Delta E$  value corresponds to the high kinetic stability of a molecule, while the molecule with a low  $\Delta E$  value is considered a more polarizable molecule and is associated with high chemical reactivity and low kinetic stability. The compound that has the lesser negative eigenvalue either for HOMO or LUMO is considered the best. Compound KAM 11 showed the best HOMO eigenvalue of -5.19329 eV for amide compounds while C13 gave the best HOMO eigenvalue of -5.95494 eV for ester compounds suggestive of these compounds having the best electron-donating capability. For the amide compounds, this was then followed by compound KAM 3 which showed the HOMO eigenvalue of -5.31547 eV which also showed good donating capability. Furthermore, compound KAM 4 from the amides showed the best LUMO eigenvalue of -1.2174 eV, while compound C13 from the esters was still the best candidate with a LUMO eigenvalue of -1.2874 eV, suggesting the best electron-accepting capability as well. C13 showed that it can act as an electron donor and electron acceptor at the same time. The amides acceptable LUMO values were followed by those for compound KAM 11, KAM 1, and KAM 3 which showed the LUMO eigenvalues of -1.2623, -1.2917, and -1.4607 eV respectively based on the highest to lowest eigenvalue suggesting moderately high electron-withdrawing capability. In addition, on the ester compounds C12 had the second best LUMO eigenvalue of -1.4912 eV. The frontier molecular gap ( $\Delta E$ ) on the other hand was high for compound KAM 1 and KAM 4 with the energy gap values of 4.54 and 4.55 eV respectively for amides. Esters that showed a large energy gap were compounds C4 and C13 with the energy gap values of 4.66 and 4.67 eV respectively, indicating high kinetic stability and low chemical reactivity. Moderately high  $\Delta E$  values were obtained for compounds KAM 11, KAM 3 and KAM 6 with the values 3.93, 3.85 and 3.44 eV respectively for amides and C12 and C9 with energy gap values of 4.52 and 4.54 eV respectively, indicative of moderately high kinetic stability. However, compounds KAM 2 and C8 showed low  $\Delta E$  value of 2.83 eV and 4.50 eV, suggesting high chemical reactivity.

**Table 4. 7:** Frontier molecular orbitals results of the selected amide compounds (KAM 1, KAM 2, KAM 3, KAM 4, KAM 6, and KAM 11).

| Compound | HOMO (eV) | LUMO (eV) | $\Delta E$ (eV) |
|----------|-----------|-----------|-----------------|
| KAM 1    | -5.83113  | -1.2917   | 4.54            |
| KAM 2    | -7.93865  | -5.1103   | 2.83            |
| KAM 3    | -5.31547  | -1.4607   | 3.85            |
| KAM 4    | -5.77180  | -1.2174   | 4.55            |
| KAM 6    | -8.53186  | -5.0871   | 3.44            |
| KAM 11   | -5.19329  | -1.2623   | 3.93            |

**Table 4. 8:** Frontier molecular orbitals results of the selected ester compounds (C4, C8, C9, C12, and C13).

| Compound | HOMO (eV) | LUMO (eV) | $\Delta E$ (eV) |
|----------|-----------|-----------|-----------------|
| C4       | -6.31794  | -1.6528   | 4.66            |
| C8       | -6.17644  | -1.6773   | 4.50            |
| C9       | -6.09099  | -1.5546   | 4.54            |
| C12      | -6.00882  | -1.4912   | 4.52            |
| C13      | -5.95494  | -1.2874   | 4.67            |

The diagram in Figure 4.5 and Figure 4.6 shows the frontier molecular descriptors for the compounds that showed high HOMO or LUMO or  $\Delta E$  values. Compound KAM 4 ( $\Delta E = 4.55$  eV) in Fig. 4.5 (a) was the most kinetically stable and least reactive compound, while KAM 2 ( $\Delta E = 2.83$  eV) in Fig. 4.5 (b) was the most chemically reactive compound according to DFT calculations. Furthermore, compound KAM 11 (-5.19329 eV) in Fig. 4.5(c) was the best electron-donating compound when compared to other compounds on the molecular descriptors calculations. Concerning the ester compounds shown in Figure 4.6 only compounds C8 and C13 (shown in Figure 4.6 (a) and (b) respectively) showed favourable frontier molecular descriptors. Compound C8 had the lowest  $\Delta E$  value of 4.50 eV, while C13 showed the best HOMO and LUMO values of 5.95494 and -1.2874 respectively. In addition, C13 also had the highest  $\Delta E$  value of 4.67 eV suggesting high kinetic stability. The red and green colours in the HOMO and LUMO phases signify negative and positive phases respectively of the molecular

frontier orbital wave function<sup>276</sup>. Results of other compounds are shown in the supporting information. In all the compounds, compound C13 was found to be the overall consistent compound with potentially desirable bioactivity throughout the *in-silico* tests done. Therefore, because of the high binding affinity, MM-GBSA, HOMO, LUMO and  $\Delta E$  values, it was selected for molecular dynamic simulations and then compared with topotecan.

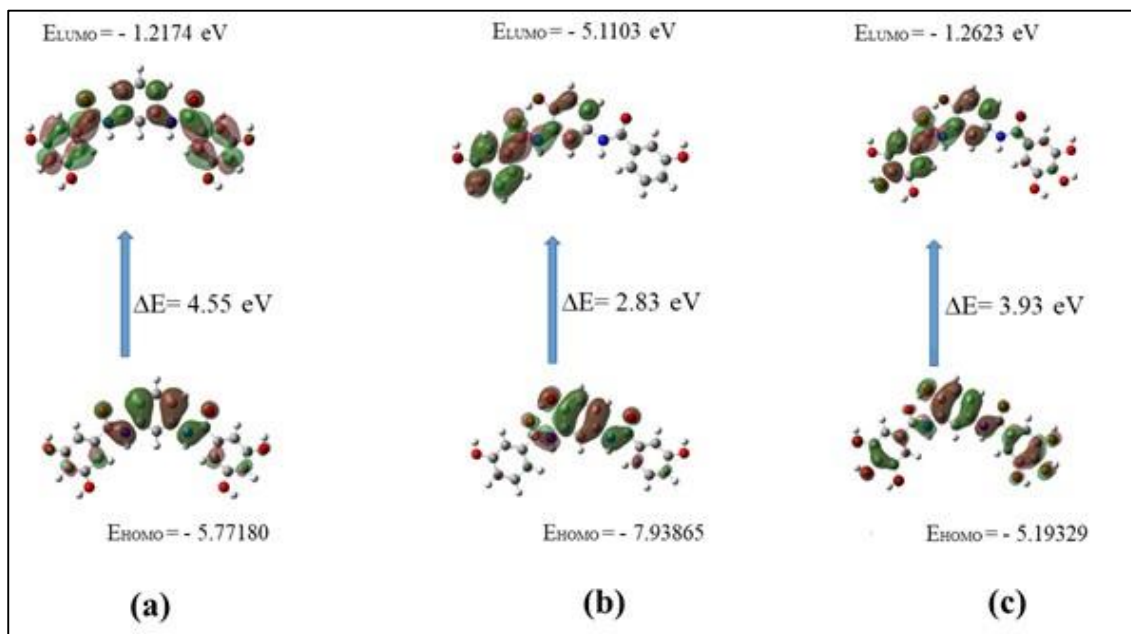


**Figure 4. 5:** Frontier molecular orbital descriptors of amide compounds (a) KAM 4 which showed the best LUMO value and high  $\Delta E$  value, (b) KAM 2 which showed the lowest  $\Delta E$  value and (c) KAM 11 which showed the best HOMO eigenvalue.

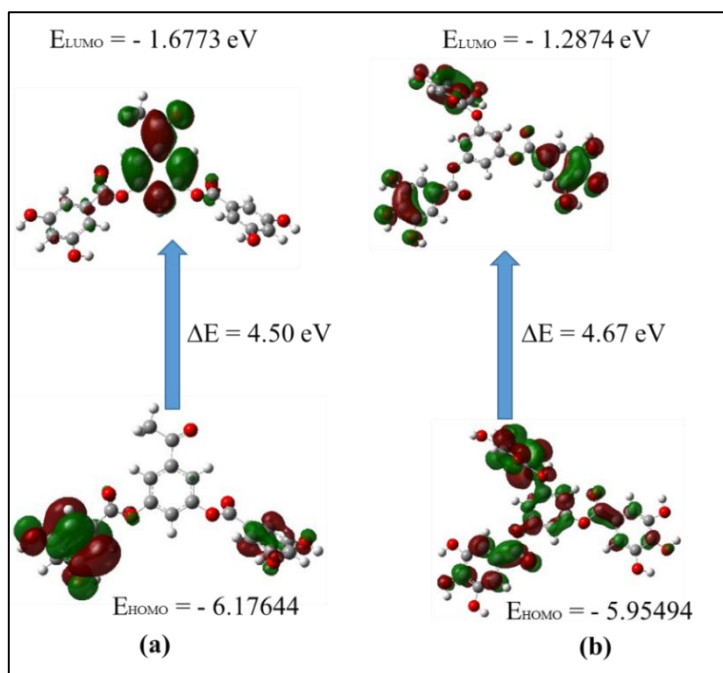


**Figure 4. 6:** Frontier molecular orbital descriptors of ester compounds (a) C8 which showed the lowest  $\Delta E$  value and (b) C13 which showed the best HOMO and LUMO values and also the highest  $\Delta E$  value.

#### 4.3.4 Molecular Dynamics (MD)

The dynamic nature of the protein–ligand complex has been widely studied by MD simulations for scrutinizing the conformational stability, conformational changes, internal motions and protein-ligand interactions shown in Figures 4.7, 4.8, and 4.9 in no particular order. Root-mean-square deviation (RMSD) analysis highlighted below in Figure 4.7, is crucial for quantifying the structural stability of the protein or protein-ligand complexes within a regular time frame. The analysis shows that the compound C13 (CE12) forms stable complexes with the receptors 1K4T and 7NEZ as observed with RMSD values around 3Å. Together with Topotecan (TTC) having RMSD around 2Å which indicates stronger interaction compared to compound CE12. The plot also reveals that the complexes with CE12 form within 5 nanoseconds with minor fluctuation around 70 nanoseconds. Topotecan (TTC) stabilizes around 30 nanoseconds and continues to stabilize until minor fluctuations are observed around 70 nanoseconds.

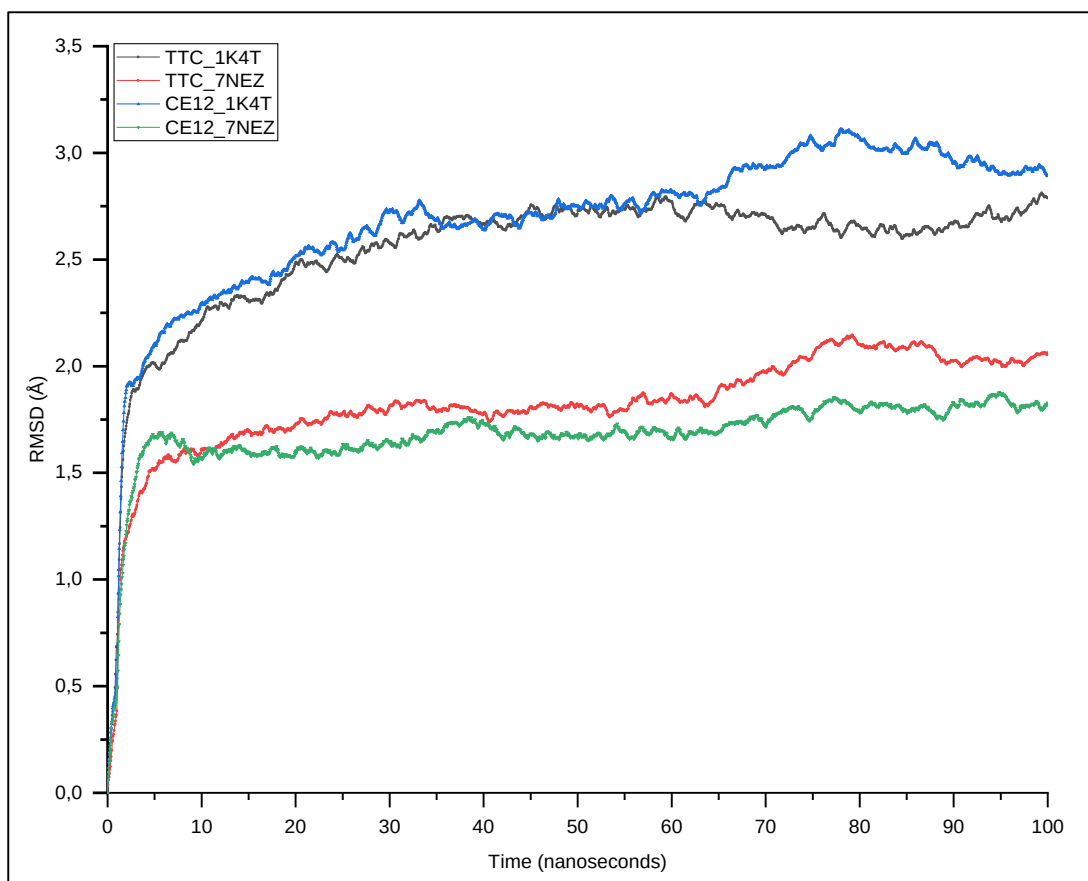


**Figure 4. 7:** Root-mean-square deviation (RMSD) graph of compound C13 (CE12) and TTC complexes with 1K4T and 7NEZ proteins.

Intermolecular H-bonding shown in Figure 4.8, is also one of the most crucial parameters for understanding the binding affinity of the ligand to the protein molecule. Formation or distortion of hydrogen bonds is a principal component during the MD simulation. The presence of many H-bonds between the protein and ligand signifies a stronger binding affinity. Herein, TTC-1K4T is observed to have around 4 hydrogen bonds which drop to 3 stable hydrogen bonds throughout the simulation. Similarly, C13-1K4T forms 3 hydrogen bonds which remained stable throughout the simulation. On the other hand, TTC-7NEZ formed 2 stable hydrogen bonds whilst C13-7NEZ formed 3 stable hydrogen bonds. These results therefore, justify the consistently high binding affinity of compound C13 (CE12) with both studied proteins.



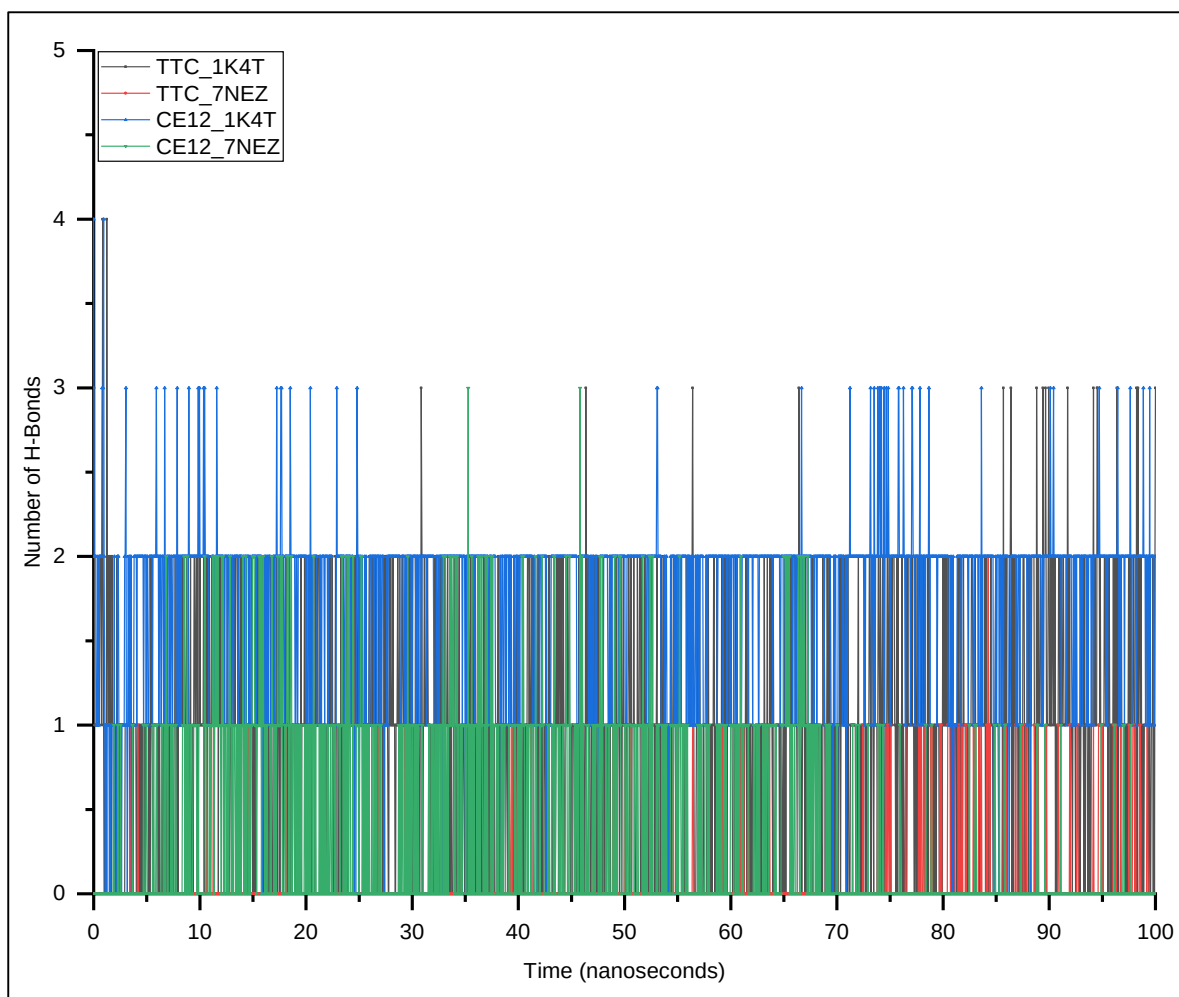


**Figure 4. 8:** H-bonds graph of compound C13 (CE12) and TTC complexes with 1K4T and 7NEZ proteins.

The root-mean-square fluctuation (RMSF) analysis is another essential parameter for identifying the rigid and flexible regions of the protein structure. It is a standard measure of the deviation of the particle from its original position. Furthermore, it can also be used to assess the flexibility of the backbone elements of the protein structure as well as the ligand <sup>277</sup>. The RMSF was analyzed for the entire protein structure and ligand combined demonstrating each atom's mean displacement <sup>278</sup>. The interaction with 1K4T results in a major fluctuation around the 450-500 residue region. Whereas for 7NEZ (655), major fluctuations were observed around 500-510 and 1079-1090 residue regions as depicted in Figure 4.9. Thus, fluctuations corresponded to residues that were far from the ligand active site. In contrast, the residues in the active site interacting with the ligands were approximately stable and showed a little fluctuation during the simulation trajectory. This narrow range of RMSFs for the active site

residues for the compounds analyzed demonstrated that the molecules could form stable interactions with the protein during MD simulation<sup>278,279</sup>.

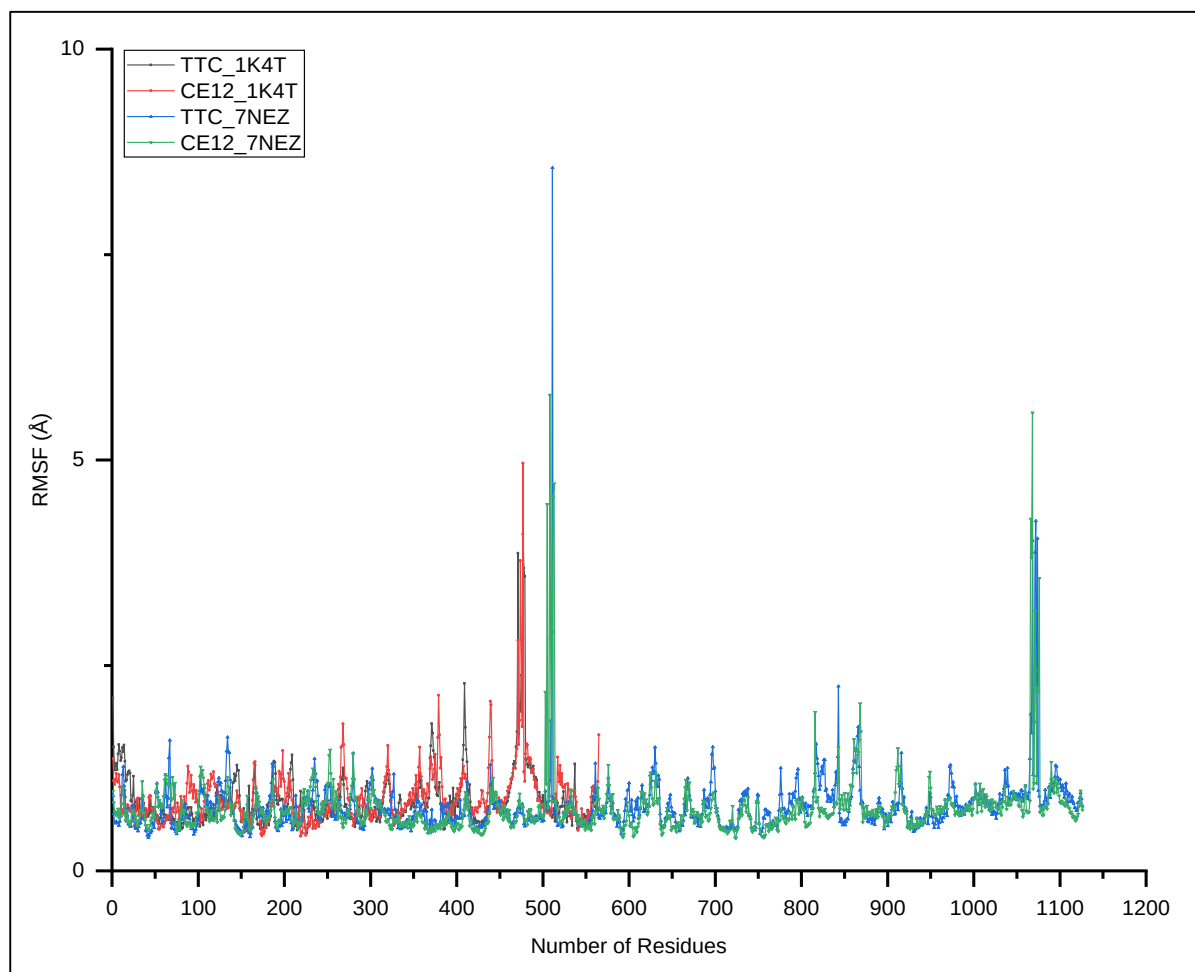


**Figure 4. 9:** The root-mean-square fluctuation (RMSF) graph of compound CE12 and TTC complexes with 1K4T and 7NEZ proteins.

#### 4.4 Discussion

Several authors have used the *in silico* approach to determine the bioactivity properties of some hydroxybenzoic acid derivatives, particularly gallic acid derivatives. Arsianti and co-workers<sup>280</sup>, used *in-silico* docking to determine the inhibition potential of gallic acid ester derivatives against malarial dihydrofolate reductase (malaria DHFR), and octyl gallate was determined to be the most active compound amongst those studied. The activity was attributed to the lipophilicity (Log P value) which adhered to Lipinski's rule of five resulting in high stability, affinity, and hydrophobicity properties<sup>280</sup>. Al-Shawabkeh et al.<sup>281</sup> reported an *in silico* and *in vitro* study on a series of aryl amides, aryl esters, and sulfonamide compounds against type 2 diabetes hormone-sensitive lipase (HSL). According to Al-Shawabkeh and co-workers, the

most active compounds were aryl amides and aryl ester compounds. The observed activity was attributed to the hydrophobic nature of the compounds and their interaction with the hydrophobic active site of HSL <sup>281</sup>. Hence, we designed aryl amides and esters in this study and tested their toxicity via the *in silico* route.

A measure of undesirable and/or adverse effects of chemicals is referred to as toxicity <sup>282</sup>. Toxicity tests are mainly to identify the harmfulness of a substance towards either plants, animals, or humans and they constitute the most important step in drug design <sup>282</sup>. Several factors such as ADME (absorption, distribution, metabolism, and excretion), biological and chemical properties contribute significantly to the toxicity of material <sup>282</sup>. The designed compounds did not show any toxicity risk based on the predictions undertaken.

#### **4.4.1 *In silico* drug-likeness and toxicity studies**

OSIRIS Property Explorer (OPE) a web-based system used to calculate drug properties such as toxicity risk assessment and predictions of fragment-based drug-likeness and overall drug-score <sup>270</sup>, was applied in this study. The OPE toxicity predictions showed no putative mutagenic, tumorigenic, irritant and reproductive toxic effect of the designed compounds and topotecan (Table 4.1 and 4.2 previously shown), suggestive of a good candidate for *in-vitro* or *in-vivo* drug design. The fragment-based drug-likeness, overall drug score (combining drug-likeness, clogP, clogS, molecular weight, and toxicity risk factors in one single value), and toxicity of the designed compounds were determined and compared to the values for topotecan (standard DNA topoisomerase I inhibitor). The *in silico* drug-likeness was determined by comparing fragments of the designed compounds to the approved drugs and non-drugs of the Fluka company's database <sup>270</sup>. The fragments referred to, are moieties and backbone structure of the designed compound that are similar to the commercially available drug or at-least a drug that is recognised in the Fluka company database. A positive drug similarity value (0.1-10) indicates that a molecule contains fragments found in commercial drugs <sup>270</sup>. Compounds KAM 9, KAM 5, KAM 1, KAM 6, KAM 2 and KAM 11 from the amide counterparts showed positive drug-likeness (DL), while only compound C6 and C15 from the ester counterparts showed the positive DL value indicating that these compounds contain fragments of the commercially available drugs. However, only compound C9 and C15 showed drug similarity values higher than 3, which is less than half of the value obtained for topotecan (TTC) (6.64). The negative drug-likeness (DL) of other compounds KAM 7, KAM 10, KAM 12, KAM 4 and KAM 3 from the amide compounds and C1, C2, C7, C11, C3, C8, C12, C10, C4, C5, C14, C9 and C13 from

the ester compounds supposedly indicates non-drug likeness as previously stated according to the Fluka company database <sup>270</sup>.

The compounds which possessed the galloyl moiety (KAM 9, KAM 5, KAM 6, KAM 11, and C15) showed positive values for drug-likeness because the galloyl moiety is found in some of the known phytochemical compounds such as epigallocatechin and ellagitannin and these phytochemicals have been used in several applications that involve inhibition of signaling pathways for cancer application. Compounds KAM 8, KAM 1, KAM 2, and C6 also showed positive values for drug-likeness and the reason could be that these compounds bear the 3-hydroxy moiety also found in 3-Hydroxy-4-methoxymandelate a known compound (a compound that is used to screen for inherited metabolic diseases) <sup>283</sup>.

The drug score blends druglikeness, cLogP, logS, molecular weight, and toxicity risks into a single useful number (0.0-1.0) which can be used to assess a compound's overall potential to be approved as a drug. Compound C9 showed the highest overall drug score (0.92) compared to topotecan and other designed compounds. All the compounds showed moderate to high overall drug scores thus signifying their potential use as drugs. To further test the designed compounds for their potential bioactivity, programs such as Molinspiration can be used to check for the potential bioactivity against signalling pathways that are found on diseases such as cancer amongst others.

Molinspiration predictions showed possible bioactivity values of the designed compounds against druggable targets such as G protein-coupled receptors ligands (GL), kinase inhibitors (KI), ion channel modulators (ICM), nuclear receptor ligands (NRL), and enzyme inhibitors (EI). From the amide compounds, KAM 1, KAM 4, KAM 6, KAM 2, KAM 3 and KAM 11 showed positive bioactivity values for kinase inhibition (KI) indicating possible KI capabilities of the designed compounds. However, the KI values for the aforementioned compounds were less than those obtained for topotecan indicating less bioactivity compared to topotecan. The commonality of these compounds (KAM 1, KAM 4, KAM 6, KAM 2, KAM 3 and KAM 11) is that they are bis/di-amide compounds and they bear the hydroxybenzoic acids in their backbone structure. The ester compounds (Table 4.2) on the other hand, showed positive bioactivity value for only nuclear receptor ligands. The compounds that showed positive NRL values were compounds C3, C8, C4, and C9. The commonality of these compounds is that they are all bis-ester compounds and also that their backbone structure is either the 3-hydroxybenzoic acid motif (C3 and C9) or the 3,5-dihydroxybenzoic acid motif (C8 and C4).

The overall bioactivity predictions of the designed compounds gave satisfactory results for some of the compounds, necessitating further *in silico* tests.

#### 4.4.2 Physicochemical properties

In order for a drug to be viewed as effective, a potent compound must reach its target at a sufficient concentration and remain in its bioactive form long enough for the expected biological events to occur<sup>284</sup>. The early estimation of ADME in the discovery phase has been shown to significantly reduce the fraction of pharmacokinetic-related failure in the clinical phases<sup>285</sup>. During drug development, some parameters assessed include absorption, distribution, metabolism, and excretion (ADME), for determination of the pharmacokinetics of orally bioactive compounds<sup>284</sup>. Computer-based ADME predictions have revolutionized the drug development setting, since they have been successfully used as an alternative to experimental-based ADME studies<sup>286</sup>. The ADME parameters were obtained (Table 4.3 and Table 4.4) for the designed compounds. The Lipinski's "rule of five"(LRO5) was applied to achieve correlation between pharmacokinetic and physicochemical parameters<sup>284,287</sup>. This rule of five (RO5) is used to evaluate the drug-likeness of the designed compounds<sup>288</sup>. The rule is grounded on the notion that, for a compound to be active orally, it should not violate more than one of the Lipinski's rules for any of the parameters<sup>287,288</sup>. The amide compounds KAM 4, KAM 6, KAM 3 and KAM 11 (Table 4.3) violated one or two of the LRO5. Compound KAM 11 violated two of the LRO5 parameters (surface area and H-bond donors), while compounds KAM 4, KAM 6 and KAM 3 violated only one of the parameters as shown in Table 4.3. All other designed compounds except the aforementioned ones obeyed the LRO5. Ester compounds on the other hand showed higher violation number in comparison to the amide compounds. C12, C4, C9, and C13 violated 1 or more of the LRO5 parameters. C12 violated 3 of the parameters (surface area, H-bond donors, and H-bond acceptors), C4 violated 1 (XLog P3), C9 and C13 both violated 4 and similar parameters (molar mass, surface area, H-bond donors, and H-bond acceptors). The amide compound KAM 11 was the only compound that violated two of the LRO5 suggesting that this compound might not be used as an oral drug but can be used with another form of administering a drug such as the intravenous, intramuscular, and other varied routes, while compounds KAM 4, KAM 6 and KAM 3 violated only one of the RO5 parameters. Compound KAM 11 violated the polar surface area and H-bond donor, where the surface area correlates with the compound membrane permeation capability, while the presence of many H-bond donors in a compound can affect the compound from getting out of the gut wall into the blood thereby reducing drug potency.

Esters also showed similar but added violations to the amides, for instance, C12 also violated the surface area and H-bond donors but additionally, it violated the H-bond acceptor as well. Compounds C9 and C13 had an additional violation of molar mass greater than the 500 Dalton cut-off value in addition to the violations obtained for compound C12. The molar mass affects the material's permeability as well. However, the ability of a compound to obey the Lipinski rule does not necessarily mean that a compound is bioactive, but it hints at a higher probability for the compound to be active orally<sup>289</sup>.

In this case, compounds KAM 11, KAM 6, KAM 5, and KAM 2 would exhibit poor solubility and/or poor permeability hence they would fail to satisfy the criteria for orally active compounds.

#### **4.4.3 Molecular docking and protein-ligand interaction**

The results clearly show that the galloyl moiety played a crucial role in the hydrogen bond interaction of the designed compounds with the studied proteins. Hydrogen bond interactions are important in designing the drugs since they stabilize the protein-ligand complex together with the hydrophobic interactions. Against 1K4T, amide compounds with the galloyl moiety showed superior number of hydrogen bond interactions. The same trend was observed with the 7NEZ protein with compound C15 showing two hydrogen bond interactions with ThrA402. Furthermore, esters also expressed similar behavior against 1K4T with compounds CE6 and CE12 showing more hydrogen bond interactions than other compounds (see Fig. S3 and Fig. 4). However, for the 7NEZ protein, this was only the case for compound CE06 which showed two hydrogen bond interactions with AsnA436, one of the key amino acid in ABCG2 proteins as previously reported by Ibrahim and co-workers<sup>268</sup>. Most of the hydrogen bond interactions occurred between the amino acids and the hydroxyl groups of the designed compounds, and to a lesser extent they occurred either with the carbonyl group of the ester compounds or an amine group of the amide counterparts, thus signifying the importance of the pendant hydroxyl groups when designing potential DNA topoisomerase I and ABCG2 dual active compounds. Apart from the hydrogen bond interactions all the compounds showed a significant amount of hydrophobic interactions with the active site amino acids. Results relating to the protein-ligand interactions for all the designed compounds are presented in Appendix A2 (Figure S1 to Figure S4).

## 4.5 Conclusion

An *in-silico* study of amide and ester derivatives of hydroxybenzoic acid compounds was carried out. The designed compounds showed no toxicity effects and the prediction studies showed potential bioactivity for the designed compounds. From the designed compounds, di-amide compounds KAM 1, KAM 2, KAM 3, KAM 4, KAM 6, and KAM 11 from the amides and C4, C8, C9, C12, and C13 from the ester counterparts showed consistent high binding affinity when docked against both the human DNA topoisomerase I and the multidrug-resistant ABCG2 proteins. From the docked compounds, compound C13 showed the overall best MM-GBSA and docking score. Moreover, protein-ligand interactions showed that compound C13 was binding with the active site amino acids through putative polar and hydrophobic interactions via the pendant hydroxyl groups for hydrogen bond interactions. Compound C13 (CE 12) was further subjected to MD simulations which corroborated the overall best binding scores and the DFT results. Compared with all the compounds analyzed, C13 showed potential for application as DNA topoisomerase I and ABCG2 inhibitor or as a lead compound to be further modified since its bioactivity parameters were comparable and in some instances way better than those for topotecan based on the *in silico* results. However, *in vitro* and *in vivo* studies are required to validate the *in silico* findings. Therefore, the *in silico* tested compounds were taken for further tests via *in vitro* anticancer and antibacterial studies.

## Chapter 5: Synthesis and characterization of hydroxybenzoic acid amide and ester derivatives

---

### 5.1 Introduction

The synthesis and characterization of new compounds are critical in organic chemistry for expanding the scope of chemical knowledge and exploring applications in various fields <sup>290</sup>. One such intriguing class of compounds is hydroxybenzoic acid derivatives, which have garnered attention for their diverse biological and industrial significance <sup>291</sup>.

Hydroxybenzoic acids constitute a versatile group of compounds widely recognized for their pharmaceutical, cosmetic, and industrial applications <sup>292</sup>. Furthermore, the incorporation of amide and ester functionalities into the hydroxybenzoic acid scaffold introduces additional structural diversity, potentially leading to compounds with enhanced properties <sup>293</sup>.

The synthesis of hydroxybenzoic acid amides and esters involves multi-step processes, including acylation, amidation, and esterification reactions. These reactions are often catalyzed by specific reagents (such as boronic acid, boric esters, and group (IV) metal salts among other catalysts), and under optimized conditions to achieve high yields and purity of the target compounds. Several methods have been employed in the synthesis of hydroxybenzoic acid derivatives. Many of these techniques rely on the protection/deprotection strategy, which has demonstrated promise in synthesizing non-racemized and epimerized compounds effectively <sup>294–296</sup>. However, this route is tedious, time-consuming, expensive and sometimes complex, since it requires a lot of chemical reagents to produce high-quality and pure compounds <sup>297,298</sup>. In this study, we employed a direct acylation of the hydroxybenzoic acid derivative to form an acid chloride, this method was also reported by Khatkar and co-workers <sup>175</sup>. The other advantage of this method is that only two steps are required to obtain the product in high yields as opposed to other methods that rely on harsh conditions employed in boron-derived catalysts.

Characterization of the obtained derivatives is a crucial step in understanding their chemical nature. Techniques such as nuclear magnetic resonance (NMR) spectroscopy, infrared (IR) spectroscopy, and mass spectrometry (MS) are employed to elucidate the molecular structure and confirm the successful formation of the targeted compounds.



Spectroscopic techniques play a vital role in characterizing synthesized compounds, providing valuable information about their molecular structure, composition, and chemical properties <sup>299</sup>. Several spectroscopic methods are commonly used for characterization purposes. Among the various analytical techniques available, Nuclear Magnetic Resonance (NMR), Fourier Transform Infrared (FTIR) spectroscopy, and Mass Spectrometry (MS) are commonly employed due to their versatility and effectiveness.

FTIR spectroscopy measures the absorption of infrared radiation by a sample, providing information about the molecular vibrations of functional groups. When infrared light interacts with a molecule, it excites the molecular vibrations, causing characteristic absorption bands corresponding to specific functional groups <sup>300</sup>. While NMR spectroscopy exploits the magnetic properties of atomic nuclei in a magnetic field. When subjected to a strong magnetic field and radiofrequency radiation, nuclei with an odd number of protons or neutrons (e.g., <sup>1</sup>H, and <sup>13</sup>C) absorb energy and undergo nuclear spin transitions <sup>301</sup>. The resulting NMR spectrum provides information about the chemical environment, connectivity, and dynamics of the nuclei in a molecule <sup>301</sup>. <sup>1</sup>H-NMR provides information about hydrogen atoms, including their number, chemical shifts ( $\delta$ ), integration (relative number of protons), and coupling patterns (splitting due to neighbouring protons) <sup>302</sup>. <sup>13</sup>C-NMR reveals the carbon atoms environment and can identify functional groups and molecular connectivity. Complementary, to the aforementioned techniques is the mass spectrometry (MS). Mass spectrometry measures the mass-to-charge ratio ( $m/z$ ) of ions generated from a sample. The sample is ionized, and the resulting ions are separated based on their mass-to-charge ratio using electric and magnetic fields. The mass spectrum provides information about the molecular weight, fragmentation pattern, and structural features of the compound.

## **5.2 Materials and methods**

### **5.2.1 Materials and characterization**

All reagents and solvents used in the study were of analytical grade and they were used without further purification unless otherwise stated. The progress of the reaction was monitored by thin-layer chromatography (TLC) performed on a silica gel TLC Al foil with 60 Å medium pore diameter. Silica gel high purity grade 60 Å pore size, 70 – 230 mesh was used for column chromatography.

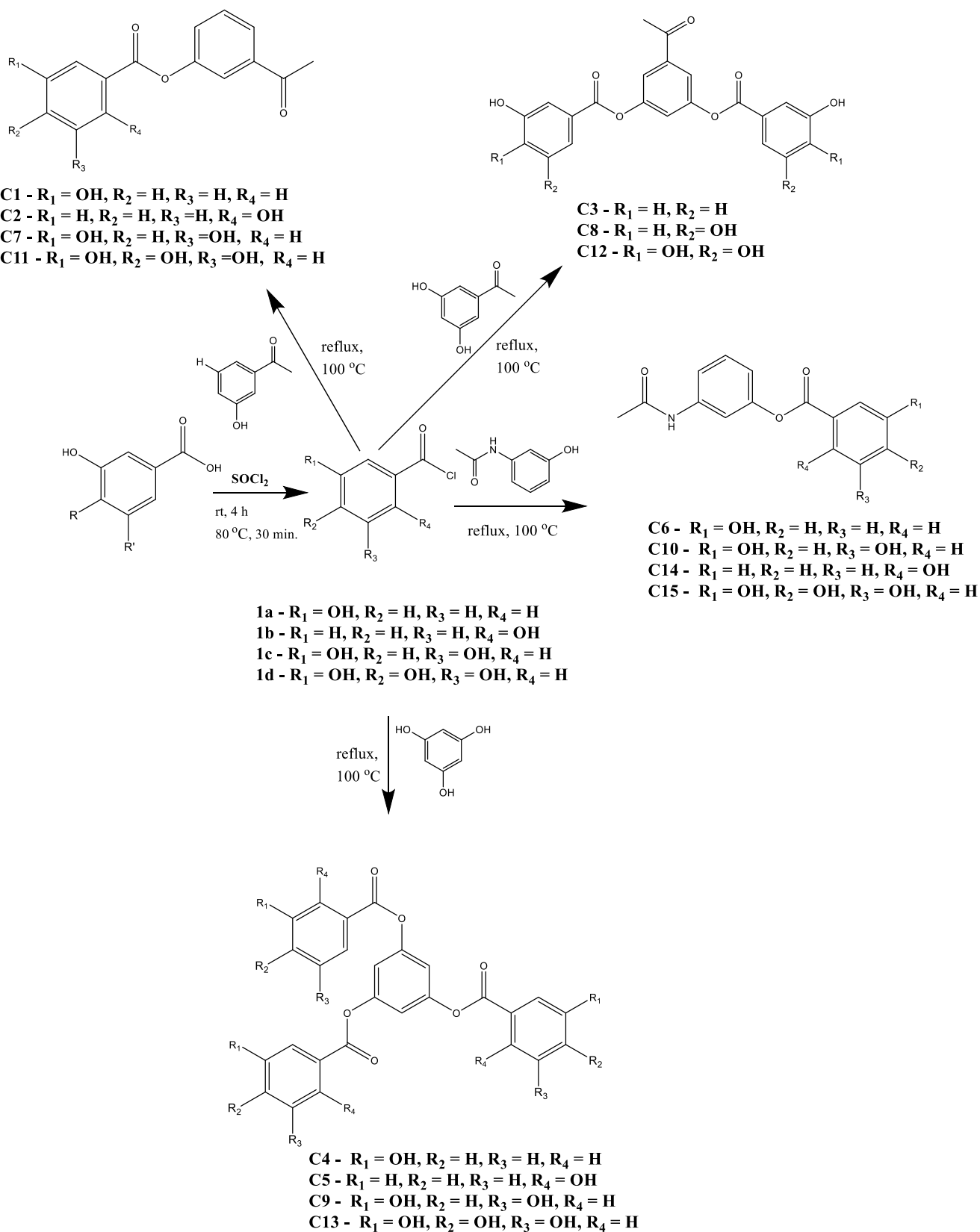
The synthesized compounds/products formed were purified by either recrystallization or column chromatography and formation of the compounds was checked by UHPLC HRMS mass spectrometry. Mass spectral analysis was conducted using a Loop injection technique with a UHPLC HRMS mass spectrometer. The samples were dissolved in HPLC grade methanol and adjusted to an appropriate concentration (typically 10 ppm). A volume of 10  $\mu$ L of sample was directly injected into the UHPLC HRMS system, which utilized a Gradient elution method over a period of 15 minutes. The elution solvent mixture initially consisted of 95% solvent A (0.1% formic acid/H<sub>2</sub>O) and 5% solvent B (0.1% formic acid/methanol), gradually transitioning to 5% solvent A and 95% solvent B. The separation was performed using an Acclaim RSLC 120 C18 column (2.2  $\mu$ m, 2.1 x 50 mm).

The Fourier-Transform infrared spectroscopy analysis was performed on a Shimadzu IRSpirit Fourier-Transform infrared spectrophotometer that utilizes a QATR-S single-reflection ATR accessory. In ATR-FTIR a small amount of sample (solid, powder or liquid) is placed in a diamond crystal. ATR-FTIR uses the reflectance of the sample when it is placed on the crystal.

<sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra of the products were recorded at room temperature (20 °C) on a Bruker Avance 500 NMR spectrometer (500 MHz) using 5 mm diameter tubes. Samples were dissolved in DMSO-*d*<sub>6</sub>. Chemical shift values ( $\delta$ ) were given in parts per million (ppm) and abbreviations were used to designate chemical shift multiplicities: s = singlet, bs = broad singlet, d = doublet, dd = double doublet, t = triplet, td = triplet doublet, q = quartet, m = multiplet.

### 5.2.2 Synthesis of ester derivative compounds

Ester derivative compounds were synthesized according to the method previously reported by Khatkar et al.<sup>303</sup>. As shown in scheme 5.1 the synthesis proceeds via the acylation of the hydroxybenzoic acid compounds, activating the carboxyl moiety to form an acid chloride (intermediate compounds 1a-d). The formation of hydroxybenzoate compounds is achieved by reacting the acid chloride with either 3-hydroxyacetophenone to form a mono-ester compound or with N-3-hydroxyphenylacetamide to yield another class of mono-esters. The di-substituted ester compounds are formed by reacting the acid chlorides with 3,5-dihydroxyacetophenone, while the tri-substituted ester compounds are as a result of reacting acid chlorides with phloroglucinol. The product compounds are mono-, di- and tri-substituted hydroxybenzoate derivatives.



**Scheme 5.1:** The synthetic procedure of hydroxybenzoic acid ester derivatives (**C1-C15**).

#### 5.2.2.1 General synthesis of intermediate hydroxybenzoyl chlorides

The preparation method was adapted from a method reported by Khatkar and co-workers<sup>175</sup>. Briefly, thionyl chloride (0.12 mol) was added gradually to hydroxybenzoic acids (0.1 mol) in a flat round bottom flask. After the addition of thionyl chloride, the mixture was stirred for 4 h and heated to 80 °C for 30 min in an oil bath. The excess of thionyl chloride was removed by rotary vapor at 40 °C. The hydroxybenzoyl chlorides were used on the next step without further purification. The following acid chlorides were synthesized; 2-hydroxybenzoyl chloride (**1a**), 3-hydroxybenzoyl chloride (**1b**), 3,5-dihydroxybenzoyl chloride (**1c**) & 3,4,5-trihydroxybenzoyl chloride (**1d**).

#### 5.2.2.2 General synthesis of ester compounds of hydroxybenzoic acid derivatives from hydroxyacetophenone derivatives

This section shows the preparation methods for the synthesis of ester compounds from the above-prepared acid chlorides with acetophenone derivatives (3-hydroxyacetophenone and 3,5-dihydroxyacetophenone). The reaction between hydroxybenzoyl chloride with 3-hydroxyacetophenone was a 1:1 ratio that yielded first-class mono-ester compounds, while the reaction of hydroxybenzoyl chlorides with 3,5-dihydroxyacetophenone was a 1:2 ratio.

The method used in this study is a modified method reported by Khatkar and co-workers<sup>303</sup>. To the prepared acid chloride (5 mmol, 0.783 g) in 25 mL acetonitrile and 25 mL diethyl ether. In a separate beaker 3-hydroxyacetophenone (for the first class of mono-ester compounds) (6 mmol, 0.820 g) was dissolved in 50 mL diethyl ether. The two solutions were mixed and refluxed at 100 °C overnight. The solvent was removed using a rotary vapor and the solid residue was purified by column and recrystallized in ethanol. Compounds **C1**, **C2**, **C7**, and **C11** are the first class of monoester compounds that are obtained in the aforementioned reaction.

A similar method was followed for di-substituted compounds. Di-substituted compounds are obtained by reacting acid chlorides (**1b**, **1c**, and **1d**) with 3,5-dihydroxyacetophenone in a 1:2 mole ratio to yield compounds **C3**, **C8**, and **C12**.

#### 5.2.2.3. General synthesis of ester compounds of hydroxybenzoic acid derivatives from *N*-3-hydroxyphenylacetamide (second class monoester compounds)

The 3-aminophenol was firstly protected using a method prepared by Leaym and colleagues<sup>304</sup>. Typically to a solution of 3-aminophenol (5.0 g, 45.9 mmol) in anhydrous THF (200 mL) was added Ac<sub>2</sub>O (4.4 mL, 0.0459 mol) and the mixture was stirred at room temperature (rt) for 2 h. The solvent was then removed by *vacuo* and the residue was recrystallized from toluene.

*N*-3-hydroxyphenylacetamide (*N*-3HPA) was obtained as brownish white needle-like powder (6.86 g, ~100% yield). *N*-3HPA was then reacted with 5 mmol acid chlorides (**1a-d**). Briefly acid chloride (5 mmol, 0.783 g) was dissolved in 25 mL acetonitrile and 25 mL diethyl ether. In a separate beaker *N*-3HPA (6 mmol, 0.907 g) was dissolved in 25 mL diethyl ether and 5 mL THF. The two solutions were then mixed and refluxed at 100 °C overnight. The solvent was removed using a rotary vapor and the solid residue was purified by column and recrystallization in ethanol. Compounds **C6**, **C10**, **C14** and **C15** are a product of this reaction and are a second class of monoester compounds.

#### 5.2.2.4. General synthesis of ester compounds of hydroxybenzoic acid derivatives from phloroglucinol

Acid chloride (7.5 mmol, 1.174 g) was dissolved in 25 mL THF and 10 mL diethyl ether. In a separate beaker phloroglucinol (0.25 mmol, 0.315 g) was dissolved in 30 mL diethyl ether. The two solutions were then mixed and refluxed at 100 °C overnight. The solvent was removed using a rotary vapor and the solid residue was purified by recrystallization in ethanol or column chromatography to yield compounds **C4**, **C5**, **C9**, and **C15**.

#### 5.2.2.5. Summarized characterization results of ester derivative compounds

**3-acetylphenyl 3-hydroxybenzoate (C1).** (hexane/ethyl acetate/methanol (5/4/1)): Y = 82%, **FTIR** (cm<sup>-1</sup>): 3220 (OH stretch), 1684 (C=O), 1590 (C=C, aromatic), 1201 (C-O, stretch); **<sup>1</sup>H-NMR** [DMSO-*d*<sub>6</sub>, 500 MHz] δ<sub>H</sub> (ppm): 9.96 (s, 1H, OH), 9.74 (s, 1H, OH), 8.0 - 8.14 (dd, J=6.1, 1H, Ar), 7.67 – 7.94 (m, 3H, Ar), 7.59 (tt, J=6.6, 1H, Ar), 2.48 (s, 3H, OCH<sub>3</sub>); [**<sup>13</sup>C-NMR** DMSO-*d*<sub>6</sub>, 100 MHz] δ<sub>C</sub> (ppm): 197.80, 167.34, 157.58, 150.54, 138.23, 132.03, 129.96, 129.76, 127.01, 123.30, 120.25, 119.24, 115.86, 114.25, 26.77; **MS** [M + H<sup>+</sup>] (*m/z*): Calc.: 256.28, Found: 256.28; **Formula:** C<sub>15</sub>H<sub>12</sub>O<sub>4</sub>

**3-acetylphenyl 2-hydroxybenzoate (C2).** (hexane/ethyl acetate/methanol (5/4/1)): Y = 82%, **FTIR** (cm<sup>-1</sup>): 3120 (OH stretch), 1695 (C=O), 1585 (C=C, aromatic), 1210 (C-O, stretch); **<sup>1</sup>H-NMR** [DMSO-*d*<sub>6</sub>, 500 MHz] δ<sub>H</sub> (ppm): 9.96 (s, 3H, OH), 7.99 (td, J=7.9, 1H, Ar), 7.77 (dd, J=7.8, 2H, Ar), 2.48 (s, 3H, OCH<sub>3</sub>); [**<sup>13</sup>C-NMR** DMSO-*d*<sub>6</sub>, 100 MHz] δ<sub>C</sub> (ppm): 180.92, 172.09, 161.07, 141.31, 130.84, 113.47, 112.08, 109.58, 24.78; **MS** [M + H<sup>+</sup>] (*m/z*): Calc.: 256.28, Found: 257.08; **Formula:** C<sub>15</sub>H<sub>12</sub>O<sub>4</sub>

**5-acetyl-1,3-phenylene bis(3-hydroxybenzoate) (C3):** (hexane/ethyl acetate/methanol (7/2/1)): Y = 81%, **FTIR** (v/cm<sup>-1</sup>): 3120 (OH stretch), 1695 (C=O), 1585 (C=C, aromatic), 1210 (C-O, stretch); N.D, **<sup>1</sup>H-NMR** [DMSO-*d*<sub>6</sub>, 500 MHz] δ<sub>H</sub> (ppm): 9.64 (s, 2H, OH

symmetrical), 7.98 – 8.10 (m, 1H, Ar), 7.72 – 7.86 (m, 2H, Ar), 6.92 – 7.03 (m, 4H, Ar), 7.40 – 7.66 (m, 3H, Ar), 2.69 – 3.06 (m, 2H, OCH<sub>3</sub>); [<sup>13</sup>C-NMR DMSO-d<sub>6</sub>, 100 MHz] δ<sub>C</sub> (ppm): 197.68, 171.94, 161.14, 158.58, 138.81, 135.67, 130.28, 119.19, 116.73, 113.17, 107.12, 106.17, 26.75; **MS** [M + H<sup>+</sup>] (*m/z*): Calc.: 392.36, Found: 393.20; **Formula**: C<sub>22</sub>H<sub>16</sub>O<sub>7</sub>

**benzene-1,3,5-triyl tris(3-hydroxybenzoate) (C4)**: (hexane/ethyl acetate/methanol (6/3/1)): Y = 80%, **FTIR** v/cm<sup>-1</sup>: 3391 (OH stretch), 1714 (C=O), 1576 (C=C, aromatic), 1050 (C-O, stretch); <sup>1</sup>H-NMR [DMSO-d<sub>6</sub>, 500 MHz] δ<sub>H</sub> (ppm): 8.95 (s, 3H, OHs symm.), 7.73 (d, J=15.5, 3H, Ar), 7.49 – 7.66 (dd, J=26.7, 1H, Ar), 7.36 – 7.45 (m, 1H, Ar), 7.14 (d, J=8.2, 1H, Ar), 5.66 (s, 3H, Ar); [<sup>13</sup>C-NMR DMSO-d<sub>6</sub>, 100 MHz] δ<sub>C</sub> (ppm): 158.89, 157.67, 150.81, 130.10, 127.86, 122.64, 120.60, 116.21, 94.03; **MS** [M + H<sup>+</sup>] (*m/z*): Calc.: 486.43, Found: 487.11; **Formula**: C<sub>27</sub>H<sub>18</sub>O<sub>9</sub>

**benzene-1,3,5-triyl tris(2-hydroxybenzoate) (C5)**. (hexane/ethyl acetate/methanol (6/3/1)): Y = 80%, **FTIR** v/cm<sup>-1</sup>: 3391 (OH stretch), 1714 (C=O), 1576 (C=C, aromatic), 1050 (C-O, stretch); <sup>1</sup>H-NMR [DMSO-d<sub>6</sub>, 500 MHz] δ<sub>H</sub> (ppm): 11.92 (s, 2H, OHs), 7.68 – 7.84 (m, 1H, Ar), 7.38 – 7.66 (m, 1H, Ar), 7.38 – 7.66 (m, 1H, Ar), 5.82 – 5.92 (m, 6H, Ar), 5.66 (s, 3H, Ar); [<sup>13</sup>C-NMR DMSO-d<sub>6</sub>, 100 MHz] δ<sub>C</sub> (ppm): 136.36, 125.13, 122.13, 99.69, 94.28, 83.21, 81.11, 76.89, 58.06; **MS** [M + H<sup>+</sup>] (*m/z*): Calc.: 486.43, Found: 487.09; **Formula**: C<sub>27</sub>H<sub>18</sub>O<sub>9</sub>

**3-acetamidophenyl 3-hydroxybenzoate (C6)**: (hexane/ethyl acetate/methanol (5/4/1)): Y = 88%, **FTIR** v/cm<sup>-1</sup>: 3324 (N-H stretch, 2° amine), 3324 (OH stretch), 1738 (C=O), 1608 (C=C, aromatic), 1218 (C-O, stretch); <sup>1</sup>H-NMR [DMSO-d<sub>6</sub>, 500 MHz] δ<sub>H</sub> (ppm): 10.55 (s, 1H, N-H), 10.18 (s, 3H, OH), 9.86 (s, 3H, Ar), 9.70 (d, 6H, Ar), 9.61 (s, 1H, Ar), 9.31 (d, 5H, Ar), 4.71 (s, 2H, OCH<sub>3</sub>) N.D; [<sup>13</sup>C-NMR [DMSO-d<sub>6</sub>, 100 MHz] δ<sub>C</sub> (ppm)]: 174.05, 170.60, 169.28, 159.97, 146.47, 139.79, 132.79, 131.98, 121.52, 117.26, 114.90, 111.32, 110.28, 20.74; **MS** [M + H<sup>+</sup>] (*m/z*): Calc.: 271.27, Found: 272.28; **Formula**: C<sub>15</sub>H<sub>13</sub>NO<sub>4</sub>

**3-acetylphenyl 3,5-dihydroxybenzoate (C7)**: (hexane/ethyl acetate/methanol (5/4/1)): Y = 84%, **FTIR** (cm<sup>-1</sup>): N.D, <sup>1</sup>H-NMR [DMSO-d<sub>6</sub>, 500 MHz] δ<sub>H</sub> (ppm): 7.40 (d, 4H, Ar), 7.32 (m, 8H, Ar), 7.02 (d, 5H, Ar), 6.81 (s, 2H, Ar), 1.73 (s, 3H, OCH<sub>3</sub>); [<sup>13</sup>C-NMR [DMSO-d<sub>6</sub>, 100 MHz] δ<sub>C</sub> (ppm): 198.2, 167.4, 158.4, 138.1, 132.4, 129.8, 120.3, 119.3, 114.1, 107.3, 67.0, 26.6; **MS** [M + H<sup>+</sup>] (*m/z*): Calc.: 272.26, Found: 273.07; **Formula**: C<sub>15</sub>H<sub>12</sub>O<sub>5</sub>

**5-acetyl-1,3-phenylene bis(3,5-dihydroxybenzoate) (C8)**: (hexane/ethyl acetate/methanol (7/2/1)): Y = **FTIR** (cm<sup>-1</sup>): 3266 (OH stretch), 1687 (C=O), 1604 (C=C, aromatic), 1160 (C-O, stretch); <sup>1</sup>H-NMR [DMSO-d<sub>6</sub>, 500 MHz] δ<sub>H</sub> (ppm): 9.65 (s, 4H, OH symmetrical), 6.76 –

6.86 (m, 5H, Ar), 6.40 – 6.50 (m, 3H, Ar), 4.28 (q, 2H, Ar), 1.32 (s, 3H, OCH<sub>3</sub>); N.D; [<sup>13</sup>C-NMR DMSO-*d*<sub>6</sub>, 100 MHz] δ<sub>C</sub> (ppm): 197.73, 167.40, 165.79, 158.60, 138.84, 132.53, 131.62, 107.74, 106.19, 60.59, 26.78; **MS** [M + H<sup>+</sup>] (*m/z*): Calc.: 424.36, Found: 425.18; **Formula**: C<sub>22</sub>H<sub>16</sub>O<sub>9</sub>

**Benzene-1,3,5-triyl tris(3,5-dihydroxybenzoate) (C9):** (hexane/ethyl acetate/methanol (6/3/1)): Y = 83%, **FTIR** ν/cm<sup>-1</sup>: 3280 (OH stretch), 1700 (C=O), 1602 (C=C, aromatic), 1146 (C-O, stretch); **<sup>1</sup>H-NMR** [DMSO-*d*<sub>6</sub>, 500 MHz] δ<sub>H</sub> (ppm): 9.69 (s, 6H, OHs symm.), 6.82 (d, J=2.3, 2H, Ar), 6.80 (d, J=2.3, 4H, Ar), 5.65 (s, 3H, Ar); [<sup>13</sup>C-NMR DMSO-*d*<sub>6</sub>, 100 MHz] δ<sub>C</sub> (ppm): 167.38, 158.41, 132.52, 107.31, 94.07, 62.56, 60.55; **MS** [M + H<sup>+</sup>] (*m/z*): Calc.: 534.43, Found: 535.09; **Formula**: C<sub>27</sub>H<sub>18</sub>O<sub>12</sub>

**3-acetamidophenyl 3,5-dihydroxybenzoate (C10):** (hexane/ethyl acetate/methanol (5/4/1)): Y = 85%, **FTIR** ν/cm<sup>-1</sup>: 3272 (N-H), 3272 (OH stretch), 1694 (C=O), 1587 (C=C, aromatic), 1155 (C-O, stretch); **<sup>1</sup>H-NMR** [DMSO-*d*<sub>6</sub>, 500 MHz] δ<sub>H</sub> (ppm): 9.76 (s, 1H, N-H), 9.47 (s, 2H, OH symm.), 7.18 (s, 2H, Ar), 7.03 (t, 1H, Ar), 6.92 (d, 1H, Ar), 6.83 (d, 1H, Ar), 6.66 (d, 2H, Ar), 2.01 (s, 3H, OCH<sub>3</sub>); [<sup>13</sup>C-NMR DMSO-*d*<sub>6</sub>, 100 MHz] δ<sub>C</sub> (ppm): 168.3, 167.4, 140.2, 134.9, 132.5, 130.5, 129.3, 113.5, 112.2, 109.8, 107.3, 106.8, 24.0; **MS** [M + H<sup>+</sup>] (*m/z*): Calc.: 287.27, Found: 288.08; **Formula**: C<sub>15</sub>H<sub>13</sub>NO<sub>5</sub>

**3-acetylphenyl 3,4,5-trihydroxybenzoate (C11):** (hexane/ethyl acetate/methanol (5/4/1)): Y = 81%, **FTIR** (cm<sup>-1</sup>): 3140 (OH stretch), 1740 (C=O), 1642 (C=C, aromatic), 1207 (C-O, stretch); **<sup>1</sup>H-NMR** [DMSO-*d*<sub>6</sub>, 500 MHz] δ<sub>H</sub> (ppm): 9.74 (s, 1H, OHs symmetrical), 9.16 (s, 1H, OH asymmetrical), 7.38 (d, J=2.5, 1H, Ar), 7.31 (t, J=4.7, 2H, Ar), 7.00 (d, J=7.90, 1H, Ar), 6.93 (s, 2H, Ar); [<sup>13</sup>C-NMR [DMSO-*d*<sub>6</sub>, 100 MHz] δ<sub>C</sub> (ppm): 197.8, 167.5, 157.5, 145.4, 138.2, 129.7, 120.5, 119.2, 114.2, 108.7, 67.0, 26.7; **MS** [M + H<sup>+</sup>] (*m/z*): Calc.: 288.26, Found: 289.07; **Formula**: C<sub>15</sub>H<sub>12</sub>O<sub>6</sub>

**5-acetyl-1,3-phenylene bis(3,4,5-trihydroxybenzoate) (C12):** (hexane/ethyl acetate/methanol (7/2/1)): Y = 87%, **FTIR** (cm<sup>-1</sup>): 3353 (OH stretch), 1735 (C=O), 1600 (C=C, aromatic), 1222 (C-O, stretch); **<sup>1</sup>H-NMR** [DMSO-*d*<sub>6</sub>, 500 MHz] δ<sub>H</sub> (ppm): 9.64 (s, 4H, OHs symm.), 9.23 (s, 2H, OHs asymm.), 6.97 (s, 6H, Ar), 6.82 (s, 3H, Ar), 6.50 (s, 1H, Ar), 2.54 (s, 2H, OCH<sub>3</sub>); [<sup>13</sup>C-NMR DMSO-*d*<sub>6</sub>, 100 MHz] δ<sub>C</sub> (ppm): 197.7, 167.4, 158.5, 145.4, 137.9, 125.6, 120.4, 108.7, 107.1, 106.1, 26.7; **MS** [M + H<sup>+</sup>] (*m/z*): Calc.: 456.36, Found: 457.03; **Formula**: C<sub>22</sub>H<sub>16</sub>O<sub>11</sub>

**Benzene-1,3,5-triyl tris(3,4,5-trihydroxybenzoate) (C13).** (hexane/ethyl acetate/methanol (6/3/1)): Y = 87%, **FTIR**  $\nu/\text{cm}^{-1}$ : 3252 (OH stretch), 1729 (C=O), 1604 (C=C, aromatic), 1202 (C-O, stretch);  **$^1\text{H-NMR}$**  [DMSO- $d_6$ , 500 MHz]  $\delta_{\text{H}}$  (ppm): 8.39 – 10.35 (m, 9H, OHs symm and unsymm.), 7.14 – 7.00 (m, 2H, Ar), 6.93 (d, 5H, Ar); [ **$^{13}\text{C-NMR}$**  DMSO- $d_6$ , 100 MHz]  $\delta_{\text{C}}$  (ppm): 168.66, 145.42, 137.35, 120.43, 108.71, 98.68, 59.50; **MS** [ $\text{M} + \text{H}^+$ ] ( $m/z$ ): Calc.: 582.43, Found: 583.15; **Formula:**  $\text{C}_{27}\text{H}_{18}\text{O}_{15}$

**3-acetamidophenyl 2-hydroxybenzoate (C14):** (hexane/ethyl acetate/methanol (5/4/1)): Y = 88%, **FTIR**  $\nu/\text{cm}^{-1}$ : 3324 (N-H stretch, 2° amine), 1738 (C=O), 1608 (C=C, aromatic), 1218 (C-O, stretch);  **$^1\text{H-NMR}$**  [DMSO- $d_6$ , 500 MHz]  $\delta_{\text{H}}$  (ppm): 9.76 (s, 1H, N-H), 9.96 (s, 3H, OH & Ar), 7.47 – 7.64 (m, 1), 7.38 (d, 5H, Ar), 7.31 (s, 1H, Ar), 7.27 – 7.29 (m, 4H, Ar), 7.00 (m, 4H, Ar), 3.38 (s, 3H,  $\text{OCH}_3$ ); [ **$^{13}\text{C-NMR}$**  DMSO- $d_6$ , 100 MHz]  $\delta_{\text{C}}$  (ppm): 197.82, 167.35, 164.52, 157.59, 150.86, 138.24, 132.40, 129.77, 126.68, 120.59, 119.26, 115.83, 114.26, 26.78, **MS** [ $\text{M} + \text{H}^+$ ] ( $m/z$ ): Calc.: 271.27, Found: 272.28; **Formula:**  $\text{C}_{15}\text{H}_{13}\text{NO}_4$

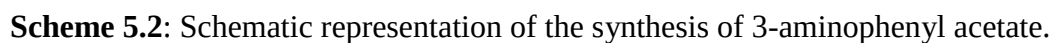
**3-acetamidophenyl 3,4,5-trihydroxybenzoate (C15):** (hexane/ethyl acetate/methanol (5/4/1)): Y = 90%, **FTIR**  $\nu/\text{cm}^{-1}$ : 3321 (NH stretch, 2° amine), 1729 (C=O), 1604 (C=C, aromatic), 1022 (C-O, stretch);  **$^1\text{H-NMR}$**  [DMSO- $d_6$ , 400 MHz]  $\delta_{\text{H}}$  (ppm): 9.78 (s, 1H, N-H), 9.50 (s, 1H, OH) 7.20 (m, 1H, Ar), 7.04 (m, 1H, Ar), 6.94 (m, 3H, Ar), 6.43 (d, 1H, Ar), 2.01 (s, 3H,  $\text{OCH}_3$ ); [ **$^{13}\text{C-NMR}$**  DMSO- $d_6$ , 100 MHz]  $\delta_{\text{C}}$  (ppm): 168.3, 167.5, 157.5, 140.3, 138.0, 129.3, 120.5, 109.8, 108.7, 106.2, 24.0; **MS** [ $\text{M} + \text{H}^+$ ] ( $m/z$ ): Calc.: 303.27, Found: 304.07; **Formula:**  $\text{C}_{15}\text{H}_{13}\text{NO}_6$

### 5.2.3 Synthesis of amide/anilide compounds

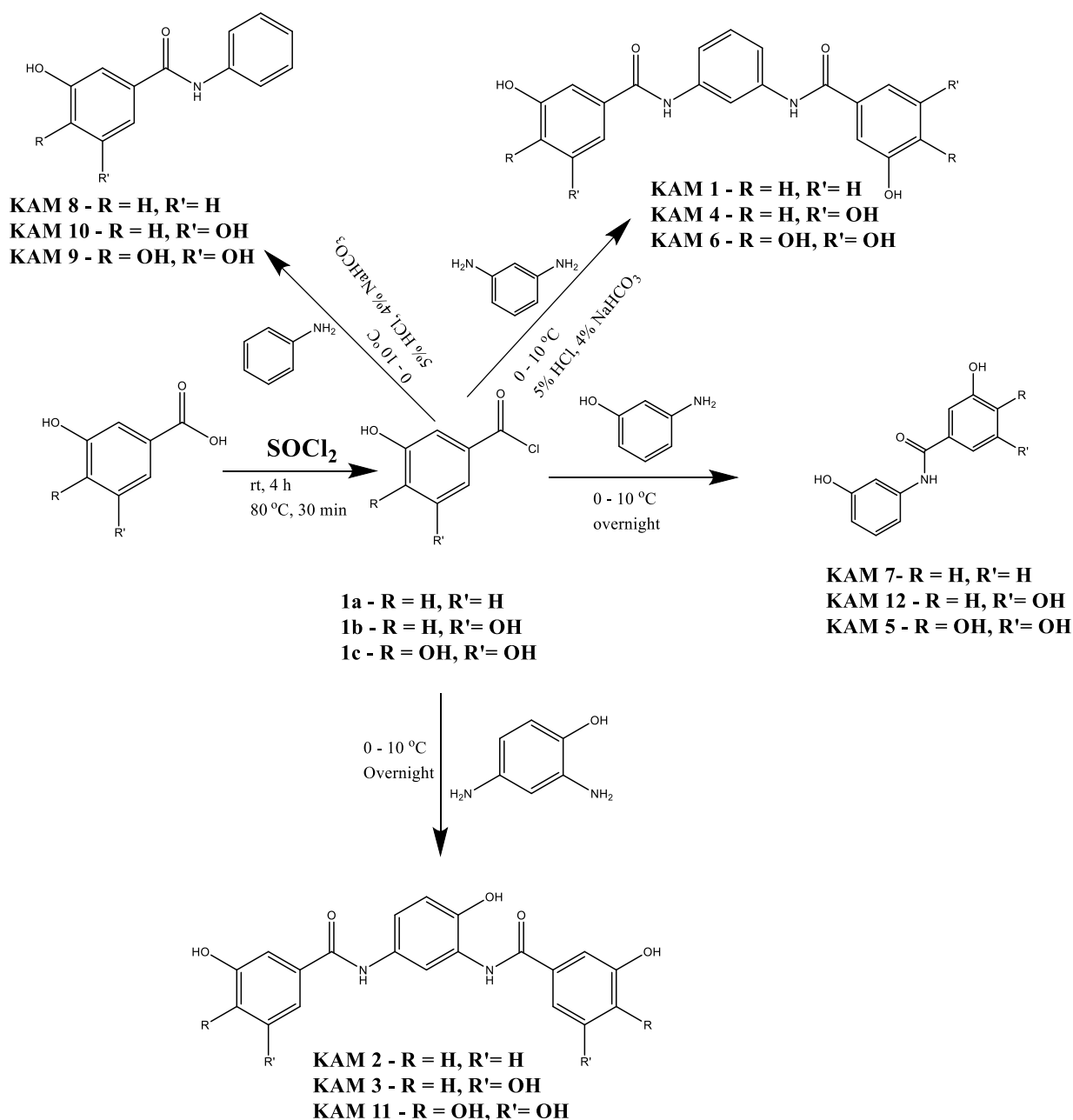
#### 5.2.3.1 Synthesis of intermediate 3-aminophenyl acetate

The reaction scheme 5.2 shows a reaction pathway of 3-aminophenyl acetate intermediate compound that was used to synthesize compounds **KAM 5**, **KAM 7** and **KAM 12**. Briefly, to a mixture of 3-aminophenol (1.2 g, 5.0 mmol) and 4-dimethylaminopyridine (60 mg) was added to a solution of triethylamine (700  $\mu\text{L}$ , 5.0 mmol) and acetic anhydride (480  $\mu\text{L}$ , 5.0 mmol) in anhydrous dichloromethane (50 mL). The reaction mixture was stirred at room temperature for 2 hours and was then concentrated in *vacuo*. The desired product was used in other reactions without further purification. Yield = 97%





Reaction scheme 5.3 shows a pathway to the synthesis of amide/anilide compounds. Similar, to ester compounds amide compounds proceed via intermediate hydroxybenzoic acid chloride (**1b-d**), which are subsequently reacted with mono- or di-amine compounds in lower temperatures (0 – 10 °C). The mono-amides are synthesized via a 1:1 molar ratio of acid chlorides with either aniline or 3-aminophenyl acetate (which was then acetate de-protected in ethanol and 4M KOH). While the di-amide compounds are formed from the reaction of acid chlorides with either 1,3-diaminobenzene or 2,4-diaminophenol.



**Scheme 5.3:** The synthetic procedure of hydroxybenzoic acid amide derivatives (**KAM 1-KAM 12**).

A solution of corresponding amine/aniline (0.1 mol) in diethyl ether (50 mL) was added dropwise to a solution of previously prepared acid chlorides (0.1 mol) in diethyl ether (50 mL) and the reaction was kept at 0-10 °C. After stirring the solution for 30 minutes, the precipitated amide/anilide was separated by filtration. The crude amide was purified by column and recrystallized in ethanol. In the case of anilides, the precipitated crude anilide was treated with

5% hydrochloric acid, 4% sodium carbonate, and water to remove residual aniline, and the resulting anilide was recrystallized with alcohol.

### 5.2.3.3 Summary of characterization results of amide compounds

***N,N'*-(1,3-phenylene)bis(3-hydroxybenzamide) (KAM1):** Hexane: Ethyl Acetate (9:1 to 7:3): Y = 91%, FTIR  $\nu/\text{cm}^{-1}$ : 3274 (NH, stretch), 3274 (OH stretch), 1652 (C=O), 1609 (C=C, aromatic), 1547 (NH, bend), 1367 (C-N, stretch); **<sup>1</sup>H-NMR** [DMSO-*d*<sub>6</sub>, 500 MHz]  $\delta$  (ppm): 9.94 (s, 2H, NHs), 9.69 (s, 1H, OH), 7.38 (t, 1H, Ar), 7.36 (s, 2H, Ar), 7.34 (m, 3H, Ar), 7.28 (d, 2H, Ar), 7.26 (s, 1H, Ar), 7.00 (d, 1H, Ar), 6.98 (d, 1H, Ar); [**<sup>13</sup>C-NMR** DMSO-*d*<sub>6</sub>, 100 MHz]  $\delta$  (ppm): 172.00, 167.31, 157.41, 157.30, 149.64, 139.56, 132.01, 129.56, 129.29, 119.94, 115.80; **MS** [M + H<sup>+</sup>] (*m/z*): Calc. : 348.11, Found: 349.09; **Formula:** C<sub>20</sub>H<sub>16</sub>N<sub>2</sub>O<sub>4</sub>

***N,N'*-(4-hydroxy-1,3-phenylene)bis(3-hydroxybenzamide) (KAM2):** Dichloromethane: Ethyl Acetate (9:1 to 7:3): Y = 89%, FTIR  $\nu/\text{cm}^{-1}$ : 3209 (NH, stretch), 3209 (OH stretch), 1728 (C=O), 1580 (C=C, aromatic), 1510 (NH, bend), 1367 (C-N, stretch); **<sup>1</sup>H-NMR** [DMSO-*d*<sub>6</sub>, 500 MHz]  $\delta$  (ppm): 10.04 (s, 1H, NH<sub>9</sub>), 9.94 (s, 2H, OHs), 9.41 (s, 1H, NH<sub>8</sub>), 9.74 (d, 4H, Ar), 8.15 (d, 1H, Ar), 7.38 (dt, 3H, Ar), 7.30 (d, 2H, Ar), 7.26 (d, 1H, Ar), 7.05 (d, 1H, Ar), 6.89 (s, 1H, Ar); [**<sup>13</sup>C-NMR** DMSO-*d*<sub>6</sub>, 100 MHz]  $\delta$  (ppm): 169.67, 167.88, 167.25, 157.91, 150.89, 133.70, 132.71, 129.94, 127.05, 126.41, 123.07, 120.22, 116.31; **MS** [M + H<sup>+</sup>] (*m/z*): Calc. : 364.11, Found: 365.10; **Formula:** C<sub>20</sub>H<sub>16</sub>N<sub>2</sub>O<sub>5</sub>

***N,N'*-(4-hydroxy-1,3-phenylene)bis(3,5-dihydroxybenzamide) (KAM3):** Dichloromethane : Ethyl Acetate (9:1 to 7:3): Y = 83%, FTIR  $\nu/\text{cm}^{-1}$ : 3452 (NH, stretch), 3452 (OH stretch), 1739 (C=O), 1592 (C=C, aromatic), 1439 (NH, bend), 1369 (C-N, stretch); **<sup>1</sup>H-NMR** [DMSO-*d*<sub>6</sub>, 500 MHz]  $\delta$  (ppm): 9.94 (s, 1H, NH<sub>9</sub>), 9.68 (s, 1H, OHs), 9.88 (s, 1H, NH<sub>8</sub>), 9.55 (s, 4H, Ar), 8.54 (m, 1H, Ar), 6.79 (d, 4H, Ar), 6.40 (d, 2H, Ar); [**<sup>13</sup>C-NMR** DMSO-*d*<sub>6</sub>, 100 MHz]  $\delta$  (ppm): 169.98, 167.42, 159.34, 151.66, 150.49, 134.32, 133.26, 120.89, 113.36, 108.30, 107.21; **MS** [M + H<sup>+</sup>] (*m/z*): Calc. : 396.36 , Found: 396.10; **Formula:** C<sub>20</sub>H<sub>16</sub>N<sub>2</sub>O<sub>7</sub>

***N,N'*-(1,3-phenylene)bis(3,5-dihydroxybenzamide) (KAM4):** Hexane: Ethyl Acetate (9:1 to 7:3): Y = 79%, FTIR  $\nu/\text{cm}^{-1}$ : 3452 (NH, stretch), 3452 (OH stretch), 1739 (C=O), 1592 (C=C, aromatic), 1439 (NH, bend), 1369 (C-N, stretch); **<sup>1</sup>H-NMR** [DMSO-*d*<sub>6</sub>, 500 MHz]  $\delta$  (ppm): 10.10 (s, 1H, NH), 9.68 (s, 2H, OH), 9.94 (s, 1H, Ar), 7.43 (dd, 1H, Ar), 7.35 (t, 1H, Ar), 6.78 (d, 1H, Ar), 6.70 (s, 1H, Ar); [**<sup>13</sup>C-NMR** DMSO-*d*<sub>6</sub>, 100 MHz]  $\delta$  (ppm): 167.97, 167.43, 165.72, 158.35, 154.62, 149.00, 137.15, 107.28, 105.84; **MS** [M + H<sup>+</sup>] (*m/z*): Calc. : 380.10, Found: 381.10; **Formula:** C<sub>20</sub>H<sub>16</sub>N<sub>2</sub>O<sub>6</sub>

**3,4,5-trihydroxy-*N*-(3-hydroxyphenyl)benzamide (KAM5):** Hexane: Ethyl Acetate: Methanol (7:2:1): Y = 81%, FTIR  $\nu/\text{cm}^{-1}$ : 3457 (NH, stretch), 3101 (OH stretch), 1739 (C=O), 1536 (C=C, aromatic), 1432 (NH, bend), 1361 (C-N, stretch);  $^1\text{H-NMR}$  [DMSO-*d*<sub>6</sub>, 500 MHz]  $\delta$  (ppm): 9.68 (bs, 1H, NH & OH), 7.24 (t, 2H, Ar), 6.72 – 6.82 (m, 6) N.D; [ $^{13}\text{C-NMR}$  DMSO-*d*<sub>6</sub>, 100 MHz]  $\delta_{\text{C}}$  (ppm): 167.94, 158.77, 146.82, 145.92, 142.04, 138.40, 127.81, 120.95, 115.66, 109.34; **MS** [ $\text{M} + \text{H}^+$ ] ( $m/z$ ): Calc. : 261.06, Found: 262.10; **Formula:** C<sub>13</sub>H<sub>11</sub>NO<sub>5</sub>

***N,N'*-(1,3-phenylene)bis(3,4,5-trihydroxybenzamide) (KAM6):** Hexane: Ethyl Acetate (9:1 to 7:3): Y = 93%, FTIR  $\nu/\text{cm}^{-1}$ : 3457 (NH, stretch), 3212 (OH stretch), 1739 (C=O), 1648 (C=C, aromatic), 1544 (NH, bend), 1369 (C-N, stretch);  $^1\text{H-NMR}$  [DMSO-*d*<sub>6</sub>, 500 MHz]  $\delta$  (ppm): 9.94 (s, 2H, NH), 9.19 (s, 2H, OHs symm.), 8.84 (s, 1H, OH asymm.), 7.97 (s, 1H, Ar), 7.87 (t, 1H, Ar), 7.25 (d, 1H, Ar), 6.91 (s, 2H, Ar); [ $^{13}\text{C-NMR}$  DMSO-*d*<sub>6</sub>, 100 MHz]  $\delta$  (ppm): 171.98, 167.47, 165.45, 154.07, 149.62, 145.37, 137.39, 120.45, 108.64; **MS** [ $\text{M} + \text{H}^+$ ] ( $m/z$ ): Calc. : 412.09, Found: 413.22; **Formula:** C<sub>20</sub>H<sub>16</sub>N<sub>2</sub>O<sub>8</sub>

**3-hydroxy-*N*-phenylbenzamide (KAM7):** Hexane: Ethyl Acetate: Methanol (7:2:1): Y = 87%, FTIR  $\nu/\text{cm}^{-1}$ : 3457 (NH, stretch), 3079 (OH stretch), 1746 (C=O), 1586 (C=C, aromatic), 1439 (NH, bend), 1369 (C-N, stretch);  $^1\text{H-NMR}$  [DMSO-*d*<sub>6</sub>, 500 MHz]  $\delta$  (ppm): 9.96 (bs, 1H, NH & OH), 7.59 (d, 2H, Ar), 7.28 (t, 2H, Ar), 7.02 (t, 1H, Ar), 6.95 (s, 1H, Ar); [ $^{13}\text{C-NMR}$  DMSO-*d*<sub>6</sub>, 100 MHz]  $\delta$  (ppm): 168.77, 162.79, 149.05, 145.90, 139.80, 129.09, 123.42, 119.45, 116.11, 114.34, 109.24; **MS** [ $\text{M} + \text{H}^+$ ] ( $m/z$ ): Calc. : 229.07, Found: 230.07; **Formula:** C<sub>13</sub>H<sub>11</sub>NO<sub>3</sub>

**3-hydroxy-*N*-(3-hydroxyphenyl)benzamide (KAM8):** Hexane: Ethyl Acetate: Methanol (7:2:1): Y = 87%, FTIR  $\nu/\text{cm}^{-1}$ : 3457 (NH, stretch), 3079 (OH stretch), 1739 (C=O), 1536 (C=C, aromatic), 1439 (NH, bend), 1369 (C-N, stretch);  $^1\text{H-NMR}$  [DMSO-*d*<sub>6</sub>, 500 MHz]  $\delta$  (ppm): 10.05 (s, 1H, NH), 9.89 (s, 1H, OH), 7.35 (d, 4H, Ar), 7.19 (t, 1H, Ar), 6.96 – 6.98 (m, 3H, Ar), 6.76 (t, 1H, Ar), 6.43 (dd, 1H, Ar); [ $^{13}\text{C-NMR}$  DMSO-*d*<sub>6</sub>, 100 MHz]  $\delta$  (ppm): 168.22, 167.58, 165.58, 158.11, 157.43, 149.83, 140.40, 129.43, 129.22, 119.92, 119.59, 118.15, 115.88; **MS** [ $\text{M} + \text{H}^+$ ] ( $m/z$ ): Calc. : 229.07, Found: 230.07; **Formula:** C<sub>13</sub>H<sub>11</sub>NO<sub>3</sub>

**3,4,5-trihydroxy-*N*-phenylbenzamide (KAM9):** Hexane: Ethyl Acetate: Methanol (7:2:1): Y = 73%, FTIR  $\nu/\text{cm}^{-1}$ : 3530 (NH, stretch), 3319 (OH stretch), 1520 (C=O), 1443 (C=C, aromatic), 1401 (NH, bend), 1341 (C-N, stretch);  $^1\text{H-NMR}$  [DMSO-*d*<sub>6</sub>, 500 MHz]  $\delta$  (ppm): 9.95 (bs, 1H, NH & OH overlap), 7.57 (d, 2H, Ar), 7.27 (t, 2H, Ar), 7.01 (t, 1H, Ar), 6.88 (s, 3H, Ar); [ $^{13}\text{C-NMR}$  DMSO-*d*<sub>6</sub>, 100 MHz]  $\delta$  (ppm): 166.98, 158.01, 136.78, 132.10, 128.58,

128.15, 117.23, 114.19, 106.91, 106.41; **MS** [ $M + H^+$ ] ( $m/z$ ): Calc. : 245.07, Found: 246.07;

**Formula:**  $C_{13}H_{11}NO_4$

**3,5-dihydroxy-N-phenylbenzamide (KAM10):** Hexane: Ethyl Acetate: Methanol (7:2:1): Y = 69%, FTIR  $\nu/cm^{-1}$ : 3282 (NH, stretch), 3226 (OH stretch), 1753 (C=O), 1592 (C=C, aromatic), 1502 (NH, bend), 1348 (C-N, stretch);  **$^1H$ -NMR** [DMSO- $d_6$ , 500 MHz]  $\delta$  (ppm): 9.58 (bs, 2H, NH & OH overlap), 7.32 (t, 1H, Ar), 7.08 (t, 3H, Ar), 6.77 (d, 1H, Ar), 6.59 – 6.72 (m, 4H, Ar), 6.42 (s, 1H, Ar); [ **$^{13}C$ -NMR** DMSO- $d_6$ , 100 MHz]  $\delta$  (ppm): 168.28, 145.34, 139.31, 137.01, 136.93, 128.61, 122.92, 118.93, 108.73; **MS** [ $M + H^+$ ] ( $m/z$ ): Calc. : 229.07, Found: 230.07; **Formula:**  $C_{13}H_{11}NO_3$

**N,N'-(4-hydroxy-1,3-phenylene)bis(3,4,5-trihydroxybenzamide)(KAM11):**

Dichloromethane : Ethyl Acetate (9:1 to 7:3): Y = 72%, FTIR  $\nu/cm^{-1}$ : 3290 (NH, stretch), 3280 (OH stretch), 1753 (C=O), 1600 (C=C, aromatic), 1530 (NH, bend), 1334 (C-N, stretch);  **$^1H$ -NMR** [DMSO- $d_6$ , 500 MHz]  $\delta$  (ppm): 10.32 (bs, 3H, NH & OH overlap), 10.08 (bs, 5H, OH & Ar overlap), 7.24 (t, 1H, Ar), 6.92 (s, 1H, Ar), 6.80 (d, 1H, Ar), 6.78 (d, 1H, Ar); [ **$^{13}C$ -NMR** DMSO- $d_6$ , 100 MHz]  $\delta$  (ppm): 167.50, 158.38, 145.47, 137.99, 132.95, 130.51, 120.49, 114.95, 113.33, 110.14, 108.82; **MS** [ $M + H^+$ ] ( $m/z$ ): Calc. : 428.09, Found: 429.09; **Formula:**  $C_{20}H_{16}N_2O_9$

**3,5-dihydroxy-N-(3-hydroxyphenyl)benzamide (KAM12):** Hexane: Ethyl Acetate: Methanol (7:2:1): Y = 81%, FTIR  $\nu/cm^{-1}$ : 3457 (NH, stretch), 3220 (OH stretch), 1757 (C=O), 1551 (C=C, aromatic), 1448 (NH, bend), 1363 (C-N, stretch);  **$^1H$ -NMR** [DMSO- $d_6$ , 500 MHz]  $\delta$  (ppm): 10.31 (s, 1H, NH), 10.07 (s, 1H, OH), 9.96 (s, 1H, OH-phenylbenzamide), 7.84 – 7.94 (m, 3H, Ar), 7.52 – 7.66 (m, 2H, Ar), 7.33 – 7.45 (m, 1H, Ar), 7.08 – 7.31 (m, 1H, Ar), 6.76 (t, 1H, Ar), 6.68 – 6.73 (m, 1H, Ar); [ **$^{13}C$ -NMR** DMSO- $d_6$ , 100 MHz]  $\delta$  (ppm): 170.12, 168.67, 158.35, 140.83, 138.93, 129.63, 110.64, 110.12, 107.79, 106.70, 105.15; **MS** [ $M + H^+$ ] ( $m/z$ ): Calc. : 245.07, Found: 246.07; **Formula:**  $C_{13}H_{11}NO_4$

## 5.3 Results

### 5.3.1 Elucidation of ester compounds using FTIR spectroscopy

Ester derivatives of hydroxybenzoic acid were successfully synthesized, as outlined in Scheme 5.1. The physicochemical properties and spectral findings of these derivatives are detailed in Table 5.1. For the characterization of these compounds, Fourier-transform infrared (FTIR)

spectroscopy was employed, confirming the conjugation and formation of the ester compounds. The distinctive absorption peaks of these hydroxybenzoic acid (HBA) derivatives are illustrated in Figure 5.1 and summarized in Table 5.1.

The FTIR spectra for all ester compounds exhibited nearly identical vibrational peaks, albeit with slight shifts corresponding to the specific compounds conjugated onto the hydroxybenzoic acid backbone. Notably, a broad FTIR spectrum in the range of  $2500 - 3500\text{ cm}^{-1}$  was observed for all compounds, attributed to the hydroxyl groups of the hydroxybenzoic acid derivatives. The broadness of this peak varied among the ester derivatives, and in cases where conjugation with *N*-3-Hydroxyphenyl *N*-phenylacetamide occurred, the hydroxyl peak overlapped with the  $\nu\text{-N-H}$  and  $\text{sp}^2\text{ }\nu\text{-CH}$  peaks, displaying a single spike indicative of a secondary amine.

Carbonyl peaks were observed in the range of  $1684 - 1740\text{ cm}^{-1}$ , varying based on the type of ester formed. Taking compound C1 as an example, the characteristic bands for hydroxyl ( $\nu\text{-OH}$ ), carbonyl ( $\nu\text{-C=O}$ ), aryl ( $\nu\text{-C=C}$ ), and ester ( $\nu\text{-C-O}$ ) bonds were identified. The hydroxyl peak manifested as a broad band at  $3220\text{ cm}^{-1}$ , while the carbonyl stretching vibration occurred at  $1684\text{ cm}^{-1}$ . The aryl and ester peaks ( $\nu\text{-C-O}$ ) appeared at  $1590\text{ cm}^{-1}$  and  $1201\text{ cm}^{-1}$ , respectively. Typically, the  $\text{-C=O}$  stretching vibration of a ketone/ester absorbs above  $1700\text{ cm}^{-1}$  in IR spectroscopy. However, the absorption at a lower frequency can be explained by conjugation with adjacent double bonds or aromatic rings, which can delocalize the electrons and reduce the frequency of the carbonyl stretching vibration. Additionally, hydrogen bonding interactions involving the carbonyl group can also result in a shift to a lower frequency

These results collectively suggest the successful conjugation of reactants and the formation of the anticipated ester compounds. The detailed characteristic bands for each compound are comprehensively presented in Table 5.1, providing a thorough overview of the spectroscopic fingerprints associated with the synthesized hydroxybenzoic acid ester derivatives.

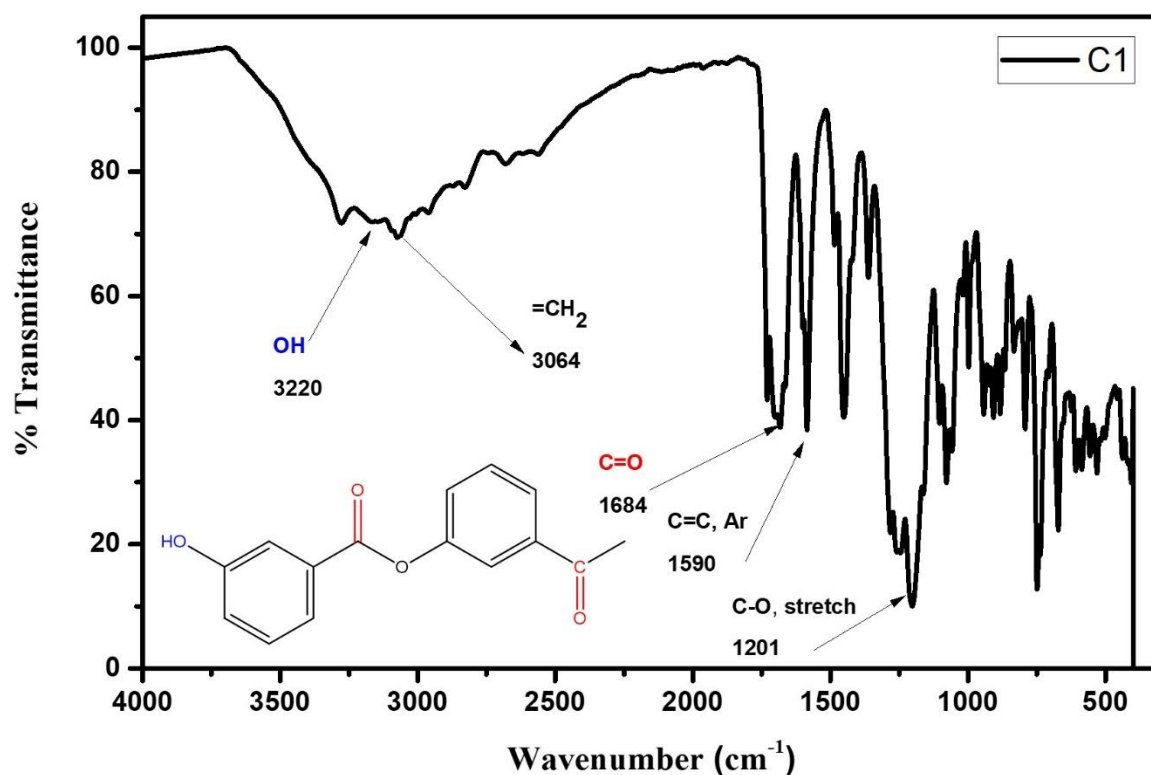


**Figure 5. 1:** FTIR graph of 3-acetylphenyl 3-hydroxybenzoate (C1)

**Table 5. 1:** Characteristic peaks of ester compounds (C1-C15)

| Compound | MW<br>(g/mol) | Yield<br>(%) | IR $\nu$ (cm <sup>-1</sup> ) |      |      |      |      |
|----------|---------------|--------------|------------------------------|------|------|------|------|
|          |               |              | N-H                          | OH   | C=O  | C=C  | C-O  |
| C1       | 256.26        | 82           | -                            | 3220 | 1684 | 1590 | 1201 |
| C2       | 256.26        | 84           | -                            | 3120 | 1695 | 1585 | 1210 |
| C3       | 392.36        | 81           | -                            | 3260 | 1693 | 1601 | 1171 |
| C4       | 486.43        | 87           | -                            | 3391 | 1714 | 1576 | 1050 |
| C5       | 486.43        | 72           | -                            | 3281 | 1701 | 1587 | 1032 |
| C6       | 271.27        | 71           | 3324                         | 3324 | 1738 | 1608 | 1218 |
| C7       | 272.26        | 88           | -                            | 3263 | 1730 | 1600 | 1201 |
| C8       | 424.36        | 85           | -                            | 3266 | 1687 | 1604 | 1160 |

|     |        |    |      |      |      |      |      |
|-----|--------|----|------|------|------|------|------|
| C9  | 534.43 | 90 | -    | 3280 | 1700 | 1602 | 1146 |
| C10 | 287.27 | 80 | 3272 | 3272 | 1694 | 1587 | 1155 |
| C11 | 288.26 | 83 | -    | 3140 | 1740 | 1642 | 1207 |
| C12 | 456.36 | 87 | -    | 3353 | 1735 | 1600 | 1222 |
| C13 | 582.43 | 77 | -    | 3252 | 1729 | 1604 | 1202 |
| C14 | 271.27 | 82 | 3321 | 3321 | 1741 | 1602 | 1205 |
| C15 | 303.27 | 81 | 3321 | 3321 | 1729 | 1604 | 1022 |

### 5.3.2 Elucidation of amide compounds using FTIR spectroscopy

FTIR spectroscopy played a crucial role in characterizing the amide compounds derived from hydroxybenzoic acid, offering valuable insights into the successful formation of amide bonds. The resulting spectra exhibited distinctive vibrational peaks, providing clear indications of the chemical transformations.

To determine the formation of amide derivatives of hydroxybenzoic acids, various vibrational peaks were analyzed, encompassing amine ( $2^\circ$  NH, stretch), hydroxyl ( $\nu$ -OH), carbonyl ( $\nu$ -C=O), aryl ( $\nu$ -C=C), amide ( $\nu$ -NH, bend), and amide ( $\nu$ -C-N, stretch). Taking KAM 1 (shown in Fig 5.2) as an illustrative example, characteristic peaks were observed at  $3274\text{ cm}^{-1}$  ( $\nu$ -NH stretch),  $3274\text{ cm}^{-1}$  ( $\nu$ -OH),  $1652\text{ cm}^{-1}$  ( $\nu$ -C=O),  $1609\text{ cm}^{-1}$  ( $\nu$ -C=C),  $1547\text{ cm}^{-1}$  ( $\nu$ -NH bend), and  $1367\text{ cm}^{-1}$  ( $\nu$ -C-N stretch), as depicted in Fig. 5.2.

The secondary amine ( $\nu$ -NH, stretch) peak for all compounds fell within the range of  $3217 - 3457\text{ cm}^{-1}$ , while the  $\nu$ -OH peak of the hydroxybenzoic acid backbone compounds spanned from  $3079$  to  $3452\text{ cm}^{-1}$ . Carbonyl ( $\nu$ -C=O) peaks were identified within the range of  $1520 - 1757\text{ cm}^{-1}$ , and aromatic  $\nu$ -C=C peaks appeared between  $1443$  and  $1648\text{ cm}^{-1}$ . The amide II ( $\nu$ -NH, bend) peak emerged in the range of  $1401 - 1592\text{ cm}^{-1}$ , whereas the amide  $\nu$ -CN stretch manifested within the  $1334 - 1369\text{ cm}^{-1}$  range. These vibrational peaks were attributed to specific moieties integrated into the hydroxybenzoic acid backbone structure.

Summarizing the findings, Table 5.2 outlines the vibrational peaks for all the compounds, shedding light on the distinct characteristics associated with amide bond formation in hydroxybenzoic acid derivatives.



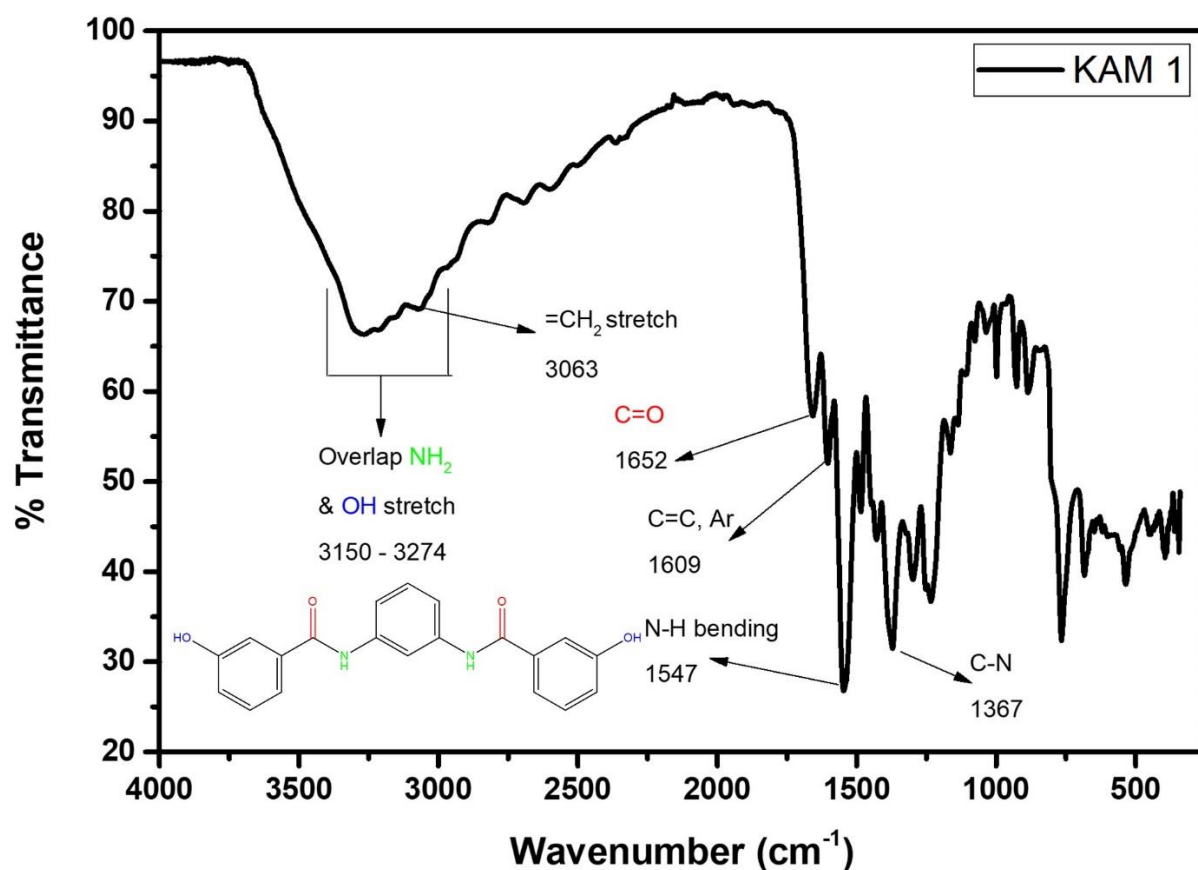


**Figure 5. 2:** FTIR graph of N,N'-(1,3-phenylene)bis(3-hydroxybenzamide) (**KAM 1**)

**Table 5. 2:** Summary of the characteristic peaks of amide compounds (KAM 1-KAM 12)

|          |               |              | IR $\nu$ (cm <sup>-1</sup> ) |      |      |      |              |      |
|----------|---------------|--------------|------------------------------|------|------|------|--------------|------|
| Compound | MW<br>(g/mol) | Yield<br>(%) | N-H                          | OH   | C=O  | C=C  | N-H,<br>bend | C-N  |
| KAM 1    | 348.36        | 91           | 3274                         | 3274 | 1652 | 1609 | 1547         | 1367 |
| KAM 2    | 364.36        | 89           | 3209                         | 3209 | 1728 | 1580 | 1510         | 1367 |
| KAM 3    | 396.36        | 83           | 3452                         | 3452 | 1739 | 1592 | 1439         | 1369 |
| KAM 4    | 380.36        | 79           | 3217                         | 3217 | 1739 | 1648 | 1592         | 1369 |
| KAM 5    | 261.23        | 81           | 3457                         | 3101 | 1739 | 1536 | 1432         | 1361 |
| KAM 6    | 412.35        | 93           | 3457                         | 3212 | 1739 | 1648 | 1544         | 1369 |

|        |        |    |      |      |      |      |      |      |
|--------|--------|----|------|------|------|------|------|------|
| KAM 7  | 229.24 | 87 | 3457 | 3079 | 1746 | 1586 | 1439 | 1369 |
| KAM 8  | 213.24 | 82 | 3457 | 3079 | 1739 | 1536 | 1439 | 1369 |
| KAM 9  | 245.23 | 73 | 3530 | 3319 | 1520 | 1443 | 1401 | 1341 |
| KAM 10 | 229.24 | 69 | 3282 | 3226 | 1753 | 1592 | 1502 | 1348 |
| KAM 11 | 428.35 | 72 | 3290 | 3280 | 1753 | 1600 | 1530 | 1334 |
| KAM 12 | 245.23 | 81 | 3457 | 3220 | 1757 | 1551 | 1448 | 1363 |

### 5.3.3 Elucidation of ester compounds using $^1\text{H}$ -NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy was also used to corroborate the FTIR results that were obtained. Since the conjugation of the ester compounds was through different conjugates, the compounds were classified as follows; mono-ester, bis-substituted/di-substituted, tri-substituted and mono-substitution with *N*-3-hydroxyphenylacetamide moiety that possess both the ester (shown in Fig. 5.3 (a) – (d)). The monoesters are divided into two classes; the first is the monoesters derived from 3-hydroxyacetophenone, shown in Fig. 5.3 (a). Then the second class of monoesters is derived from *N*-3-hydroxyphenylacetamide and these compounds possess both the ester and amide linkages as shown in Fig. 5.3 (b). The compounds were then grouped according to their conjugates.

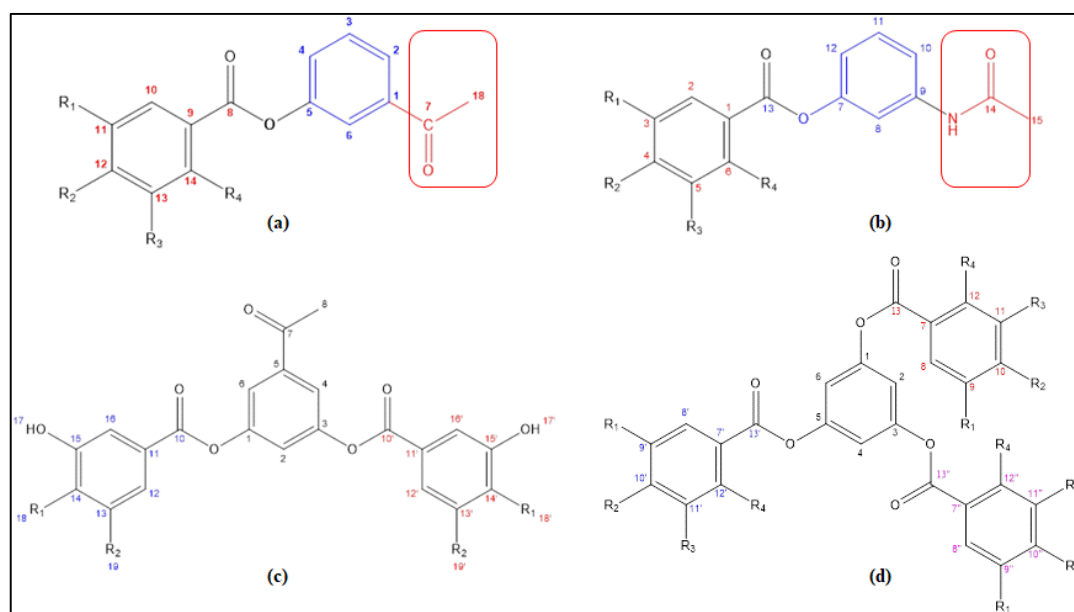


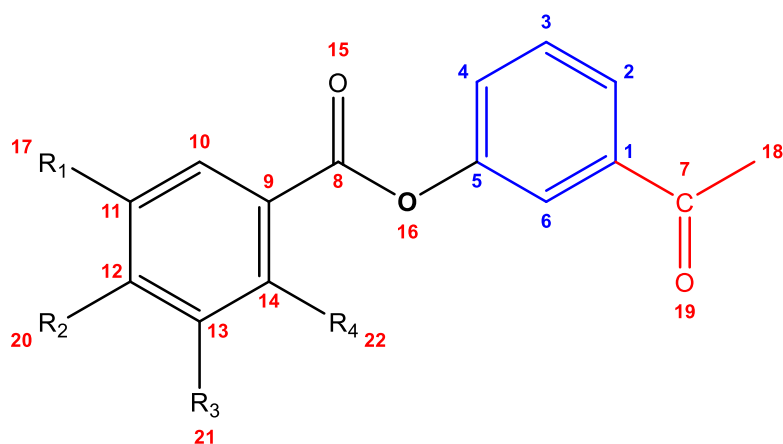
**Figure 5. 3:** Classifications of ester according to their substitutions (a) mono-ester from acetophenone, (b) mono-ester from *N*-phenylacetamide, (c) di-esters, and (d) tri-esters.

#### 5.3.3.1 $^1\text{H}$ and $^{13}\text{C}$ NMR results of mono-substituted ester compounds derived from 3-hydroxyacetophenone (first class of monoester compounds)

Scheme 5.4 outlines the labelling process for mono-substituted hydroxybenzoic acid ester compounds derived from 3-hydroxyacetophenone (first class monoester compounds). The proton NMR spectral results, as detailed in Table 5.3 (Appendix D1), display signals corresponding to the conjugation of 3-hydroxybenzoic acid (**C1**), salicylic acid (**C2**), 3,5-dihydroxybenzoic acid (**C7**), and gallic acid (**C11**) with 3-hydroxyacetophenone. The upfield most signal was attributed to the methyl moiety of acetophenone and it varied with conjugated hydroxybenzoic acid compounds, these methyl protons ranged from  $\delta_{\text{H}}$ -1.73 to 2.48 ppm, and on  $^{13}\text{C}$  from  $\delta_{\text{C}}$ -24.78 – 26.80 ppm (Table 5.4 in Appendix D1). Notably, compounds **C2** and **C11** lack the  $-\text{CH}_3$  proton signal, possibly due to spin-related reasons or overlap with solvent signals upon conjugation.

The downfield signal corresponds to the hydroxyl proton and the carbon bonded to the hydroxyl group. These signals vary with the conjugated hydroxybenzoic acid pendant group, appearing between  $\delta_{\text{H}}$  9.16 and 9.96 ppm in proton NMR, and between  $\delta_{\text{C}}$  145.50 and 161.07 ppm in  $^{13}\text{C}$  NMR (Tables 5.3 and 5.4 respectively in Appendix D1). Taking compound **C11** for example as shown in Fig. 5.4 (a) and (b), the upfield signal for  $-\text{CH}_3$  protons on the acetophenone moiety may overlap with the solvent  $\text{DMSO}-d_6$  peak, but is observed on the carbon spectra at  $\delta_{\text{C}}$ -26.80 ppm, downfield compared to other derivatives. Aromatic ring protons appear in the downfield region ( $\delta_{\text{H}}$ -6.93 – 7.38 ppm), with chemically equivalent protons ( $\text{H}_{10}$  and  $\text{H}_{14}$ ) exhibiting the most upfield singlet signal at  $\delta$ -6.93 ppm (shown in Fig. 5.4 (a)). The 3-acetylphenyl moiety contributes doublet for  $\text{H}_2$  at 7.38 ppm with a J-coupling of 2.5 Hz and integration of 1 and  $\text{H}_4$  at 7.00 ppm with a J-coupling of 7.90 Hz with integration of 1. Furthermore, the triplet signal in the acetophenone motif appeared at 7.30 ppm with a J-coupling of 4.7 Hz, while the gallic acid moiety yields singlet signals. Downfield signals at  $\delta_{\text{H}}$ -9.74 (symmetrical) and 9.16 ppm (asymmetrical) correspond to the hydroxyl moiety of the hydroxybenzoic acid motif, appearing as singlet signals.

Carbon spectra shown in Fig. 5.4 (b) confirm successful conjugation, showing signals for both acetophenone and hydroxybenzoate moieties. The most downfield signal at 197.90 ppm corresponds to the acetate carbonyl ( $\text{C}_7$ ) signal, while the carbon signal ( $\text{C}_8$ ) at 167.57 ppm indicates successful ester conjugation.



**C1** - R<sub>1</sub>= OH, R<sub>2</sub>= H, R<sub>3</sub>= H, R<sub>4</sub>= H

**C2** - R<sub>1</sub>= H, R<sub>2</sub>= H, R<sub>3</sub>= H, R<sub>4</sub>= OH

**C7** - R<sub>1</sub>= OH, R<sub>2</sub>= H, R<sub>3</sub>= OH, R<sub>4</sub>= H

**C11** - R<sub>1</sub>= OH, R<sub>2</sub>= OH, R<sub>3</sub>= OH, R<sub>4</sub>= H

**Scheme 5.4:** <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy labelling of monoesters derived from 3-hydroxyacetophenone to make first class of monoesters (**C1, C2, C7 and C11**)

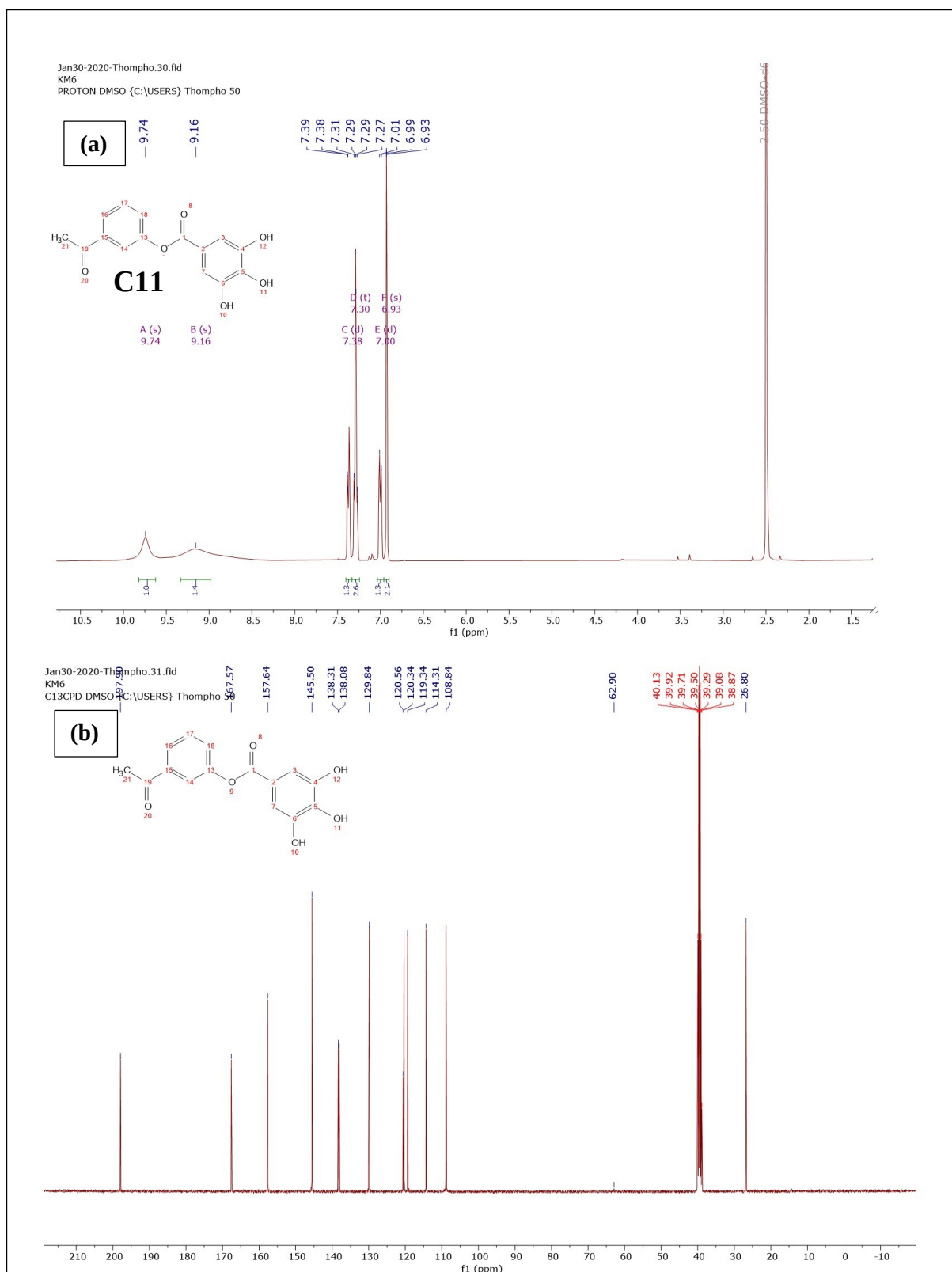


**Figure 5. 4:** NMR spectrum of 3-acetylphenyl 3,4,5-trihydroxybenzoate (**C11**) in DMSO-d<sub>6</sub> (a) <sup>1</sup>H at 500 MHz and (b) <sup>13</sup>C at 100 MHz

### 5.3.3.2 2D NMR results of mono-ester compounds derived from *N*-3-hydroxyphenylacetamide (second class of monoester compounds)

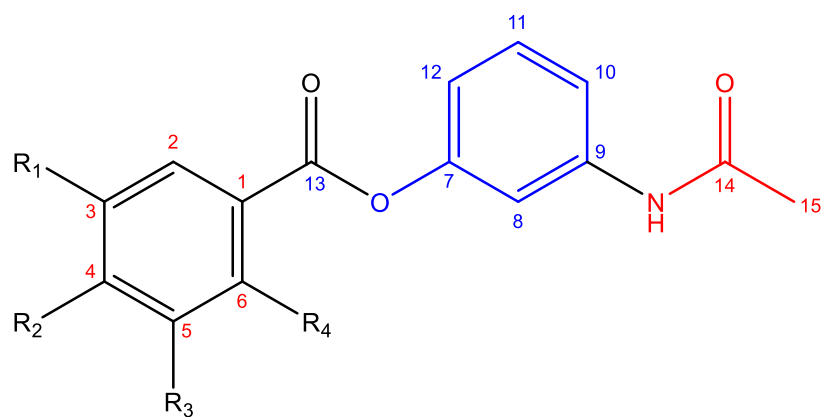
In scheme 5.5, the schematic labelling of *N*-3-hydroxyphenylacetamide-based monoester compounds is depicted, with *N*-3-hydroxyphenylacetamide serving as the core molecule for the substitution of hydroxybenzoic acid derivatives.

The 2D results for compound **C15** were used as an example to show the correlation between protons and carbons. Correlation Spectroscopy (COSY) that involves correlation between the protons which are coupled to each other<sup>305</sup>. Additionally, Heteronuclear Multiple Bond Correlation (HMBC) results reveals correlations between carbons and protons that are separated by two, three, and occasionally four bonds, especially in conjugated systems<sup>305</sup>.

The COSY results showed that the singlet proton H<sub>8</sub> showed a weak correlation with the solvent peak at  $\delta_{\text{H}}$ -6.45 ppm. Moreover, the H<sub>8</sub> also correlated strongly with protons H<sub>10</sub> and H<sub>11</sub>, a doublet and a triplet respectively as shown in Fig 5.5 (a). Protons H<sub>10</sub> and H<sub>12</sub> both showed strong correlation with the H<sub>11</sub> proton, suggesting possible fast overlapping protons, since they resonate in closely related chemical environments.

The HMBC results, on the other hand, showed the correlation between the protons and carbons as previously stated. The H<sub>8</sub> singlet proton was again correlated with both of the  $\alpha$ -carbons (C<sub>9</sub> and C<sub>7</sub> refer to scheme 5.5 for reference in structural labelling). In addition, the H<sub>8</sub> proton strongly correlated with C<sub>12</sub> as shown in Fig. 5.5 (b). The triplet signal ascribed to H<sub>11</sub> strongly with both its  $\beta$ - carbons (C<sub>7</sub> and C<sub>9</sub>). For confirming successful conjugation the H<sub>11</sub> further showed a strong correlation with the hydroxybenzoate carbonyl carbon (C<sub>13</sub>).

The <sup>1</sup>H and <sup>13</sup>C NMR spectra results are shown in Appendix B1 for reference on the proton and carbon. The results of all the first class and second monoester compounds are summarized shown in Appendix D1.



**C6** - R<sub>1</sub> = OH, R<sub>2</sub> = H, R<sub>3</sub> = H, R<sub>4</sub> = H  
**C10** - R<sub>1</sub> = OH, R<sub>2</sub> = H, R<sub>3</sub> = OH, R<sub>4</sub> = H  
**C14** - R<sub>1</sub> = H, R<sub>2</sub> = H, R<sub>3</sub> = H, R<sub>4</sub> = OH  
**C15** - R<sub>1</sub> = OH, R<sub>2</sub> = OH, R<sub>3</sub> = OH, R<sub>4</sub> = H

**Scheme 5.5:** 2D NMR spectroscopy labelling of monoester compounds derived from *N*-3-hydroxyphenylacetamide to make the second class monoester compounds (**C6**, **C10**, **C14** and **C15**).

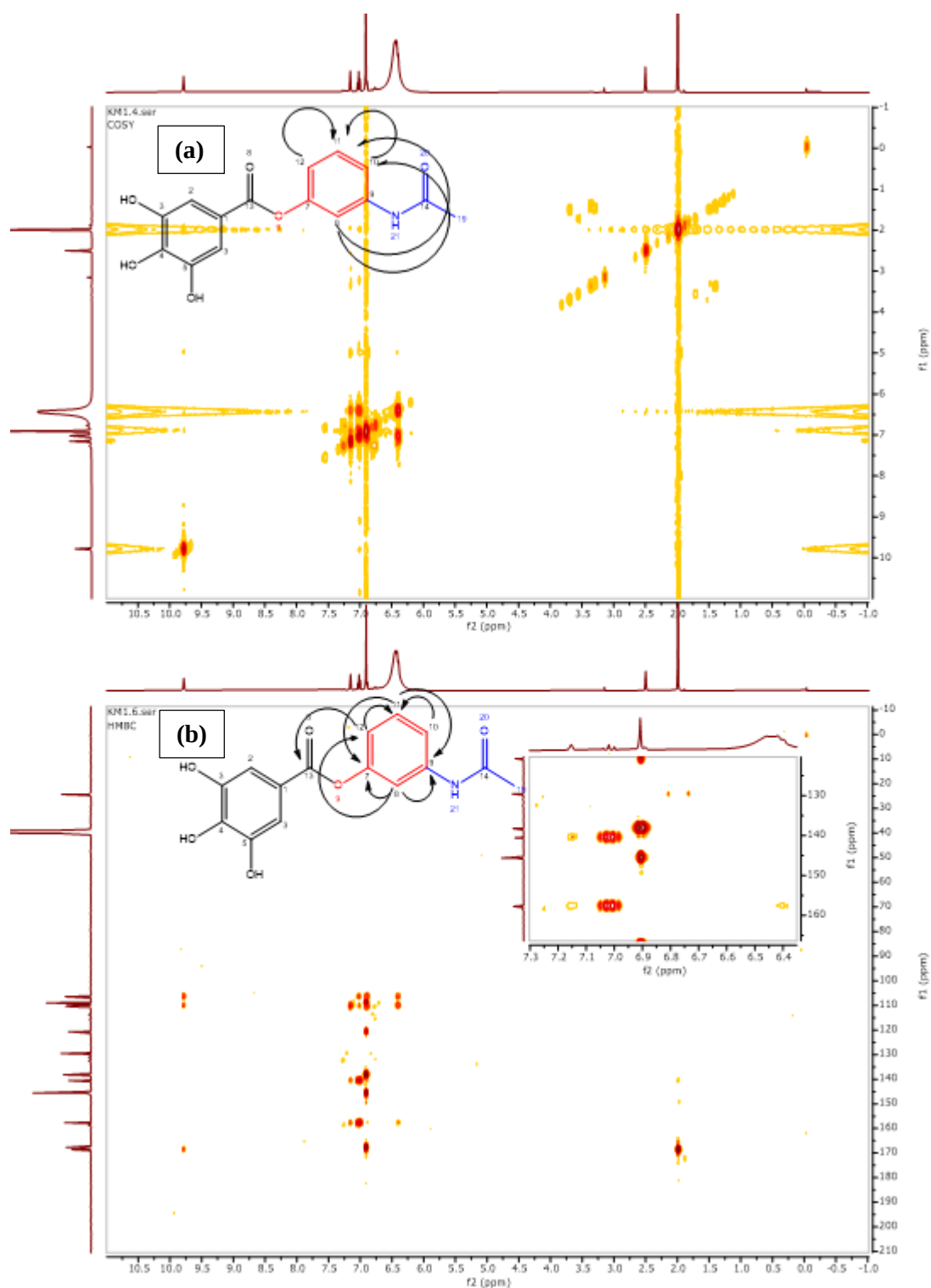


**Figure 5. 5:** 2D NMR of 3-acetamidophenyl 3,4,5-trihydroxybenzoate (a) COSY and (b) HMBC in DMSO- $d_6$ .



### 5.3.3.3 $^1\text{H}$ and $^{13}\text{C}$ NMR results of di-substituted ester derivative compounds

The central molecule employed in the synthesis of bis-substituted/di-substituted compounds was 3,5-dihydroxyacetophenone, illustrated in Scheme 5.5. Tables 5.6 and 5.7 (Appendix D2) summarizes the results of the di-ester compounds and their proton shifts.

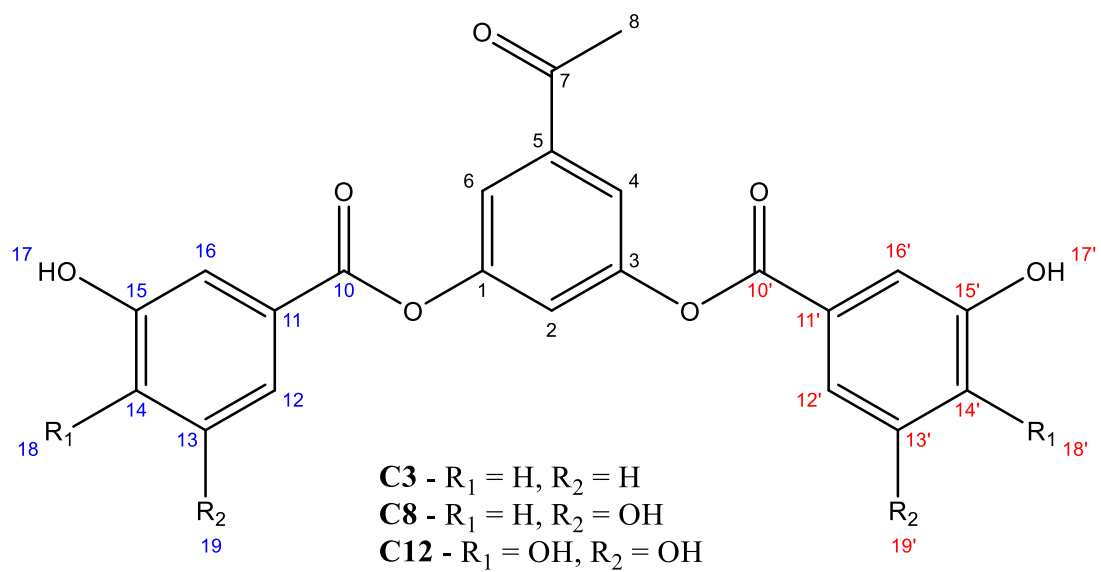
The NMR spectra of the di-substituted ester compounds showed similarities to mono-esters regarding signal shifts influenced by conjugated hydroxybenzoic acid compounds. Methyl ( $-\text{CH}_3$ ) protons in the acetophenone motif experienced variations due to conjugation, ranging from  $\delta_{\text{H}}$ -1.32 to 3.06 ppm. Di-substitution with 3-hydroxybenzoic acid (**C3**) resulted in a de-shielded multiplet signal with an integration of 1. Conversely, conjugation with 3,5-dihydroxybenzoic acid (**C8**) produced a shielded and upfield singlet signal with an integration of 3. Gallic acid (**C12**) conjugation led to a downfield singlet signals.

Carbon results indicated the acetyl methyl carbon as the most upfield signal across di-ester compounds, ranging between  $\delta_{\text{C}}$ -26.70 and 26.78 ppm. Hydroxyl signals also varied with different hydroxybenzoic acid conjugations, with the most de-shielded compounds exhibiting signals ranging from  $\delta_{\text{H}}$ -9.64 to 9.74 ppm. Compound **C12** displayed two hydroxyl signals due to different chemical environments.

Using compound **C12** as a representative for di-substituted derivatives, the upfield singlet signal at  $\delta_{\text{H}}$ -2.54 ppm was attributed to the protons of the methyl group of the acetophenone central molecule, as observed in Figure 5.6 (a). Then the corresponding methyl carbon signal appeared at  $\delta_{\text{C}}$ -26.70 ppm as shown in Fig. 5.6 (b).

The two carbonyl carbon signals that appeared in the downfield regions confirmed successful synthesis of the di-ester compounds. The carbonyl ( $\text{C}_7=\text{O}$ ) signal from the acetyl group of the acetophenone motif appeared as most downfield signal at  $\delta_{\text{C}}$ -197.67 ppm. While the carbonyl carbon signal from the hydroxybenzoate appeared at  $\delta_{\text{C}}$ -173.77 ppm. Furthermore, the symmetrical carbon bonded to the hydroxyl appeared at  $\delta_{\text{C}}$ -167.43 ppm. The asymmetrical carbon bonded to hydroxyl group appeared at  $\delta_{\text{C}}$ -145.37 ppm.

The proton and carbon NMR spectra results for all the di-ester compounds is shown in Appendix B2.



**Scheme 5.5:**  $^1\text{H}$  NMR spectroscopy labelling of hydroxybenzoic acids compounds conjugated to 3,5-dihydroxy acetophenone (**C3**, **C8**, and **C12**).

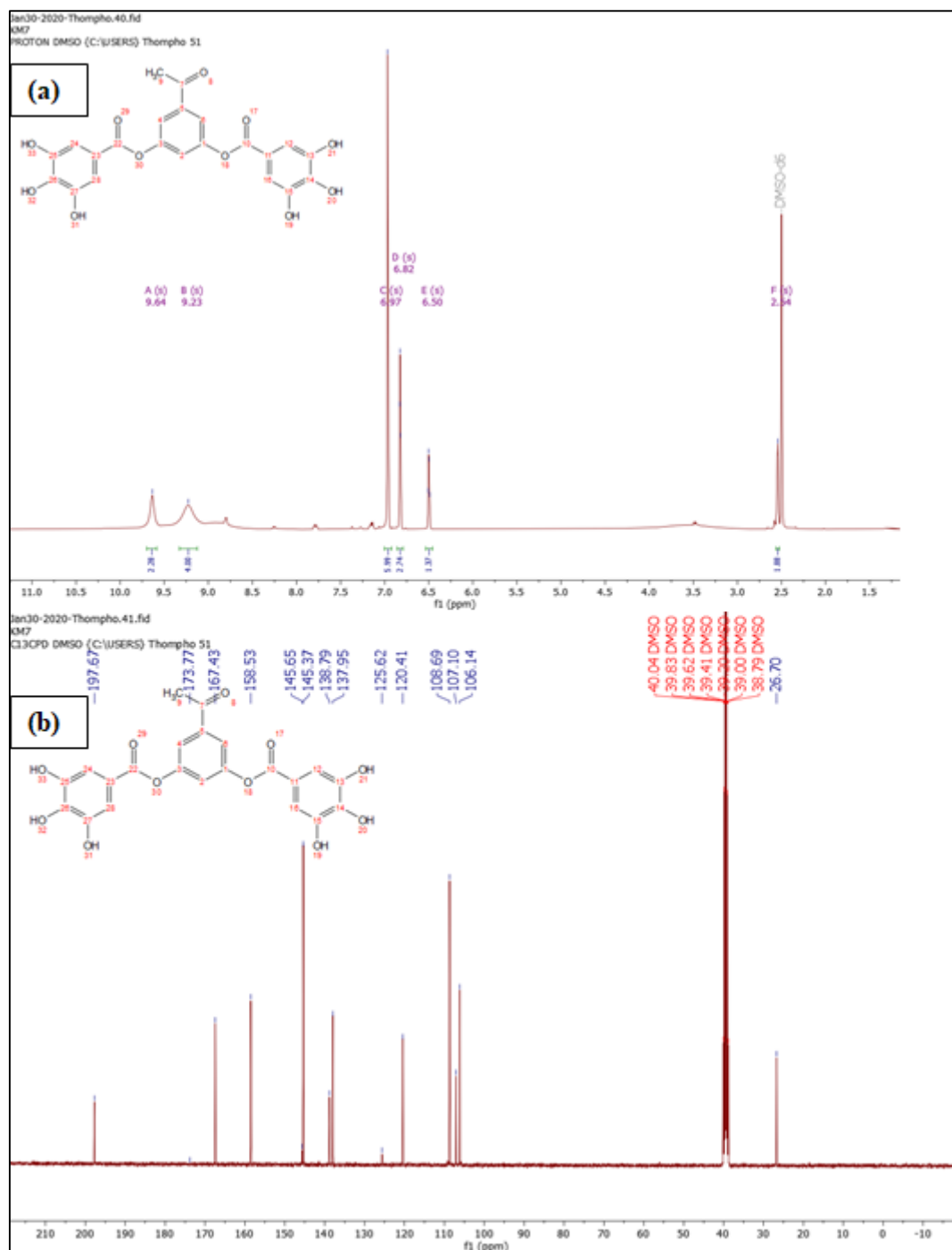


**Figure 5. 6:** NMR spectrum of 5-acetyl-1,3-phenylene bis(3,4,5-trihydroxybenzoate) (**C12**) in DMSO- $d_6$  (a)  $^1\text{H}$  at 500 MHz and (b)  $^{13}\text{C}$  at 100 MHz.

#### 5.3.3.4 $^1\text{H}$ and $^{13}\text{C}$ NMR results of tri-substituted ester derivative compounds

Scheme 5.6 outlines a proton labelling scheme for tripodally conjugated compounds, utilizing phloroglucinol as the core molecule for synthesizing tri-substituted hydroxybenzoic acid derivatives. Tables 5.8 and 5.9 in Appendix D3 highlight proton and carbon signals in selected

structural regions from the hydroxybenzoic acid motif to elucidate the nature of the conjugated compounds.

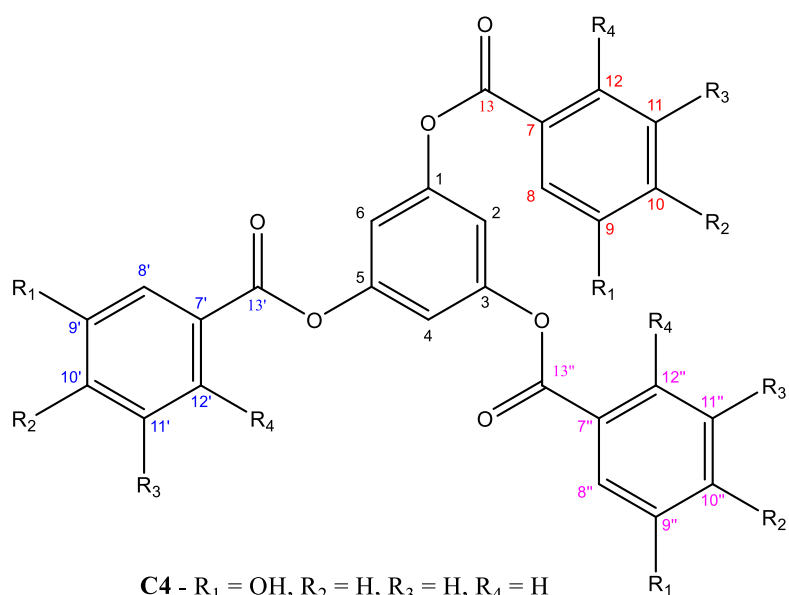
H<sub>12/12'/12''</sub> emerges as the most de-shielded proton, while H<sub>8/8'/8''</sub> ranks as the second most de-shielded proton within the aromatic rings of the hydroxybenzoic acid motif. Both protons exhibit downfield shifts, with H<sub>8/8'/8''</sub> ranging from  $\delta_{\text{H}}$ -6.93 to 7.84 ppm, appearing either as a doublet or a multiplet depending on the substituted hydroxybenzoic acid. Compound **C5** displays a multiplet in H<sub>8/8'/8''</sub>, attributed to the high electron density around the proton due to the proximity of the hydroxyl group to the carbonyl in salicylic acid.

Hydroxyl protons emerge as the most de-shielded in the entire structure, with chemical shifts ranging from  $\delta_{\text{H}}$ -8.39 to 11.29 ppm. Corresponding carbon results for carbons directly bonded to the hydroxyl appear between  $\delta_{\text{C}}$ -81.11 and 158.47 ppm. Compound **C5**, based on salicylic acid, exhibits the most de-shielded hydroxyl proton at  $\delta_{\text{H}}$ -11.29 ppm due to electron density effects.

Despite having the most de-shielded hydroxyl protons, compound **C5** features the most shielded H<sub>11/11'/11''</sub> proton, positioned as the most upfield proton in the aromatic rings, ranging from  $\delta_{\text{H}}$ -5.82 to 5.92 ppm, appearing as a multiplet with an integration of 11. A similar pattern is observed for compound **C9**, where H<sub>10/10'/10''</sub> also appears as a singlet at  $\delta_{\text{H}}$ -5.65 ppm.

Compound **C9** serves as an illustrative example for tripodal/tri-substituted ester compounds. The furthest downfield signal, assigned to the hydroxyl moiety of the hydroxybenzoic acid on proton, while on carbon, the carbonyl signal appears as the most downfield signal at  $\delta_{\text{C}}$ -167.38 ppm as shown in Fig. 5.7 (b). The hydroxyl signal appears as a six-proton singlet signal at  $\delta_{\text{H}}$ -9.98 ppm as shown in Fig. 5(a), with the carbon signal of carbon bonded to the *meta* symmetrical hydroxyl groups at  $\delta_{\text{C}}$ -158.41 ppm.

Proton NMR showed signals not from the compounds, possibly due to conformational isomers since the tri-substituted compounds are bulky and prone to different structural conformations. Other impurity peaks could be due to compounds interacting with silica because of their high polarity.



**C4** -  $R_1 = \text{OH}$ ,  $R_2 = \text{H}$ ,  $R_3 = \text{H}$ ,  $R_4 = \text{H}$   
**C5** -  $R_1 = \text{H}$ ,  $R_2 = \text{H}$ ,  $R_3 = \text{H}$ ,  $R_4 = \text{OH}$   
**C9** -  $R_1 = \text{OH}$ ,  $R_2 = \text{H}$ ,  $R_3 = \text{OH}$ ,  $R_4 = \text{H}$   
**C13** -  $R_1 = \text{OH}$ ,  $R_2 = \text{OH}$ ,  $R_3 = \text{OH}$ ,  $R_4 = \text{H}$

**Scheme 5.6:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy labelling of hydroxybenzoic acid compounds conjugated to phloroglucinol (**C4**, **C5**, **C9** and **C13**).

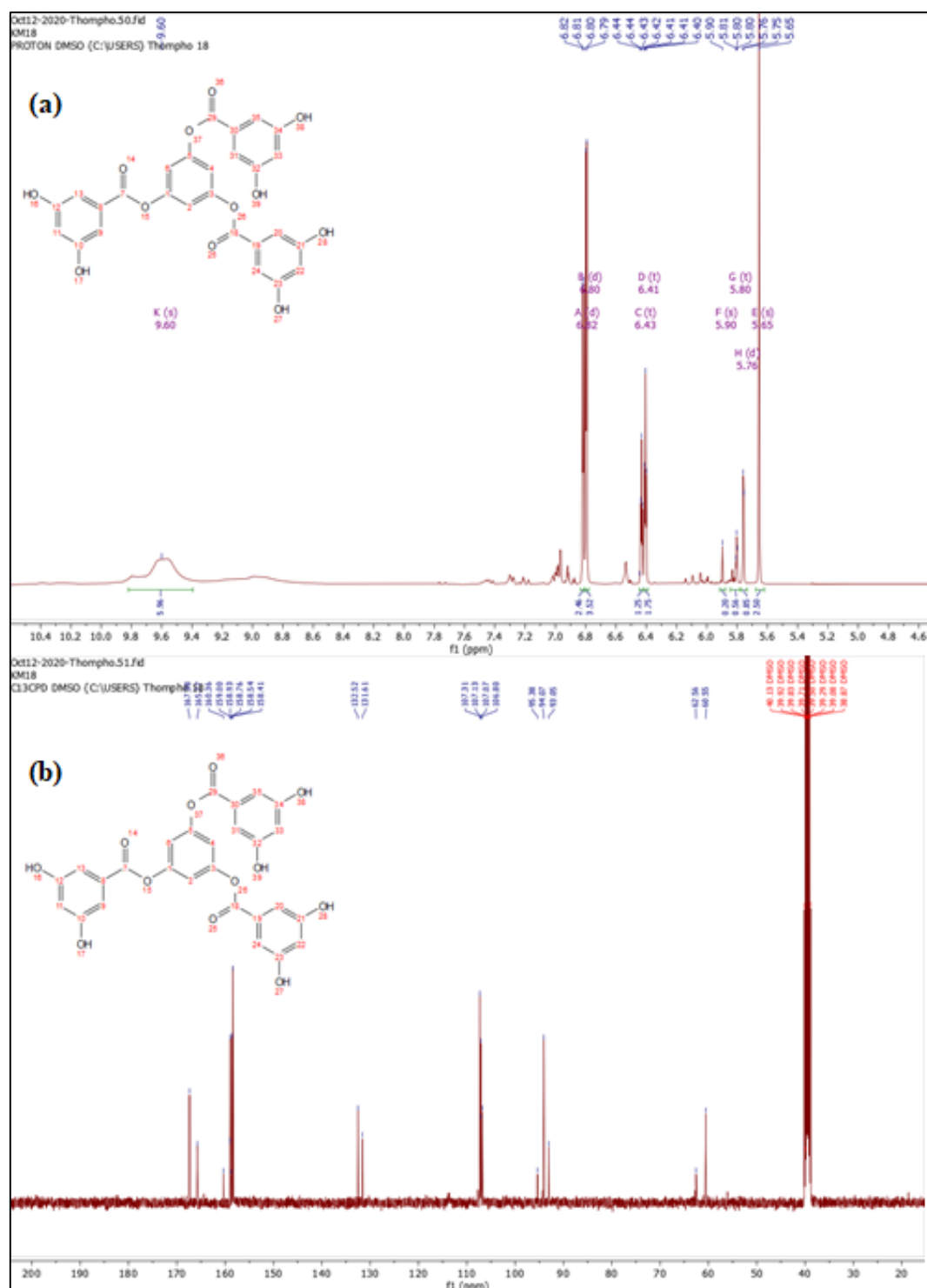


**Figure 5. 7:** NMR spectrum of benzene-1,3,5-triyl tris(3,5-dihydroxybenzoate) (C9) in DMSO-d<sub>6</sub> (a) <sup>1</sup>H at 500 MHz and (b) <sup>13</sup>C at 100 MHz.

### 5.3.4 Elucidation of amide compounds using NMR spectroscopy

The amide compounds, similar to the ester compounds, underwent characterization through proton and carbon NMR spectroscopy. The application of NMR spectroscopy served as a complementary technique to FTIR spectroscopy.

In contrast to the ester compounds, the amides exhibited a simpler classification, consisting of two sets of compounds known as mono-amides and bis/di-amides. However, within the mono-amides and di-amides also had two classes. The first classes of mono-amides was the compounds derived from aniline shown in Fig. 5.8 (a), while the second class of mono-amides were the compounds derived from 3-aminophenol as shown in Fig. 5.8 (b). On the di-amide compounds, the first class di-amide compounds were derived from 1,3-diaminobenzene (Fig. 5.8 (c)), and the second class di-amide compounds were derived from 2,4-diaminophenol as shown in Fig. 5.8 (d). These distinct classes of compounds displayed varying structural compositions and substitutions.

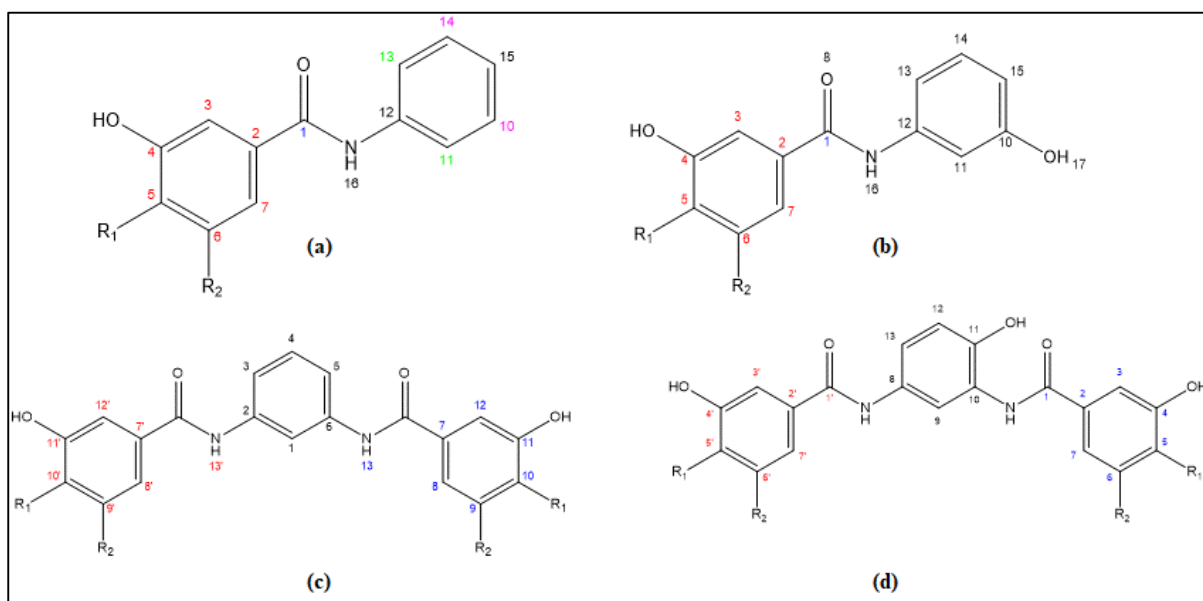


**Figure 5. 8:** Classifications of amide compounds according to their substitutions (a) mono-amides from aniline, (b) mono-amides from 3-aminophenol (c) di-amides from 1,3-diaminobenzene, and (d) di-amides from 2,4-diaminophenol.

#### 5.3.4.1 $^1\text{H}$ and $^{13}\text{C}$ NMR of first class mono-amide compounds derived from aniline

Mono-amide compounds derived from aniline proton shifts are listed in Table 5.10 (Appendix E1). These aniline based compounds are labelled as shown in scheme 5.7. The green and pink

labelling on the aniline motif indicates chemically equivalent protons and carbons. The doublet signal are anticipated for H<sub>11/13</sub> and a triplet signal for H<sub>10/14</sub> on proton, while a single carbon signals (C<sub>11/13</sub> and C<sub>10/14</sub>) are anticipated on their corresponding carbons signals to confirm symmetry in the aforementioned chemical environments.

The aniline-based mono-amide compounds showed overlapping peaks, primarily from the NH and OH chemical shifts, resulting in a broad singlet signal. This broad signal consistently appeared downfield across all three compounds, ranging from  $\delta_{\text{H}}$ -9.58 to 9.96 ppm. The corresponding carbon signal for carbons that are directly bonded to hydroxyl group (s) also appeared as second most downfield signals (that varied based on the substituted hydroxybenzamide) to carbonyl carbon that appeared as the most downfield signal and ranged from  $\delta_{\text{H}}$ -166.98 to 168.77 ppm as highlighted in Table 5.11 (Appendix E1). The downfield signals are due to direct bonding to oxygen and nitrogen atoms.

The H<sub>3</sub> signal from hydroxybenzamide moiety, on the other hand, consistently presented as an upfield singlet, ranging from  $\delta$ -6.42 to 6.95 ppm. The corresponding C<sub>3</sub> carbon signal appeared consistently upfield signal between 106.91 and 109.24 ppm. The H<sub>15</sub> proton signal from the aniline moiety consistently appeared as a triplet, ranging from  $\delta$ -7.01 to 7.32 ppm. The corresponding C<sub>15</sub> carbon signal appeared downfield compared to C<sub>3</sub> and this signal ranged from  $\delta$ -122.92 to 128.15 ppm across all three compounds.

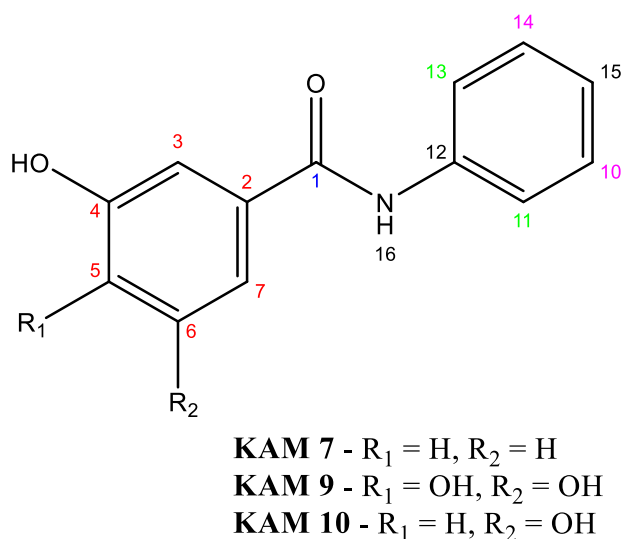
Symmetrical protons and carbons for H<sub>11/13</sub> and corresponding C<sub>11/13</sub> were slightly upfield compared to symmetrical protons and carbon of H<sub>10/14</sub> and C<sub>10/14</sub> because the former is a  $\alpha$ -carbon to the carbon directly attached to nitrogen atom. While the C<sub>10/14</sub> experiences less shielding since it is a  $\beta$ -carbon to the one attached to nitrogen atom in the aniline moiety.

For instance, in the case of **KAM 9** shown in Figure 5.9 (a) for proton and Fig. 5 (b) for carbon, the spectra showed a broad overlapping signal of NH and OH moieties appeared as the most downfield signal at  $\delta$ -9.95 ppm with an integration of 1 as shown in Fig. 5.9 (a). However, the integration of 1 in the -NH signal meant, the broad singlet could be attributed to the solvent molecule rather than the anticipated OH signal. Meanwhile, H<sub>3</sub> emerged as the most upfield singlet signal at  $\delta_{\text{H}}$ -6.95 ppm with an integration of 4 protons instead of two protons. The four protons could possibly be due to the overlapping signals with the two symmetrical OH despite the upfield signal. Moreover, corresponding carbon (C<sub>3</sub>) also appeared upfield at 106.91 ppm (Fig. 5.9 (b)). Subsequently, H<sub>15</sub> manifested as a three proton triplet signal at  $\delta_{\text{H}}$ -7.02 ppm downfield to H<sub>3</sub> signal and carbon signal appeared at  $\delta_{\text{C}}$ -128.15 ppm. The symmetrical protons



(H<sub>11/13</sub>) appeared as a three proton doublet signal at 7.59 ppm with a J-coupling of 7.4 Hz (Appendix E1). Whilst the other symmetrical protons (H<sub>10/14</sub>) appeared upfield as a three proton triplet at compared to the former. The three proton signals observed instead of either a one (for H<sub>15</sub>) or two proton signals for all symmetrical protons further indicates overlapping signals across the aromatic regions possibly due to resonance with the hydroxyl groups.

These signal patterns strongly suggest successful conjugation between gallic acid and 3-aminophenol to form compound **KAM 9**.



**Scheme 5.7:** <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy labelling of mono-amide compounds from aniline (**KAM 7**, **KAM 9**, and **KAM 10**).

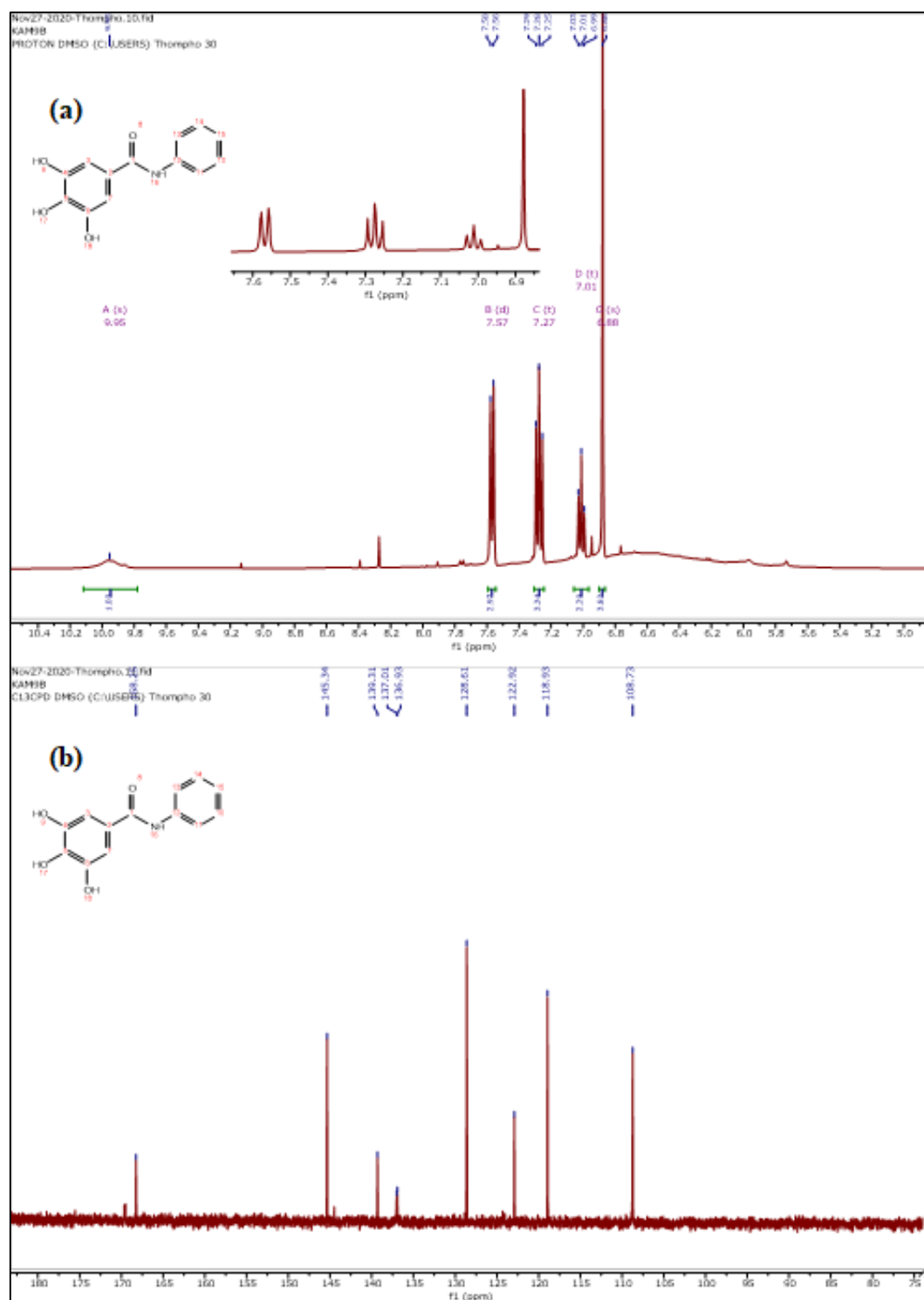


**Figure 5. 9:** NMR spectrum of 3,4,5-trihydroxy-*N*-phenylbenzamide (**KAM 9**) in DMSO-d<sub>6</sub> (a) <sup>1</sup>H at 500 MHz and (b) <sup>13</sup>C at 100 MHz.

#### 5.3.4.2 <sup>1</sup>H and <sup>13</sup>C NMR of mono-amide compounds derived from to 3-aminophenol

The second class mono-amide compounds derived from 3-aminophenol, are illustrated in Scheme 5.8. The summarized results for all the 3-aminophenol-based compounds are presented

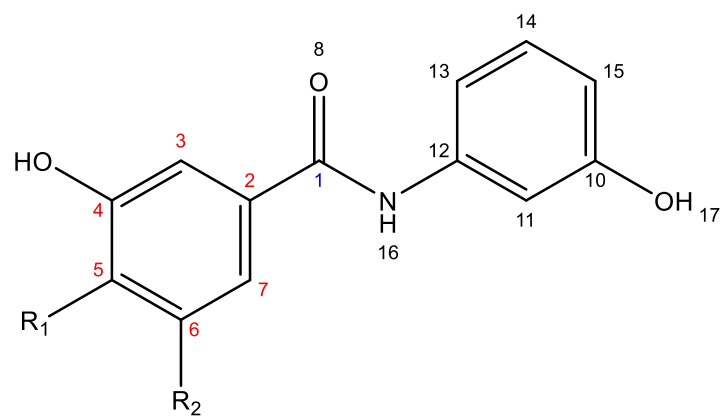
in Tables 5.12 and 5.13 in Appendix E1, revealing characteristic chemical shifts indicative of the formation of the anticipated mono-amide compounds.

Similarly to aniline-derived compounds, the downfield proton signals corresponded to the hydroxyl and amine proton signals, often appearing as broad singlet signals due to signal overlap or interactions with solvent molecules. The range of these signals depended on the substitutions present in the hydroxybenzoic acid. On the other hand, the upfield signal observed in most cases among the studied compounds was attributed to the H<sub>15</sub> proton from the 3-aminophenol motif, with its chemical shift varying based on the hydroxybenzoic acid substitutions and ranging between  $\delta$ -6.43 and 6.82 ppm. Proton H<sub>14</sub> exhibited a triplet signal across the studied compounds, with its chemical shift also influenced by the hydroxybenzoic acid substitution, ranging from  $\delta$ -6.76 to 7.24 ppm.

Figure 5.10 (a) and (b) depicts the <sup>1</sup>H NMR and <sup>13</sup>C spectra of compound KAM 5. Notably, the downfield broad singlet (bs) signal observed at  $\delta$ -9.96 ppm was attributed to the overlapping of the N-H and OH protons (see Fig. 5.11 (a)). Again, similarly to aniline-based compounds, the integration in the downfield signal was one proton, which would mean the broad singlet is only due to amine (-NH) proton interaction with solvent molecule. However, the six proton multiplet signal ranging from  $\delta$ <sub>H</sub>-6.72 – 6.82 ppm could be due to multiple signal overlaps between the aromatic protons and the hydroxyl protons from both the hydroxybenzamide motif and 3-aminophenol moiety. The carbon results showed number of signals that corresponds to the anticipated structure. In carbon spectra shown in Fig. 5.10 (b), the consistent downfield signal appearing between  $\delta$ -167.94 and 170.12 ppm was ascribed to the carbonyl carbon (O=C<sub>1</sub>) originating from the hydroxybenzamide motif.

The H<sub>14</sub> proton signal from the 3-aminophenol motif appeared as a triplet at 7.24 ppm, slightly downfield compared to those observed with 3-hydroxybenzoic acid and 3,5-dihydroxybenzoic acid (KAM 8 and KAM 12, respectively). Additionally, the singlet signal at  $\delta$ -6.92 ppm was assigned to the H<sub>3</sub> and H<sub>7</sub> protons.

Signals observed and their overlapping signal in the multiplet suggests successful conjugation since identifiable protons from both hydroxybenzamide and 3-aminophenol moieties are observed in all the spectra. The spectral data for all the mono-amide compounds is presented in Appendix C1 and the summarized results are given in Appendix E1.



**KAM 5** -  $R_1 = \text{OH}$ ,  $R_2 = \text{OH}$

**KAM 7** -  $R_1 = \text{H}$ ,  $R_2 = \text{H}$

**KAM 12** -  $R_1 = \text{H}$ ,  $R_2 = \text{OH}$

**Scheme 5.8:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy labelling of hydroxybenzoic acid compounds conjugated to 3-aminophenol (KAM 5, KAM 8, and KAM 12).

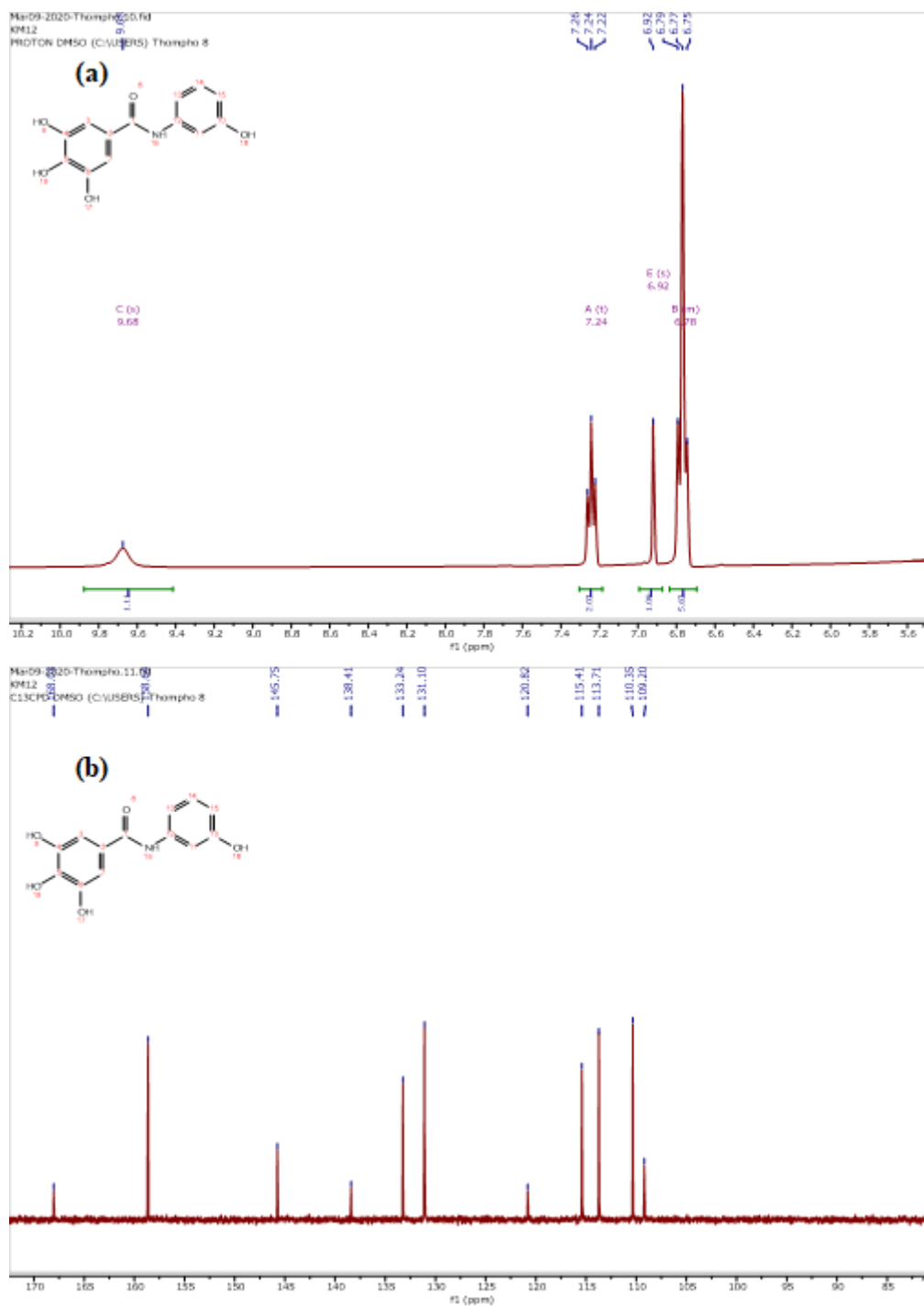


**Figure 5. 10:** NMR spectrum of 3,4,5-trihydroxy-*N*-(3-hydroxyphenyl)benzamide (**KAM 5**) in DMSO- $d_6$  (a)  $^1\text{H}$  at 500 MHz and (b)  $^{13}\text{C}$  at 100 MHz.

#### 5.3.4.3 $^1\text{H}$ and $^{13}\text{C}$ NMR of first class di-amide compounds derived from 1,3-diaminobenzene

The proton and carbon NMR results for bis/di-substituted amide compounds exhibit a consistent trend, similar to that observed in di-substituted ester compounds. In Scheme 5.9, the

annotation of these first-class di-amide compounds shows symmetrical structures. Table 5.14 and Table 5.15 show consistent chemical shifts closely related to these sets of compounds, further indicating successful conjugation.

One notable proton, the axial central H<sub>1</sub> proton from the 1,3-diaminobenzene structure, displays variation in its chemical shift between upfield and downfield signals. This variation is attributed to the presence of a different number of hydroxyl groups on the hydroxybenzamide motifs within these compounds. The range of chemical shifts for this proton spans from  $\delta$ -7.36 to 9.68 ppm, manifesting as a singlet. The corresponding carbon signal showed a consistent downfield signal for 3,5-dihydroxybenzamide (**KAM 4**) and 3,4,5-trihydroxybenzamide (**KAM 6**) at  $\delta$ <sub>C</sub>-137.15 and 137.39 ppm respectively (see Table 5.14). The 3-hydroxybenzamide based compound (**KAM 1**) showed upfield signal  $\delta$ <sub>C</sub>-115.80 ppm. The downfield signal in the former compounds could be due to the *meta* carbon C<sub>9/9'</sub> that is directly attached to the hydroxyl causing high electron density C<sub>1</sub> carbon due to resonance.

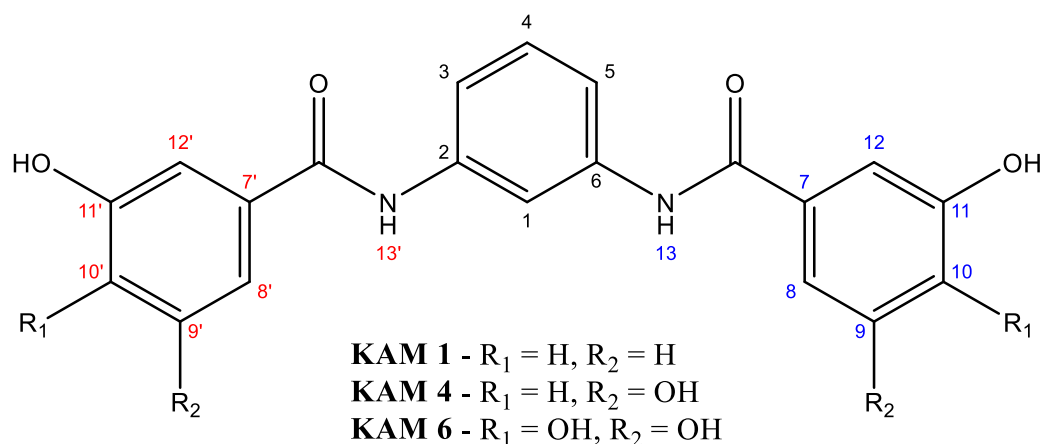
The *para* proton (H<sub>4</sub>) relative to H<sub>1</sub> appears consistently as a triplet proton across all compounds, with chemical shifts ranging from  $\delta$ <sub>H</sub>-7.38 to 7.87 ppm. On carbon, this signal appeared between  $\delta$ <sub>C</sub>-139.56 and 154.62 ppm. Similarly, the H<sub>3/5</sub> protons exhibit a doublet signal ranging from  $\delta$ <sub>H</sub>-7.28 to 7.91 ppm across the compounds. The symmetrical carbons (C<sub>3/5</sub>) central 1,3-diaminobenzene molecule mostly appeared upfield for 3-hydroxybenzamide and 3,4,5-trihydroxybenzamide based moiety that appeared at  $\delta$ <sub>C</sub>-129.56 and 120.45 ppm respectively. However, the 3,5-dihydroxybenzamide appeared downfield at  $\delta$ <sub>C</sub>-149.00 ppm.

The amide (NH<sub>13/13'</sub>) protons display the most downfield signals among the compounds, ranging from  $\delta$ <sub>H</sub>-9.94 to 10.10 ppm. The carbon signal appearing downfield was the symmetrical carbonyl signal ranging from  $\delta$ <sub>C</sub>-167.97 to 172.00 ppm. Following closely are the hydroxyl protons that showed broad singlet signals and carbon that are directly bonded to the hydroxyl groups, showing variations relative to the hydroxybenzoic acid substitutions.

Fig. 5.11 (a) and (b) illustrate a representative spectrum showcasing typical chemical shifts for bis/di-substituted amide compounds, using a gallic acid-based compound (**KAM 6**) as an example. In this spectra, the NH<sub>13/13'</sub> proton appears as a broad four-proton singlet at  $\delta$ -9.96 ppm as shown in Fig. 5.11 (a), while the *meta* hydroxyl moieties (H<sub>9/9'</sub> and H<sub>11/11'</sub>) in the same chemical environment manifest as a broad three singlet signal at  $\delta$ -9.18 ppm. Broad singlet signals are a result of overlapping signals between the N-H and OH and a trace of the solvent molecule as previously stated for the mono-amide compounds. The H<sub>4</sub> proton presents as a one

proton triplet at  $\delta_{\text{H}}$ -7.18 ppm, while the  $\text{H}_{3/5}$  proton is observed as a slightly downfield doublet signal at  $\delta_{\text{H}}$ -7.91 ppm. The symmetrical carbon corresponding to the aforementioned protons appeared upfield at  $\delta_{\text{C}}$ -120.45 ppm because it is a  $\alpha$ -carbon to the carbon that is directly attached to the -NH group. Notably, the  $\text{H}_1$  proton appears as a singlet signal, in the upfield region at  $\delta_{\text{H}}$ -6.93 ppm. The carbon that corresponds to this proton signal appeared downfield at 137.39 ppm. Downfield signal appearing at  $\delta_{\text{C}}$ -171.98 ppm was associated with the symmetrical carbonyl signal.

These consistent chemical shifts across different compounds indicate successful conjugation, further supported by FTIR results.



**Scheme 5.9:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy labelling of first-class di-amide compounds 1,3-diaminobenzene (**KAM 1**, **KAM 4**, and **KAM 6**).





#### 5.3.4.4 $^1\text{H}$ and $^{13}\text{C}$ NMR of second-class di-amide compounds derived from 2,4-diaminophenol

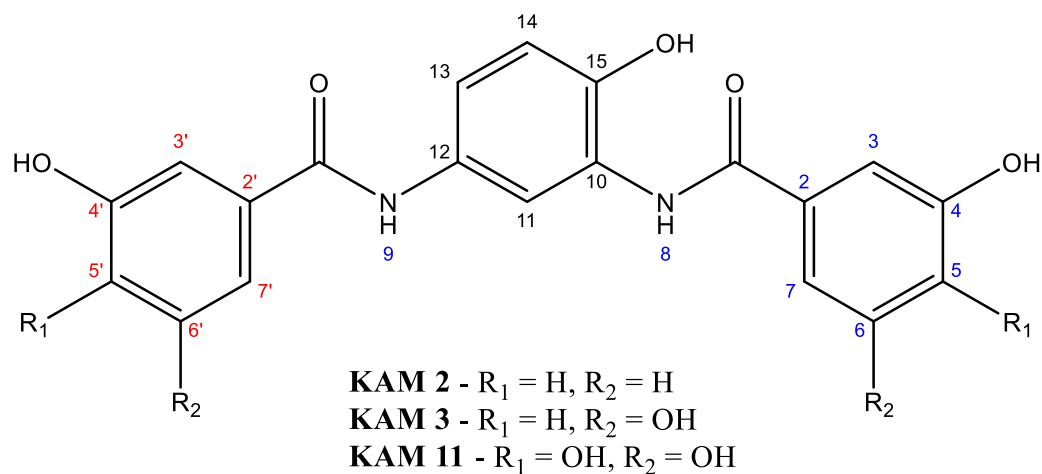
Table 5.16 and 5.17 lists all the compounds synthesized from 2,4-diaminophenol. The distinction between 1,3-diaminobenzene-based compounds and 2,4-diaminophenol-based compounds primarily lies in the availability of hydroxyl substitution in the latter compound. This substitution influences the symmetry of the central molecule, subsequently affecting splitting patterns and chemical shifts compared to 1,3-diaminobenzene-based compounds. Scheme 5.10 illustrates the proposed structure of the 2,4-diaminophenol-based compounds.

In the 2,4-diaminophenol central molecule, the  $\text{OH}_{15}$  proton signal emerges as the second most downfield signal, ranging from  $\delta_{\text{H}}$ -9.41 to 10.32 ppm, and the corresponding carbon also appeared as the second most downfield signal in compound KAM 2 and KAM 3 appearing at  $\delta_{\text{C}}$ -167.25 and 167.42 ppm respectively. Carbon results showed a deshielded and downfield signal ranging from  $\delta_{\text{C}}$ -167.50 and 169.98 ppm ascribed to the carbonyl signal. This variation is influenced by the type of hydroxybenzanide substitution on each compound. The amide protons  $\text{NH}_8$  and  $\text{NH}_9$  reside in distinct chemical environments, causing one to be upfield or downfield from the other.  $\text{NH}_8$  is slightly upfield compared to both  $\text{OH}_{15}$  and  $\text{NH}_9$  protons due to its chemical environment, and its signal ranged between  $\delta_{\text{H}}$ -9.68 and 10.32 ppm.  $\text{NH}_9$  appears as the most deshielded and downfield signal relative to all proton signals across all compounds, ranging between  $\delta_{\text{H}}$ -9.94 and 10.32 ppm.

In certain compounds like **KAM 11**, an overlap in NH and OH signals occurs due to the high substitution of electron-dense atoms, resulting in a broad singlet signal that was also observed in aniline-based compounds. Taking **KAM 11** (see Fig. 5.12 (a) and (b)) as a representative compound, the two broad singlet signals at  $\delta_{\text{H}}$ -10.32 and 10.08 ppm are attributed to the overlapping signals of  $\text{NH}_9$  and  $\text{NH}_8$ , respectively as shown in Fig 12 (a). These signals also overlap with the hydroxyl moieties from the gallic acid part and hydroxyl moieties from the 2,4-diaminophenol part. The combined integration number of 8 protons in these broad singlet signals approximates the number of protons involved in this overlapping signal. The carbonyl signal appeared at  $\delta_{\text{C}}$ -167.50 ppm as the most deshielded and downfield signal as shown Fig. 12 (b).

The  $\text{H}_{3/3'}$  and  $\text{H}_{7/7'}$  protons are predominantly found as upfield signal protons in the hydroxybenzoic acid motifs, while the  $\text{H}_{14}$  proton mostly appears as the upfield signal in the central 2,4-diaminophenol part. Consequently, all signals for all compounds are tabulated in

Table 5.16 and 5.17, while the spectra results are shown in Appendix C2 that further confirmed successful conjugation.



**Scheme 5.10:**  $^1H$  and  $^{13}C$  NMR spectroscopy labelling of second-class di-amide compounds derived from 2,4-diaminophenol (**KAM 2**, **KAM 3**, and **KAM 11**).

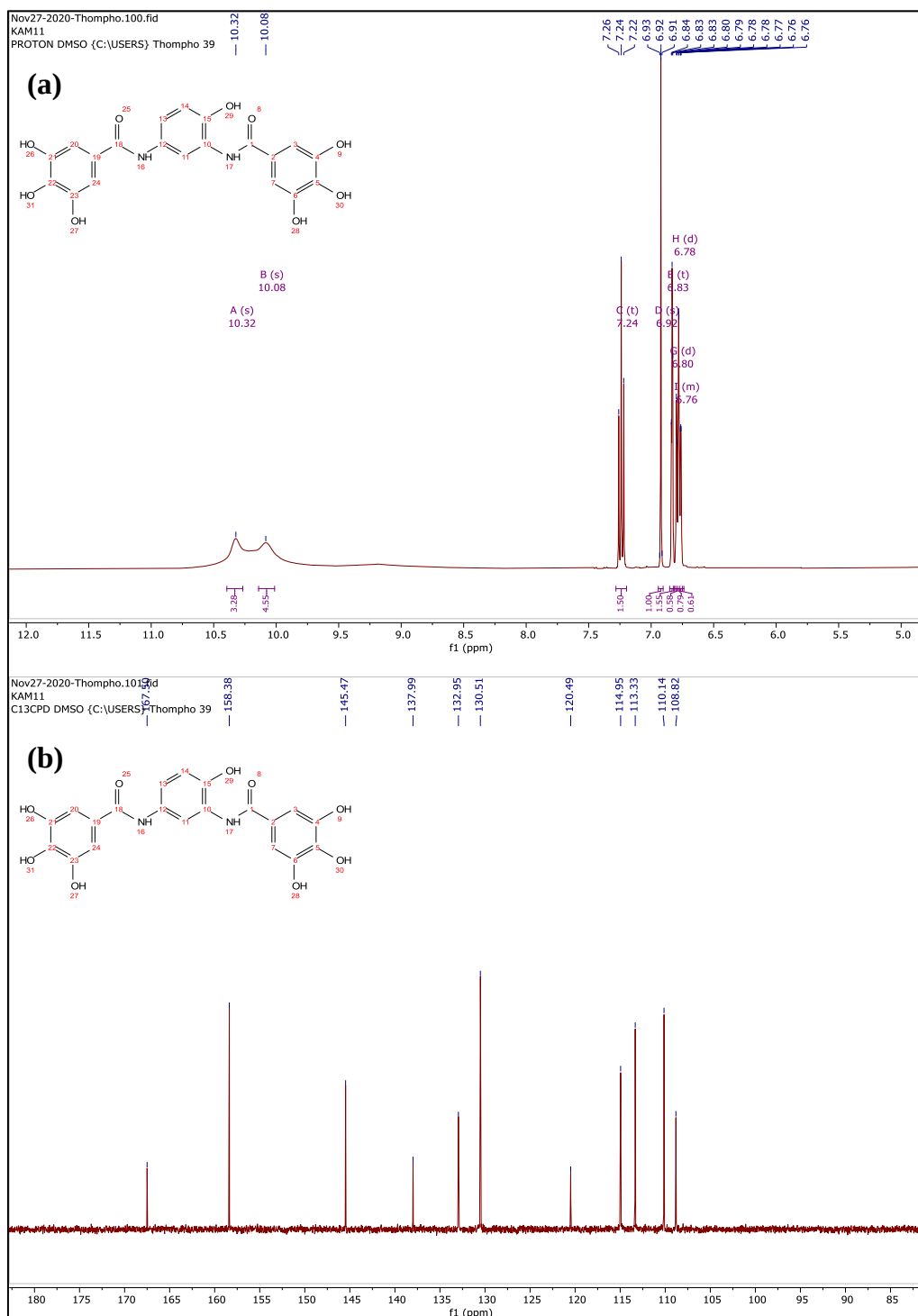


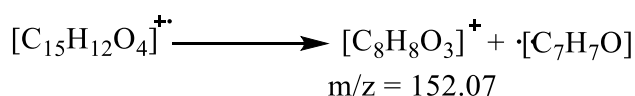
**Figure 5. 12:** NMR spectrum of *N,N'*-(4-hydroxy-1,3-phenylene)bis(3,4,5-trihydroxybenzamide) (**KAM 11**) in DMSO- $d_6$  (a)  $^1\text{H}$  at 500 MHz and (b)  $^{13}\text{C}$  at 100 MHz.

### 5.3.5 Structural confirmation of amide and ester compounds from mass spectroscopy (MS)

#### 5.3.5.1 Structural elucidation of ester compounds using MS

To piece all the information from NMR and IR spectroscopy, mass spectroscopy was used as final confirmatory technique. Table 5.19 below summarizes all the results obtained from the mass spectrum for each ester compound. Looking at the results one could deduce a common trend in all the compounds, the base peaks (tallest or most intense peak) were not the actual molecular ion peaks (compound peak) but stable fragments from the reactants or partial products. The  $\Delta$  ( $M^+$  - Base peak) value indicates the mass difference between the molecular ion peak and the base peak, providing information about the fragmentation pattern of the compound. By analyzing the  $M^+$  and  $\Delta$  values for each compound, it was possible to identify the molecular weight of the compound and infer its structural characteristics based on the fragmentation pattern observed in the mass spectrum.

Using compound **C1** as a representative compound, as seen in the MS spectrum in Fig. 5.13 the  $M^+$  peak is 256.28. However, the base peak was found to be 151.07 and this peak is attributed to the  $\lambda^3$ -methyl-3-hydroxybenzoate fragment as seen in Scheme 5.11. The  $\Delta$ -value of 105.21 was attributed to the benzaldehyl radical fragment that cleaved from the 3-acetylphenyl 3-hydroxybenzoate (**C1**) structure. These fragments further confirm the results obtained from the other spectroscopic techniques (NMR and IR) that showed successful conjugation and formation of ester compounds.



**Scheme 5.11:** Fragmentation pattern of the molecular ion peak of compound **C1**.

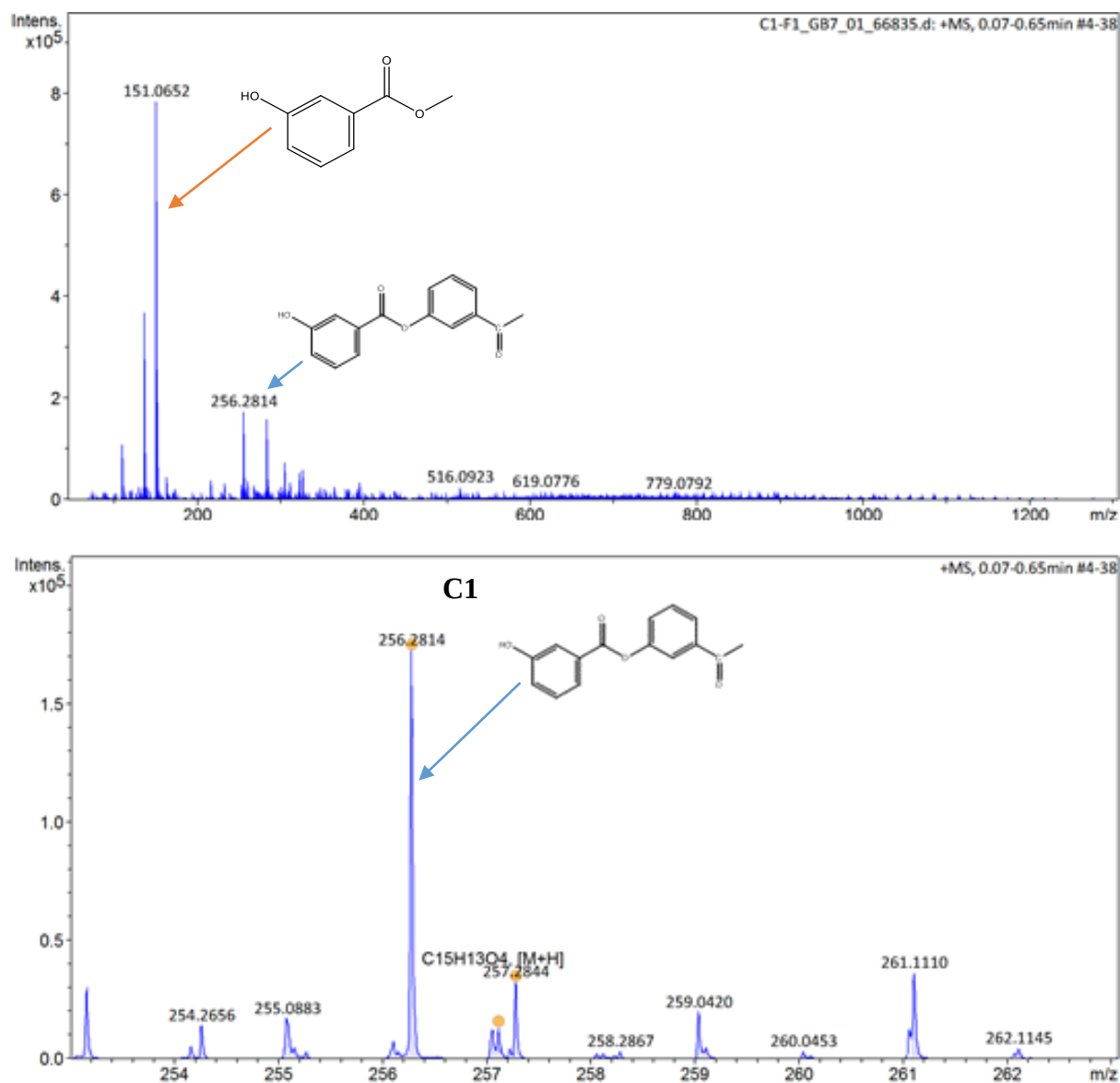


**Figure 5. 13:** Mass spectrum of 3-acetylphenyl 3-hydroxybenzoate (C1) fragments.

**Table 5.19:** Summarized mass spectrometry data of the synthesized ester compounds.

| Compound | M <sup>+</sup> | Base peak | Δ (M <sup>+</sup> - Base peak) |
|----------|----------------|-----------|--------------------------------|
| C1       | 256.28         | 151.07    | 105.21                         |
| C2       | 257.08         | 137.05    | 120.03                         |
| C3       | 393.20         | 241.04    | 152.16                         |
| C4       | 487.11         | 121.02    | 366.09                         |

|     |        |        |        |
|-----|--------|--------|--------|
| C5  | 487.09 | 127.03 | 360.06 |
| C6  | 272.28 | 152.06 | 120.22 |
| C7  | 273.07 | 137.05 | 136.02 |
| C8  | 425.18 | 152.06 | 273.12 |
| C9  | 535.09 | 155.06 | 380.03 |
| C10 | 288.08 | 152.06 | 136.02 |
| C11 | 289.07 | 137.05 | 152.02 |
| C12 | 457.03 | 102.12 | 354.91 |
| C13 | 583.15 | 183.10 | 400.05 |
| C14 | 272.28 | 152.06 | 120.22 |
| C15 | 304.07 | 152.06 | 152.01 |

#### 5.3.5.2 Structural elucidation of amide compounds using MS

The mass spectrometry (MS) results for the amide compounds exhibited similarities to the ester compounds, revealing various fragmentations that were sometimes close to the reactants and, in some cases, partial products. Similar to the ester compounds, the base peak for amides was not the molecular ion peak but rather a stable fragment of the overall structure. The  $\Delta$  ( $M^+$  - Base peak) value played a crucial role in determining the fragment that had cleaved off between the base peak and the molecular ion peak.

Compound KAM 10 was selected as a representative compound to illustrate these findings, as depicted in Fig. 5.14. The figure illustrates that the two most intense peaks were the base peak and the molecular peak, appearing at the  $m/z$  values of 94.0597 and 230.0734, respectively. The base peak was attributed to the protonated aniline fragment, while the compound fragment (molecular ion peak) also appeared as a stable fragment alongside the base peak.

These results provide further validation of the FTIR and NMR findings, indicating the successful formation of the studied compounds

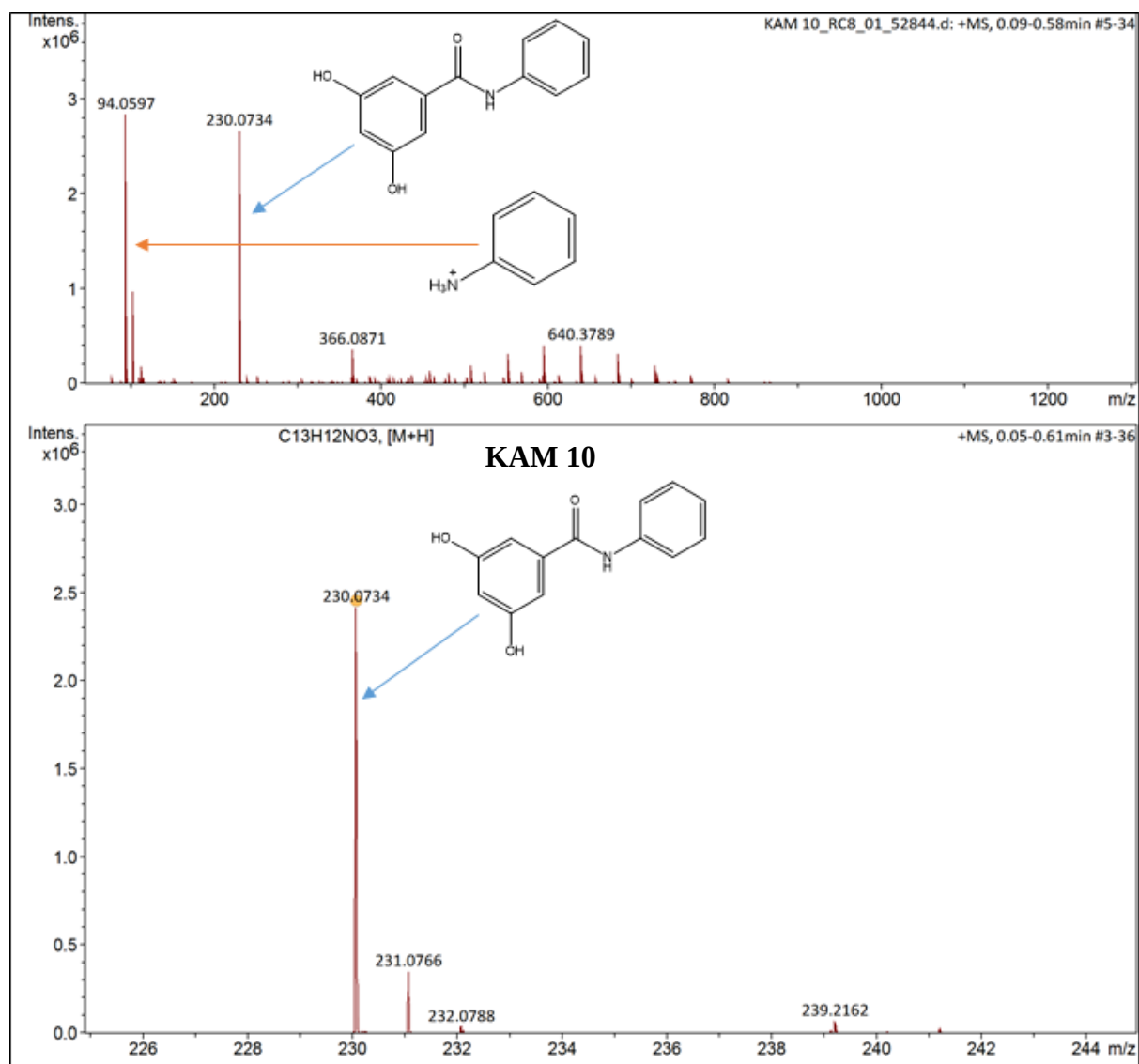


**Figure 5. 14:** Mass spectrum of 3,5-dihydroxy-N-phenylbenzamide (**KAM 10**) fragments.

**Table 5.20:** Summarized mass spectrometry data of the synthesized amide compounds

| Compound     | $M^+$  | Base peak | $\Delta (M^+ - \text{Base peak})$ |
|--------------|--------|-----------|-----------------------------------|
| <b>KAM 1</b> | 349.09 | 309.14    | 39.95                             |
| <b>KAM 2</b> | 365.10 | 113.10    | 252                               |
| <b>KAM 3</b> | 396.10 | 113.10    | 283                               |
| <b>KAM 4</b> | 381.10 | 113.10    | 268                               |

|               |        |        |        |
|---------------|--------|--------|--------|
| <b>KAM 5</b>  | 262.06 | 102.12 | 159.94 |
| <b>KAM 6</b>  | 413.22 | 115.08 | 298.14 |
| <b>KAM 7</b>  | 230.07 | 102.12 | 127.95 |
| <b>KAM 8</b>  | 230.07 | 102.12 | 127.95 |
| <b>KAM 9</b>  | 246.07 | 136.07 | 110    |
| <b>KAM 10</b> | 230.07 | 94.06  | 136.01 |
| <b>KAM 11</b> | 429.09 | 102.12 | 326.97 |
| <b>KAM 12</b> | 246.07 | 102.12 | 143.95 |

## 5.4 Discussion

### 5.4.1 FTIR, NMR and MS structural elucidation of ester compounds

The ester compounds have distinct characteristic peaks in both the FTIR and NMR spectroscopy. The carbonyl (C=O), the ester stretching vibration (C-O) and the hydroxyl group (OH) since these studied compounds are hydroxybenzoic acid. Additionally, the  $sp^2$  hybridization ( $=CH_2$ ) because of the aryl nature of these compounds and C=C bending frequency were observed in all the ester compounds.

The stretching hydroxyl group was the broadest and prominent peak on the ester compounds and as it was detailed in the results section. This peak covered a broad range in the spectrum and appeared at lower frequencies compared to free OH groups of carboxylic acids and alcohols, indicating the presence of hydrogen bonding and intermolecular interactions<sup>306,307</sup>.

The mono-, di- or tri-substitution of hydroxyl groups varied but did not show a consistent trend and were therefore, deemed insignificant. However, the *ortho* and *meta* substitutions showed a slight significance in terms of the vibrational shifts. The *ortho* position because of the close proximity of the hydroxyl group could be altered due to steric conditions and may affect the electronic effects compared to *meta* substitution, hence **C1** (3-hydroxybenzoic acid-based) and **C2** (2-hydroxybenzoic acid-based) show slightly significant shift in vibrations. The *meta*-hydroxyl vibrational peak appeared in higher frequencies than the *ortho* positioned hydroxyl group.



For *N*-phenylacetamide based compounds, there was an overlapping peak between the N-H and OH stretching vibrations. The N-H and OH overlap can be observed because these two groups are fully engaged in forming an extended H-bond network<sup>308</sup>. When OH and NH groups are involved in extensive hydrogen bonding networks, their vibrational frequencies shift may be due to the collective effects of multiple hydrogen bonds. This can lead to broadening and merging of absorption bands in the FTIR spectrum.

The carbonyl peak vibrations were within the range of ester conjugated compounds and this was the confirmation of the successful conjugation. The carbonyl peak in most cases was the most pronounced and intense peak in slightly higher frequencies ( $\sim 1700\text{ cm}^{-1}$ ) from the fingerprint region. The carbonyl peak vibrations however, varied also based on the compounds that were formed. From the results it could be noted that the *meta* substituted compounds more specifically compound **C1** and **C8** that constitutes of 3-hydroxybenzoic acid and 3,5-dihydroxybenzoic acid both showed lower frequencies than normal for ester linkages. This could be attributed to the possible resonance effects transmitted through the benzene ring, which may alter the electronic density around the carbonyl group and affect its vibrational frequency.

The C=C, vibrational peak for aryl moieties did not follow any specific trend and was consistently within the expected range and was also an intense peak. The ester confirmatory peak (C-O) occurred also at the expected range and was affected by the type of hydroxybenzoic acid that was substituted but did not follow any specific trend. The lack of specific trend of the aforementioned peaks are expected because these regions are less affected by the substituents. However, electron resonance like in the case for *N*-phenylacetamide based compounds could show a slight shift in the mentioned regions since the electrons from the amide bond could traverse in between the amine and carbonyl moieties.

The observed FTIR bands were further confirmed by NMR spectroscopy. For mono- and di-esters, there were two carbonyl signals that emanated from the acetophenone motif and the one that emanated from the hydroxybenzoate motif. These signals were consistently observed in downfield regions due to the electronegative oxygen atom in the carbonyl moieties. Meaning these signals experiences less shielding hence, they resonate at higher frequencies in the  $^{13}\text{C}$  NMR. In all the acetophenone (mono- and di-) based compounds, the carbonyl signal from the acetophenone motif appeared as the most downfield signal compared to the hydroxybenzoate carbonyl signals and this is due to the fact that this carbonyl moiety was a pendant and free

carbonyl group that was not involved in the bond formations and in most cases it was *meta* substituted in the hydroxyacetophenone motif, meaning it was far from the ester bond formations. The number of carbon signals for all the mono and di-substituted compounds corresponded well with the anticipated structures and was in full agreement with the  $^1\text{H}$ -NMR and FTIR results.

The hydroxyl moieties appeared in downfield regions in  $^1\text{H}$ -NMR also due to the electronegative oxygen atom present <sup>305</sup>. The hydroxyl moiety signal appeared as a singlet in 3-hydroxybenzoic acid and 3,5-dihydroxybenzoic acid based compounds with an integration of 1 in proton spectra. However, the different chemical environment of hydroxyl groups in gallic acid-based compounds such as **C11** that showed two singlet signals corresponding to the *meta* hydroxyl protons and the *para* hydroxyl group that experiences more shielding since its sandwiched between the two *meta* substituted hydroxyl groups. The hydroxyl signals were slightly broadened due to intermolecular hydrogen bond with possibly the solvent molecules, resulting in a wider peak shape. Similar, observation were observed by López-Martínez et al. <sup>309</sup> in their study on mango extracts that had gallic acid as a constituent. Their  $^1\text{H}$  NMR showed a broad singlet that was also ascribed to the hydrogen bond interaction with other -OH groups that are possibly water remnants. In addition, Charisiadis et al. <sup>310</sup> who studied solvent effect on phenolic acids, further confirmed via  $^1\text{H}$  NMR that there is a fast proton exchange between solvent and solute at room temperature. The di-substituted compounds showed similar trend to mono-substituted compounds due to symmetrical nature of the *meta* hydroxyl groups.

The tri-substituted compounds synthesized from phloroglucinol were showing peaks of impurities, this is was deduced from TLC plate which showed that these compounds were reacting with the silica since they were polar and sticky on the TLC plate and this made the column purification process with silica difficult. The symmetrical nature of these compounds meant that there should only be one singlet for protons in the phloroglucinol moiety and the three benzene rings with substituted hydroxyl should give similar multiplicities since they resonate in the same chemical environment. The protons from the hydroxyl moieties were observed in the downfield regions due to the electronegative oxygen atoms as previously stated for the mono- and di- substituted ester compounds and these signals appeared as broadened singlet peaks. The broadening of these peaks corresponded well with the overlapping signals and the protons corresponded well with the number of hydroxyl substituents.

The tri-substituted compounds derived from 3-hydroxybenzoic acid, showed plenty of impurity signals with some signals that corresponds to the anticipated structural peaks. The phloroglucinol protons appeared as a singlet in a slightly upfield region. While the three 3-hydroxybenzoic acid moieties aromatic signals appeared in most cases as multiplets further confirming the presence of impurities because in this compound, only singlet, doublet, and triplets signals are anticipated due to symmetry and chemical environment. The hydroxyl proton appeared as a broad singlet as anticipated ruling out the possible dimerization or trimerization via the hydroxyl group. Then the 3,5-dihydroxybenzoic acid counterparts showed less peaks of impurities compared with aforementioned compound but had a residual water peak that was broad in the upfield region which is common for DMSO- $d_6$  solubilized compounds. In 3,5-dihydroxybenzoate moiety, the downfield or the less shielded broad singlet signal with an integral of 6 was also attributed to the hydroxyl groups present which accounts for 6 protons in a tri-substituted compound. Following a similar trend was the 3,4,5-trihydroxybenzoate moiety which also showed a broad signal in the downfield region that was also associated with the hydroxyl groups and their integration of 9 (corresponding to the 9 hydroxyl protons) was also evidence of successful conjugation, however, the peaks of impurities were also observed.

The *N*-phenylacetamide based compounds also showed a similar trend to the mono-, di- and tri-substituted compounds in terms of the chemical position of the hydroxyl groups. However, this set of compounds entails the free amide pendant group. The free amide proton appeared as the most downfield signal in all the *N*-phenylacetamide based compounds and this is due to the electronegative nitrogen atom and the fact that it was not experiencing shielding because of surrounding atoms meaning it can only resonate electrons to the carbonyl moiety. In the carbon spectra this was also the case since the amide carbonyl carbon consistently appeared as the most downfield signal across all the compounds. The hydroxyl protons and carbons also appeared in downfield regions due to less shielding and an electronegative oxygen atom. However, gallic acid-based compounds would show two hydroxyl signals corresponding to the *meta* hydroxyl moieties and the second signal corresponds to the hydroxyl that is *para* to carbonyl but *ortho* to *meta* hydroxyl groups, which experiences more shielding than the *meta* hydroxyl groups<sup>309</sup>. The shielding is due to donation of electrons by resonance from the oxygen atom of the *para* hydroxyl group to the neighboring

*meta* hydroxyl groups.

The aromatic protons from the 3-acetamidophenyl moiety resonated within the expected ranges of these protons and the unsymmetrical protons were observed due to the different bonding chemical environment of the phenyl to hydroxybenzoic acid and acetamido (-NHCOCH<sub>3</sub>) moieties. These protons are spotted by two doublets, triplet and a singlet signal which covers all the expected signals on the 3-acetamidophenyl motif. The methyl protons of the acetamido moiety consistently resonated at upfield regions as sharp singlet signals as anticipated.

Complementary to the spectral techniques the high-resolution mass spectrometry coupled with ultra-high-resolution liquid chromatography (HRMS-LC) was utilized to quantify the characteristic fragmentation patterns of the synthesized ester compounds. This method offers exceptional sensitivity for the quantification of organic materials. The experiments were conducted in positive mode (electron spray ionization ESI<sup>+</sup>) and at high resolution. Upon direct injection of ester compounds at a concentration of 10 ppm, the charge-to-mass ratio corresponding to the ester compounds in question were identified, as detailed in Table 5.7.1. Importantly, there were no significant discrepancies observed between the theoretical mass and the experimental masses of the designated ester compounds. This suggests that the synthesis of the compounds was successfully achieved.

However, the fragmentation pattern of the each set of compounds was somehow similar. The base peak, which is the most intense peak, typically representing a stable fragment of the molecule rather than the intact molecular ion. The monoesters (**C2**, **C7** and **C11**) showed a similar base peak that was attributed to the cleavage of a protonated 3-hydroxyacetophenone ( $m/z = 137.05$ ) fragment and this was the most stable fragment and a common reactant in the mentioned compounds. Compound **C1** on the other hand showed a base peak that was attributed to methyl 3-hydroxybenzoate. The difference in mass between the molecular ion peak ( $M^+$ ) and the base peak ( $\Delta (M^+ - \text{Base peak})$ ) provides information about the fragmentation pattern of the compound. In most cases the  $\Delta$  value indicates a further fragmentation from the base peak, but in other instances it's a total different fragmentation leading to a new assignable fragment. Taking compound **C1** for instance the  $\Delta$  value was attributed to the de-protonated benzaldehyde radical ( $C_7H_5O^{\bullet-}$ ), which arises from the loss of acetyl and water molecule from the base peak fragment. The molecular ion peaks for the monoesters corresponded well with the predetermined empirical formula.

The di-substituted ester compounds followed similar trend to monoesters in the sense that the base peak were rather stable fragments that cleaved off. Compound **C3** (3-hydroxybenzoic acid

based compound) had a base peak of  $m/z = 241.04$  which was ascribed to 3-hydroxyphenyl-3-hydroxybenzoate radical ( $C_{14}H_9O_4^{3*}$ ) and the  $\Delta$  value was ascribed to methyl 3-hydroxybenzoate which is common in both the mono- and di-substituted ester compounds, making it the most labile fragment in ester compounds. The same methyl 3-hydroxybenzoate fragment was the base peak in **C8** (3,5-dihydroxybenzoic acid based compound). The  $\Delta$  value was attributed to 3-acetylphenyl 3,5-dihydroxybenzoate, which is a mono- conjugation of 3,5-dihydroxybenzoic acid and 3-hydroxyacetophenone. Then the gallic acid based compound (**C12**), gave a base peak that corresponded to  $(2\lambda^1\text{-ethyl})$ benzene radical ( $C_8H_7^{3*}$ ). The  $\Delta$  value was ascribe to cleavage of 1,3-phenylene bis(3-hydroxybenzoate) to give the corresponding base peak. The molecular ion peaks similar to monoesters corresponded to the calculated empirical formula.

The tri-substituted ester compounds (**C4**, **C5**, **C9** and **C13**) also followed suite like the mono- and di-substituted compounds by showing base peak that corresponds to either the reactant of partial anticipated product fragment. Compound **C4** (3-hydroxybenzoic acid based compound) gave a base peak that was attributed to the protonated acetophenone. The  $\Delta$  value was attributed to the 5-methoxy-1,3-phenylene bis(3-hydroxybenzoate) hydrate, which arises from the cleavage of 3-hydroxybenzoic acid moiety. The salicylic acid based compound (**C5**), showed a similar fragmentation pattern to 3-hydroxybenzoic acid and this was expected since the only difference in these compounds is the positioning of the hydroxyl group. The 3,5-dihydroxybenzoic acid based compound (**C9**) showed a base peak that was ascribed to protonated 3-hydroxyphenyl acetate. The cleaved off moiety was attributed to 1,3-phenylene bis(3,5-dihydroxybenzoate). The gallic acid based compound (**C13**) gave a base peak that was ascribed to methyl 3,4,5-trihydroxybenzoic acid and the cleaved off moiety or  $\Delta$  value corresponded to the protonated 3-((3,4-dihydroxybenzoyl)oxy)phenyl 3,4,5-trihydroxybenzoate fragment. The molecular ion peak for all the compounds corresponded well with the theoretical mass and the empirical formula found was in agreement with the calculated formula.

The *N*-phenylacetamide compounds (**C6**, **C10**, **C14** and **C15**) all showed a similar base peak that was attributed to the fragment of the protonated *N*-(3-hydroxyphenyl)acetamide. Compound **C6** (3-hydroxybenzoic acid based) and compound **C14** (salicylic acid based) further cleaved off the base peak to give a  $\Delta$  value that corresponded to the de-protonated *N*-phenylformamide fragment. The  $\Delta$  value for compound **C10** (3,5-dihydroxybenzoic acid based) was ascribed to *N*-ethyl-3-methylaniline due to the loss of the oxygen atom bond on the

carbonyl and the hydroxyl group of *N*-(3-hydroxyphenyl)acetamide. Furthermore, for gallic acid based compound (**C15**) the  $\Delta$  value was associated with fragment of 3,4-dihydroxy-5-methylbenzaldehyde that was due to the hydroxyl cleavage in the carboxyl acid and one *meta* positioned hydroxyl moiety.

Overall these compounds molecular ion peaks like the previously studied compounds were in agreement with the theoretical values further confirming successful conjugation of the ester compounds.

#### 5.4.2 FTIR, NMR and MS structural elucidation of amide compounds

The amide compounds just like esters had characteristic peaks to determine presumably successful conjugation. The secondary amine (NH-stretch), hydroxyl group (OH), carbonyl (C=O), aromatic C=C, NH-bend and the C-N stretch peaks are the most important peaks to reveal and amide bond formation.

The number of hydroxyl groups and the number of substituents upon bonding, either mono or di-substituted amides showed some slight shifts in the spectral results. The chemical shifts were expected due to the bulkier nature of di-amide substitution compared to mono-amide substitution<sup>311</sup>. Some compounds showed an overlapping peaks of hydroxyl and N-H stretch. The overlapping peaks are due to the previously given reason for *N*-phenylacetamide ester compounds, because these two groups are fully engaged in forming an extended H-bond network. The peaks were not specific to any consistent type of compound (mono- and di-amides). However, most of the mono-substituted compounds such as compound **KAM 5**, **KAM 7**, **KAM 8**, **KAM 9** and **KAM 12** showed higher frequency N-H stretch peaks. This could be due to differences in hydrogen bond contribution between mono-amides and di-amides. In mono-amides, the N-H group may be more readily available for hydrogen bonding interactions with solvent molecules or other functional groups in the molecule, leading to a higher stretching frequency<sup>312</sup>. In contrast, in di-amides, the presence of multiple amide groups may reduce the availability of the N-H group for hydrogen bonding, resulting in a lower stretching frequency<sup>313</sup>.

The carbonyl peak of **KAM 1** and **KAM 9** appeared in lower frequencies compared to the other compounds. While other compounds unexpectedly appeared in higher frequencies which is uncharacteristic of amide compounds. This could be attributed to the resonance effect, since aryl amide compounds typically contain a conjugated aromatic ring system, which can exhibit

resonance effects that influence the electronic distribution within the molecule. Resonance delocalization of electrons can lead to a higher effective electron density around the carbonyl carbon, resulting in a stronger C=O bond and thus a higher stretching frequency <sup>314</sup>.

The NMR results further corroborated the FTIR results since the NH and OH protons appeared as downfield signal due to the electronegative nitrogen and oxygen atoms. These bonds are affected by the attached aryl group(s) that causes them to occur downfield than in free NH and OH resonance. The carbon spectra clearly showed all carbons corresponding to the anticipated structures and their anticipated positions. The carbonyl carbon in most cases appeared as the most downfield carbon due to both sp<sup>2</sup> hybridization and to the double bond to oxygen. The hydroxyl carbon and proton also resonated at downfield regions and they occurred between the ranges of  $\delta_C$  155 – 185 ppm which is a range of amide compounds <sup>315</sup>.

In the mono-substituted amide compounds, the 3-aminophenol based compounds were unsymmetrical due to the added hydroxyl group whose proton consistently occurred in downfield regions. However, this signal was upfield compared to the NH proton but slightly downfield to the hydroxybenzoic acid hydroxyl proton(s), due to less shielding compared to these protons. There was a broad singlet signal in most cases for OH and NH shifts, the broad peak was not only due to the overlapping protons but also an exchange with solvent molecules (DMSO-*d*<sub>6</sub>). The aromatic protons for both 3-aminophenol and hydroxybenzoic acid moieties resonated in the expected region, even though they were affected by the hydroxyl substitutions in the hydroxybenzamide. This was also the case for aniline based compounds but these compounds showed symmetry in the aniline ring. Comparing the 3-aminophenol and aniline based compounds, there were differences in the chemical shifts of the aromatic protons and the hydroxyl protons due to the positional isomerism of the hydroxyl group on the phenylene ring.

The di-amides similar to mono-amides had two set of compounds, the 1,3-diaminobenzene and 2,4-diaminophenol based compounds. The former compounds showed symmetry as anticipated and the latter was unsymmetrical. The furthest downfield signal like the mono-substituted based compounds were the NH and OH protons which showed broad overlapping singlet signal due to similar attribute as the mono-substituted compounds. The chemical shifts of these peaks was influenced by hydrogen bonding and neighbouring substituents, since they shifted based on the number of hydroxyl moieties substituted in the hydroxybenzamide ring. These substituted hydroxyls also had an effect on the aromatic signals.

These findings were further supported by MS results which showed molecular ion peaks corresponding to the anticipated compounds with their  $m/z$  ratio showing protonated molecular weights. The fragmentation of these compounds was mainly giving base peaks that corresponded to the initial protonated precursor compounds or partial compound peaks. For example compound **KAM 1** a di-amide gave a base peak that was ascribed to (E)-N-(3-(2-methylpenta-2,4-dienamido)phenyl)benzamide. The other prominent peak in compound **KAM 1** that fragmented with  $m/z = 229$  was attributed to N-(3-aminophenyl)-3-hydroxybenzamide. The molecular ion peak formula for the di-amide compounds corresponded well with the calculated formula, which further indicated successful conjugation.

The mono-amide compound for instance, compound **KAM 10** showed a molecular ion peak that matched the anticipated calculated formula like any other mono-amide compounds. The base peak for mono-amides were initial precursor, in KAM 10 protonated aniline was the base peak for aniline-based compounds. While for 3-aminophenol compounds the base peak was found to be  $m/z = 102.12$  for all the 3-aminophenol based compounds and this fragment attributed to dehydrated *m*-toluidine peak. This fragment is the loss of gallic acid fragment plus dehydration of the hydroxyl group with a proton.

## 5.5 Conclusion

Spectroscopic techniques such as FTIR, NMR, and MS play crucial roles in characterizing synthesized compounds, providing valuable information about their molecular structure, composition, and chemical properties. Specifically, in the context of ester and amide compounds, these techniques offer insights into the formation of functional groups, substitution patterns, and the presence of characteristic peaks indicative of successful conjugation.

FTIR spectroscopy allowed for the identification of characteristic absorption bands corresponding to functional groups such as carbonyl (C=O), ester stretching (C-O), hydroxyl (OH), and  $sp^2$  hybridization ( $=CH_2$ ). In the case of ester compounds, variations in the vibrational frequencies of these functional groups were attributed to factors such as substitution patterns, electronic effects, and resonance phenomena within the aromatic ring. Similarly, amide compounds exhibited characteristic peaks for carbonyl (C=O), NH-stretch, OH, and C-N stretching vibrations. The presence of amide bonds is confirmed by the appearance of the carbonyl peak and the N-H stretching overlap with the hydroxyl group in the FTIR spectrum, which can be influenced by factors like resonance effects in aryl amides.



NMR spectroscopy complements FTIR analysis by providing detailed information about chemical environments, connectivity, and dynamics of nuclei within a molecule. In both ester and amide compounds, NMR spectra revealed distinctive shifts for protons and carbons associated with different functional groups. For example, in ester compounds, carbonyl carbons appeared as downfield signals due to their electronegativity and double bond character, while hydroxyl protons resonate at downfield regions as well. This was also the case for amide carbonyl carbon that appeared downfield followed by hydroxyl bonded carbons.

Mass spectrometry offered insight on the molecular weight, fragmentation patterns, and structural features of compounds. In ester compounds, the fragmentation pattern revealed the stable fragments corresponding to key precursors or partial products, allowing for confirmation of the conjugation process. Similarly, in amide compounds, the molecular ion peaks confirm the presence of the desired compounds, while fragmentation patterns provided additional structural information.

Overall, the integration of FTIR, NMR, and MS techniques provided a comprehensive understanding of the synthesized ester and amide compounds, confirming their molecular structures and elucidating their chemical properties. These spectroscopic methods are invaluable tools for chemists and researchers in the characterization and analysis of organic compounds, aiding in the development of new materials and pharmaceuticals.



## Chapter 6: Synthesis and characterization of nanoparticles

---

### 6.1 Introduction

Creating a new drug molecule is costly and time-consuming<sup>316</sup>. Despite many efforts to improve the bioavailability, selectivity, and stability of new drugs, their ability to reach the target site in sufficient dosages remains a major challenge. In theory, drug delivery systems can improve the efficacy and reduce the toxicity of drug molecules. Numerous drug delivery systems have been successfully used recently. However, some challenges still need to be resolved, and cutting-edge technology must be developed for drugs to be successfully delivered to the intended sites<sup>203</sup>.

There are currently ongoing studies on nano-based drug delivery systems that can be used and exploited to facilitate an advanced drug delivery system<sup>203</sup>. The “enhanced permeability and retention” (EPR) effect is one of the delivery system factors that can be taken advantage of by long-circulating nanocarriers like polymers for preferential extravasation from tumor vessels<sup>317</sup>. Nanoparticle-based polymer drug delivery systems have several advantages over free drugs or bulk delivery systems because they assist the drug in solubility, drug release profiles, diffusivity, bioavailability, and immunogenicity<sup>317,318</sup>. Polymer nanoparticles (PNPs) have the added advantage of being able to be modified for certain physical or chemical stimuli responses such as pH, redox, and temperature.

In this study, the polymer nanoparticles were prepared by polymerization of *N*-isopropyl acrylamide (NIPAM) and trans-cinnamic acid. Polymeric nanoparticles (NPs) are small particles with a size between 1 and 1000 nm engineered to carry active substances that have been surface-adsorbed onto the polymer or are entrapped within the polymeric core<sup>319</sup>. The choice of monomers in this study was based on inherent stimuli response in NIPAM and the potential bioactivity enhancement provided by trans-cinnamic acid.

Physicochemical properties such surface, size and morphology of nanoparticles play a crucial role in the application of nanoparticles as drug delivery systems. These physicochemical properties play an important role in cellular uptake or cell internalization. Since the cationic surface of polymeric NPs interacts with the anionic terminal of phospholipids, proteins, and glycans on the cell surface through electrostatic interactions, the positive charge of the surface may endorse more cellular attachment causing a higher uptake either through endocytosis or

by direct penetration<sup>320,321</sup>. In essence, for ideal polymeric NPs, the size should be small in the range of 20-200 nm and the surface charge should be positive for high cellular uptake<sup>322</sup>. However, other studies have shown a percentage cell uptake of up to 75% with a negatively charged surface while 65% was obtained for a neutral-charged surface<sup>323</sup>. While other studies have shown a high cellular uptake with a positively charged surface. All factors including size, shape, and surface charge are mainly dependent on parameters such as monomer concentration, temperature, pH amongst others.

## **6.2 Materials and Methods**

### **6.2.1 Materials**

#### **6.2.1.1 Chemicals**

The monomers, N-isopropylacrylamide (NIPAM), and trans-cinnamic acid were purchased at Sigma Aldrich SA and were used without further purification. The cross-linker N,N'-methylenebisacrylamide (MBA), the accelerator, N,N,N',N'-Tetramethylethylenediamine (TEMED), the surfactant sodium dodecyl sulfate (SDS) and the initiator ammonium persulfate (APS) were also obtained from Sigma Aldrich SA. Phosphate buffer saline (PBS) was used to dilute the nanoparticles for dynamic light scattering (DLS) analysis.

#### **6.2.1.2 Characterization**

##### **Transmission electron microscopy (TEM)**

Transmission electron microscopy images were captured using the FEI Technai T12 TEM equipped with an FEI eagle 4k x 4k CCD camera, operating at 120 kV. The sample images were taken at magnifications ranging from 5000x to 50000x, with a pixel size of 0.25 nm/pixel. An electron dose ranging from 1 to 45 e/Å<sup>2</sup> was utilized, while the nominal under-focus varied from -150 m (at 5000x magnification) to -2 m (at 50000x magnification).

##### **Dynamic light scattering (DLS)**

Dynamic Light Scattering technology from Malvern Panalytical was used to obtain the hydrodynamic size, zeta potential and polydispersity index (PDI). A homogeneous samples particles or molecules were dispersed in phosphate buffered saline (PBS). Samples were transferred into a clean and compatible sample cell for analysis.

## **6.2.2 Methods**

### **6.2.2.1 Synthesis of blank polymeric nanoparticles**

The nanoparticles were synthesized using a modified method that was reported by Lima et al<sup>324</sup>. In a mixture of 2.8 g of monomers (NIPAM and trans-cinnamic acid in a 50:50 ratio), dissolved in 190 mL of de-ionized water, 0.28 g of MBA (crosslinker) and 0.8 g of SDS were added to the reaction mixture. A solution containing 0.095 g of APS in 10 mL of de-ionized water was added to initiate polymerization. This was followed by the addition of a single drop of TEMED. The solution mixture was then stirred for 4 hours at 70 °C in an inert environment. The polymerization of the product was terminated by placing the mixture in a refrigerator. Acetone was added after cooling the solution to precipitate the polymer NPs from the solution. The product was obtained by evaporating and decanting the solution, and the gel-like product was purified by placing the resulting nanoparticle gel on a dialysis tube for 7 days while changing the water daily. After 7 days, attempts were made to lyophilize the product to obtain a powdery sample, but these attempts were unsuccessful.

### **6.2.2.2 Loading of compounds onto the polymeric nanoparticles**

The post-loading method was achieved by using a modified method reported by Nowaczyk and co-workers<sup>325</sup>. Briefly, the synthesized gel-like nanoparticles were dissolved in water to make an approximate 10% w/v hydrogel solution. Then the selected compounds (KAM1 and C1) in 1 mg/mL concentration were added to the hydrogel solution and mixed for 2 h. After 2 h the water trapped inside of the nanoparticles was replaced by the selected compounds solution upon heating to the upper critical temperature of NIPAM (which is ~38 °C). The heating was done for 1 hour. Then after an hour the solution was cooled to room temperature (rt) and stirred overnight. The loaded nanoparticles were also in a gel-like liquid form and were resistant to lyophilization.

It is important to provide a disclaimer at this critical juncture that the drug delivery and drug entrapment efficiency studies were almost impossible to conduct due to the gel-like nature of both the loaded and blank nanoparticles.

## 6.3 Results

### 6.3.1 FTIR results of the blank polymer and loaded nanoparticles

The ATR-FTIR spectra of NIPAM, TCA, and poly(NIPAM-co-TCA) or blank nanoparticles are shown in Figure 6.1. The results showed characteristic absorption peaks of monomers at  $\nu$ -1653/1615  $\text{cm}^{-1}$  (NIPAM) <sup>326-328</sup> and 1671/1628  $\text{cm}^{-1}$  (TCA) <sup>329</sup>. These bands corresponded to the monomer's  $\nu$ -C = C bond stretching vibration and the carbonyl group's C = O bond stretching vibration <sup>326,327</sup>. The vinyl group ( $\nu$ -CH<sub>2</sub>=CHR) in NIPAM occurred at 960  $\text{cm}^{-1}$  <sup>326</sup>. The secondary amine ( $\nu$ -N-H) stretching bond appeared at 3284  $\text{cm}^{-1}$  and the  $\nu$ -N-H bending appeared at 1546  $\text{cm}^{-1}$ . The trans-cinnamic acid (TCA) showed a peak at 1741  $\text{cm}^{-1}$  attributed to the conjugated  $\nu$ -C=C between the aryl ring and the carboxylic acid. The conjugated  $\nu$ -C=C (TCA) peak and both the  $\nu$ -N-H (stretching and bending) of NIPAM disappeared in the nanoparticles spectra. This confirms the polymerization of the monomers, however, the NIPAM conjugation was interesting because it bonded via the amine group instead of the  $\nu$ -C=C. This was confirmed by the disappearance of the amine groups. This was further confirmed by the availability of the vinyl functional group which appeared in lower wavenumber (866  $\text{cm}^{-1}$ ) for the polymer falling in the reported range of 800-1000  $\text{cm}^{-1}$  for PNIPAM copolymers <sup>326,327</sup>. The symmetric and asymmetric  $\nu$ -CH<sub>3</sub> of monomers appeared between 2824  $\text{cm}^{-1}$  and 2950  $\text{cm}^{-1}$ . While the methylene groups (=CH<sub>2</sub>) occurred between 3010  $\text{cm}^{-1}$  and 3071  $\text{cm}^{-1}$ .

The spectra of blank and loaded nanoparticles are shown in Figure 6.2. The spectra of loaded nanoparticles KAM1-L and C1-L showed similar spectra to blank nanoparticle absorption peaks indicating bonding via ionic bond. However, the emergence of two new peaks around 1734  $\text{cm}^{-1}$  and 1576  $\text{cm}^{-1}$  suggests chemical intermolecular bonding. KAM1-L only showed an emergence of a new absorption peak at 1539  $\text{cm}^{-1}$  also attributed to chemical intermolecular interaction.

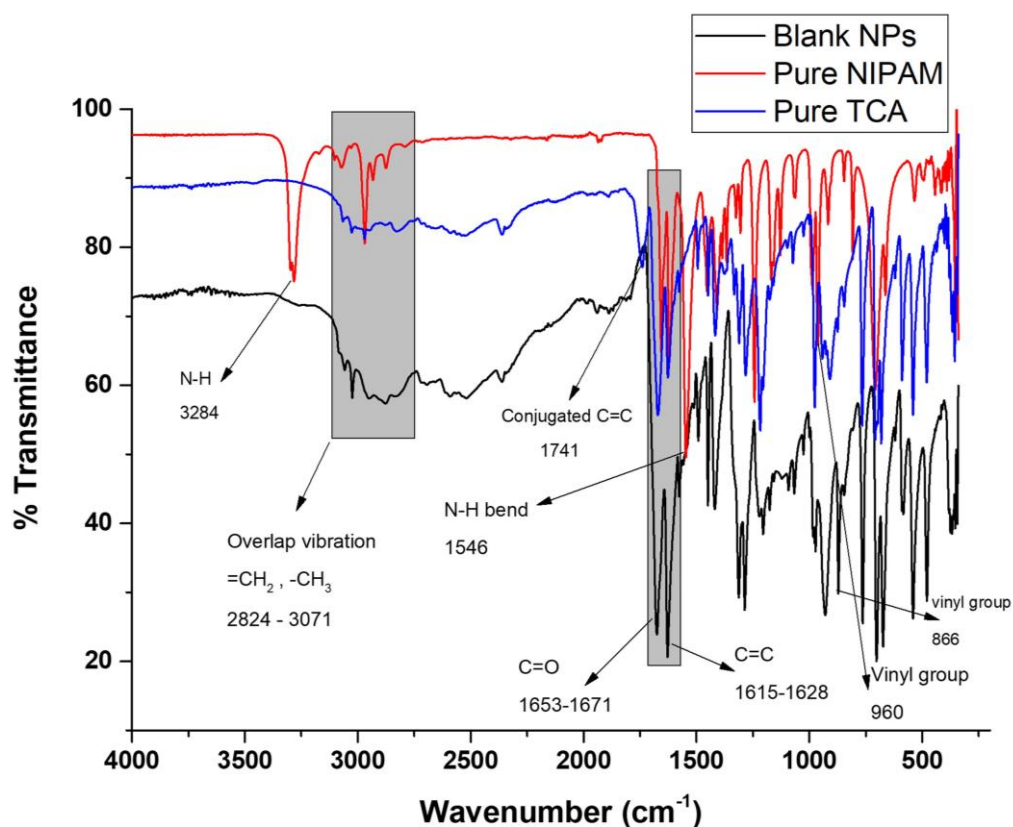


**Figure 6. 1:** Fourier transform infrared spectra of pristine NIPAM (red), pristine TCA (blue) and blank nanoparticles (black).

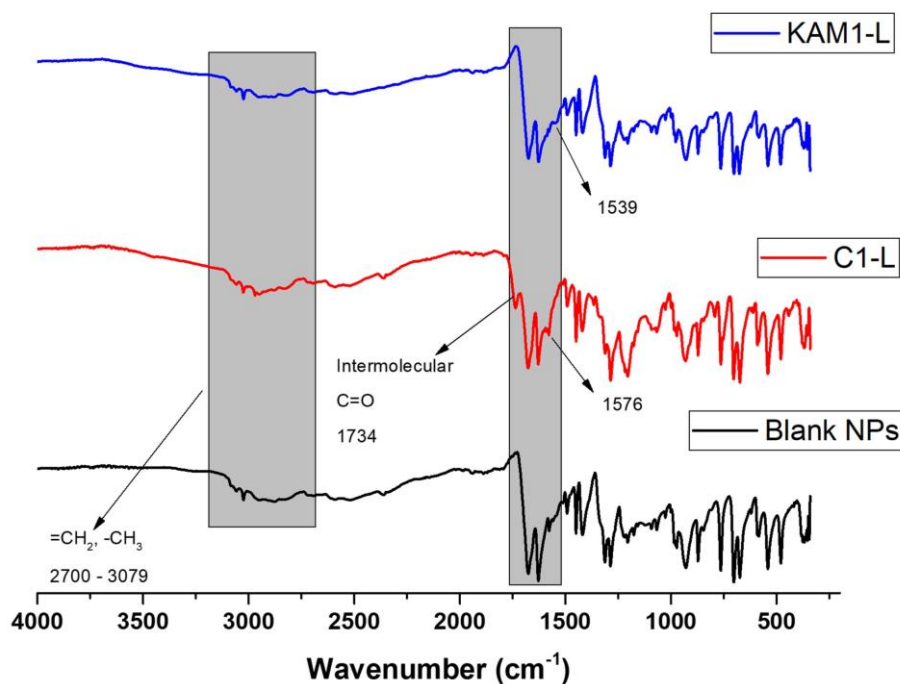
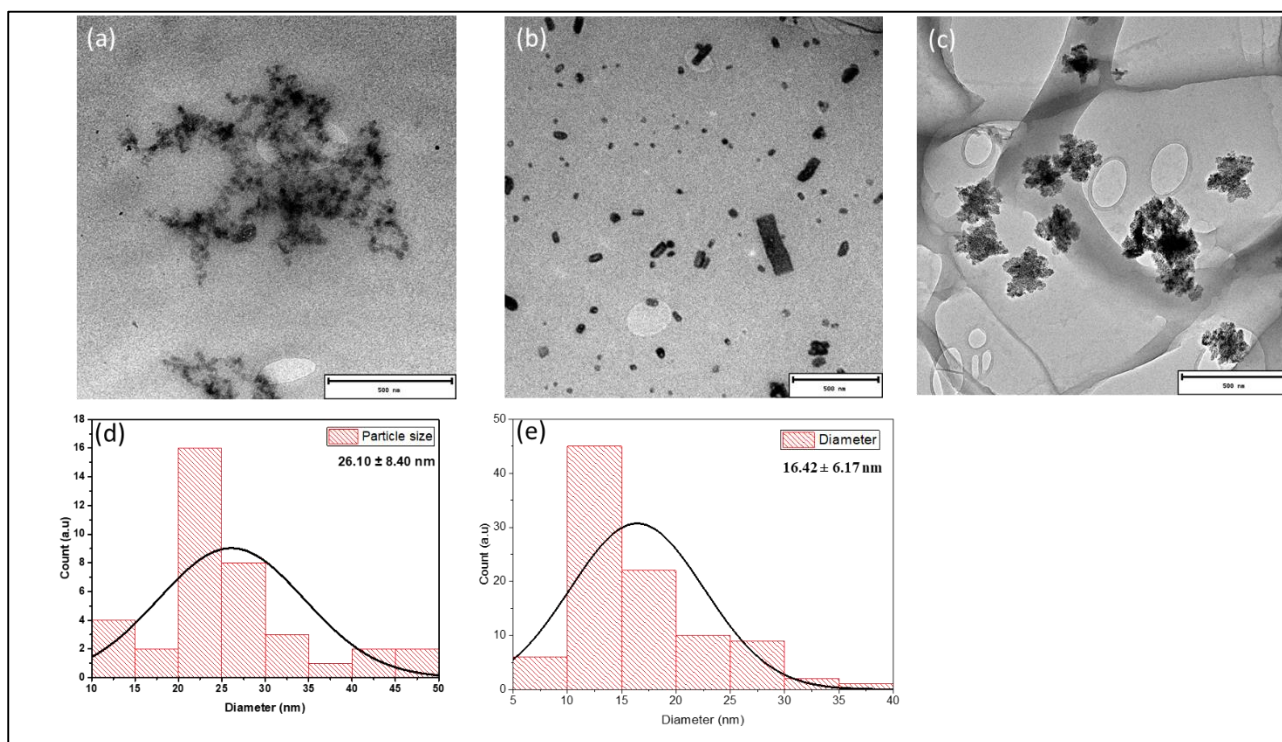


**Figure 6. 2:** Fourier transform infrared spectra of loaded nanoparticles (KAM1-L (blue), C1-L (red)) and blank nanoparticles (black).

### 6.3.2 TEM results of blank nanoparticles

The TEM images shown in Figure 6.3, reveal the effect of monomer concentrations on the size and shape of nanoparticles. Fig. 6.3 (a & d) shows the TEM image and histogram of nanoparticles prepared at 30:70 (NIPAM: TCA) monomer concentrations. The aforementioned concentration showed spherical nanoparticles that were small and clustered/agglomerated with average particle size of  $26.10 \pm 8.40$  nm dominated by particles in the range of 20 to 25 nm. The increase in the NIPAM concentration to a 50:50 ratio (Figure 6.3 b & e), showed a decrease in particles size to an average particle size of  $16.42 \pm 6.17$  nm with more particles ranging from 10 to 15 nm and the morphology also changed to mixed shapes that were mostly dominated by spherical particles but had some rod-like morphologies. The nanoparticles shown in Fig. 6.3 (c) showed particles that were big in size presenting in flower-like morphology most likely due to an increase in NIPAM concentration to a 70:30 ratio.



\*\*NIPAM – N-isopropylacrylamide, TCA – trans-cinnamic acid.

**Figure 6. 3:** TEM images of blank nanoparticles for (a-c) 30:70 monomer (NIPAM: TCA) concentration, 50:50 monomer (NIPAM: TCA), and 70:30 monomer (NIPAM: TCA) respectively and histogram micrographs of (d-e) 30:70 monomers and 50:50 monomers respectively.



### 6.3.3 TEM results of loaded nanoparticles

The TEM images and histogram of C1-loaded and KAM1-loaded are shown in Figure 6.4 are prepared from the 50:50 monomer concentrations, since this concentration was found to give well dispersed and small shaped nanoparticles. The C1-loaded nanoparticles depicted in Figure 6.4 (a) show spherical nanoparticles around the copper grid damaged areas and the histogram in Figure 6.4 (c) shows that the average particle size was  $102 \pm 32$  nm dominated by particles in the range of 80-120 nm. While KAM1-loaded only showed a few particles on the surface of the copper grid, which was unusual for most loaded nanoparticles. The average particle size was found to be  $2.40 \pm 1.30$  nm and were dominated by particles in the range of 1-3 nm.

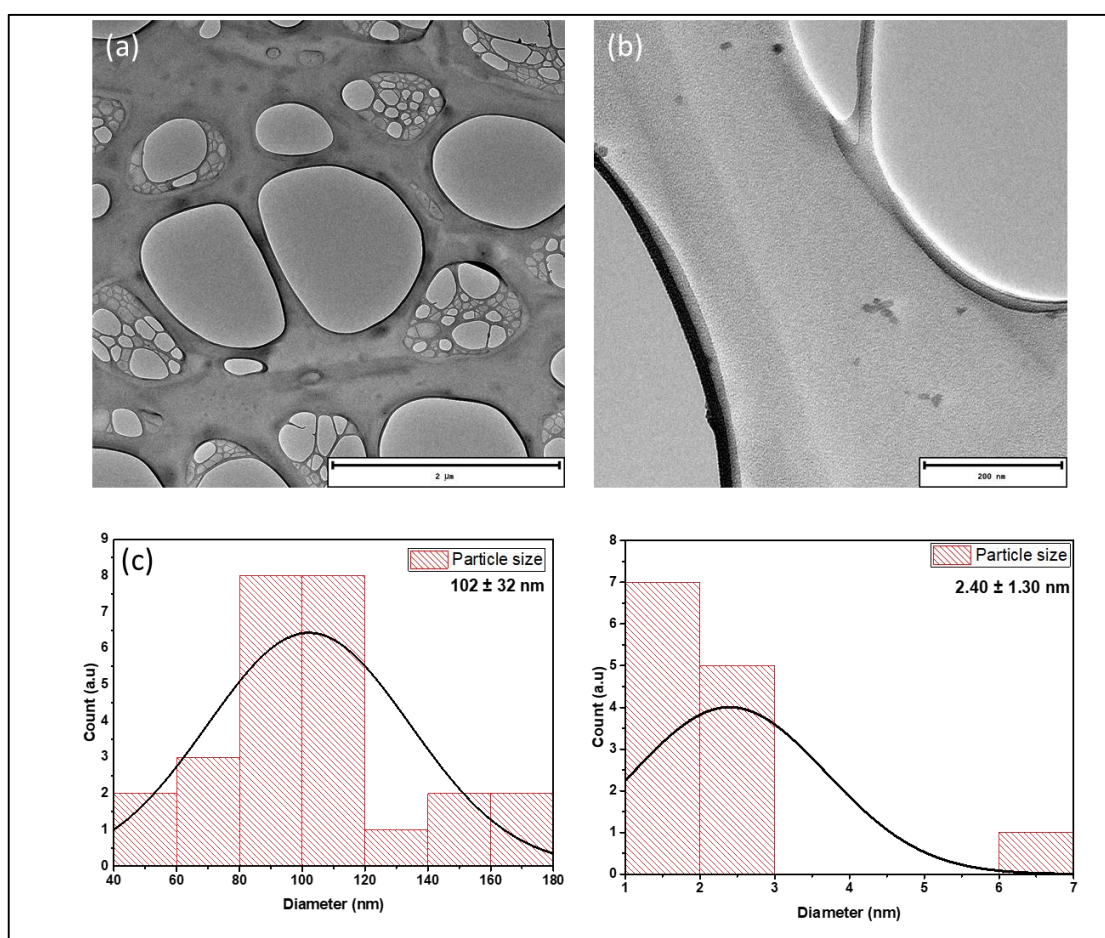


**Figure 6. 4:** TEM images of (a-b) C1-loaded and KAM1-loaded nanoparticles respectively and histogram of (c-d) C1-loaded and KAM1-loaded nanoparticles respectively.

### 6.3.4 Dynamic light scattering (DLS) results of blank and loaded nanoparticles

Results in Table 6.1 show the polydispersity index (PDI), zeta potential and hydrodynamic diameter parameter values of blank and loaded nanoparticles. The PDI of blank nanoparticles was found to be 0.239, while that for loaded nanoparticles was found to be 0.105 and 1.00 for C1-loaded and KAM1-loaded respectively. A PDI value that is  $> 0.5$  indicates polydispersity or broad particle size distribution, and a PDI value that is  $< 0.5$  indicates less particle size distribution or monodispersed particles<sup>330</sup>. Blank and C1-loaded nanoparticles PDI results suggested that these nanoparticles had lower polydispersity compared to the KAM1-loaded. Furthermore, the zeta potential for blank nanoparticles was sitting at  $-26.3 \pm 0.00$  mV while the loaded nanoparticles showed a positive surface charge of  $+16.4$  and  $+7.33$  mV for C1-loaded and KAM1-loaded respectively. Regarding the particle size, the hydrodynamic diameter determined from dynamic light scattering (DLS) was found to be 40.59, 98.70 and 3.18 nm for blank, C1-loaded and KAM1-loaded nanoparticles respectively. The particles sizes for blank and KAM1-loaded nanoparticles on DLS were slightly larger than sizes obtained from transmission electron microscope (TEM). However, C1-loaded nanoparticles were slightly smaller in size in comparison to the observed TEM sizes.

**Table 6. 1:** Dynamic light scattering (DLS) physicochemical characteristic of blank (50:50 molar ratio) and loaded nanoparticles.

| Nanomaterial | PDI   | Zeta-potential<br>(mV) | Hydrodynamic<br>size (nm) |
|--------------|-------|------------------------|---------------------------|
| Blank NPs    | 0.239 | $-26.3 \pm 0.00$       | 40.59                     |
| C1-loaded    | 0.105 | $+16.4 \pm 0.00$       | 98.70                     |
| KAM1-loaded  | 1.00  | $+7.33 \pm 0.00$       | 3.18                      |

## 6.4 Discussion

### 6.4.1 Fourier transform infrared (FTIR) spectroscopy

Confirmation of monomer interaction and the loading of compounds to nanoparticles were probed using attenuated reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. ATR-FTIR results confirmed successful polymerization and conjugation of the monomers toward polymer formation. It was observed in Figure 6.1a that certain characteristic peaks of

pristine NIPAM and TCA had disappeared from the polymer nanoparticle spectra. Absence of the  $\nu$ -N-H peak in the polymer indicated polymerization via the amine moiety which is again a rare observation since it is well documented that polymerization of NIPAM occurs via the double bond of the vinyl functional group ( $\nu$ -CHR=CH<sub>2</sub>)<sup>325–328,331</sup>. This unique case could be stemming from the bonding between C=O of TCA and N-H of NIPAM upon heating above lower critical solution temperature (LCST) forming partial intermolecular hydrogen bond C=O•••N-H as previously reported by<sup>332</sup>. As suggested by Futscher et al<sup>332</sup> above the LCST 34 °C temperature dehydrates and forms new hydrogen bonds between the neighbouring C=O and N-H moieties.

The amide I and II bands appearing between 1546 cm<sup>-1</sup> and 1653 cm<sup>-1</sup> for amide I and amide II in pristine NIPAM are attributed to  $\nu$ -N-H bending and  $\nu$ -C=O stretching respectively<sup>332,333</sup>. On the other hand, the TCA monomer bonded via the conjugated double bond since the  $\nu$ -C=C peak was absent in the polymer further proving that polymerization occurred between the monomers.

Loaded nanoparticles showed another interesting phenomena in C1-L where there was an emergence of new bands at 1734 cm<sup>-1</sup> attributed to intermolecular hydrogen bonding of C=O from the ester compound upon loading to the nanoparticles also forming C=O•••N-H bond. The second peak in C1-L appeared at 1576 cm<sup>-1</sup> also attributed to the intermolecular bonding with the amide part of the nanoparticles. This second peak was also observed in KAM1-L and could be attributed to the intermolecular N-H•••N-H interaction of the amide compound with the amide part of the nanoparticles. These new bands rule out ionic interaction of the nanoparticles with the compounds, suggesting hydrogen bonding because ionic interactions only show slight vibrational shift of peaks with no new peaks.

#### 6.4.2 Transmission electron microscopy (TEM)

Polymer nanoparticles have gained considerable attention in varied fields including biomedical engineering, material science, drug delivery, and nanotechnology. These nanoscale structures possess unique properties that make them ideal for numerous applications. The size and shape of these nanoparticles play a crucial role in determining their properties and functionalities. In recent years, researchers have been exploring the influence of monomer concentrations on the size and shape of polymer nanoparticles.

The monomer concentration effect was observed in this study, since different concentrations gave different shapes and sizes. From the results it was observed that lower monomer concentration (30%) of NIPAM and higher concentration (70%) of trans-cinnamic acid favoured spherically shaped small nanoparticles with agglomeration tendencies as seen in Fig. 6.2 (a). The smaller size of nanoparticles can be ascribed to lower monomer concentration of NIPAM and higher concentration of trans-cinnamic acid which in turn result in agglomeration. Conversely, higher monomer concentrations typically lead to larger nanoparticle sizes or aggregates attributed to the increased availability of monomers, enhancing the polymerization process and resulting in more polymer chains<sup>334</sup>. Zaki et al.<sup>335</sup> in their study commented on the contribution of an increase in temperature in addition to high monomer concentrations with both factors resulting in big nanoparticles. Lower monomer concentrations showed smaller-sized nanoparticles, but viscosity variation and limiting concentration are cited by many as affecting the particle size<sup>335</sup>. However, an increase in the percentage of NIPAM (50%) to an equal amount of trans-cinnamic acid (50%) interestingly showed a decrease in particle size as seen in Fig. 6.2 (b). This could be attributed to insufficient monomer availability which limits the polymerization process, resulting in shorter polymer chains and consequently smaller particle sizes.

The findings reported in this study in terms of the nanoparticle size for molar ratio as a parameter were however, in contrast to the results reported by Hwang et al.<sup>331</sup> who reported on a molar ratio effect of NIPAM and 2-Hydroxyethyl Methacrylate (HEMA). In their study, they found that an equal molar ratio of 5:5 of NIPAM:HEMA showed a larger particle size of approximately 500 nm, while a higher monomer concentration of NIPAM resulted in smaller nanoparticles<sup>331</sup>. The noted difference could be due to differences in the structure and interaction of HEMA and trans-cinnamic acid to NIPAM. HEMA has a hydroxyl pendant group before polymerization and a ketone group after polymerization with NIPAM, whereas trans-cinnamic acid has an aryl and a carboxylic acid group as pendant groups after polymerization.

A lower concentration of NIPAM (30%) spherical shape could be attributed to the equilibrium between nucleation and growth during particle formation, resulting in self-assembled spherical particles indicating that the nanoparticles were thermodynamically stable<sup>336</sup>. Moderate (50:50) and high NIPAM (70%) concentrations favored anisotropic particles and this is mainly due to the disruption in the equilibrium between nucleation and growth, resulting in irregular particle shapes such as rods, flower-like and other elongated shapes<sup>336,337</sup>. However, several factors

other than the precursor concentration can have effect on the overall shape of nanoparticles, parameters such as surfactant, temperature, solution pH amongst others can drive the reaction kinetically to produce anisotropic nanoparticles. Wu et al. reported a comprehensive investigation of the precursor concentration effect on the shape of nanoparticles in their review<sup>338</sup>. The higher monomer concentrations were linked with the facilitation of elongated anisotropic particle formation

Drug loading refers to the process of incorporating therapeutic agents into nanoparticles. This technique allows for controlled release, improved stability, and enhanced efficacy of drugs. In this study, compounds (C1 and KAM1) were loaded onto the nanoparticles in a 50:50 mass ratio. However, it proved essential to investigate how drug loading impacts nanoparticle size and shape. In this study, two contrasting scenarios unfolded in terms of the size and shape of the nanoparticles after incorporation of the drugs. In the first observation with compound C1-loaded, the nanoparticle size increased to  $102 \pm 32$  nm, while for compound KAM 1-loaded nanoparticles the size drastically decreased to  $2.40 \pm 1.30$  nm with an abnormal distribution of particles as observed in the histogram in Fig. 6.3 (d) and secondly, smaller numbers of particles were observed for KAM1-loaded nanoparticles.

Increase in size for C1-loaded nanoparticles is the most likely and anticipated scenario because loading of drugs or compounds onto the polymeric matrix occurs either by surface adsorption of drugs, or by encapsulation. Either of the two scenarios increases the diameter of the particles. Similar results were obtained by Varan and Bilensoy<sup>339</sup>, who reported an increase in particle size upon surface coating and drug-loading onto polymeric nanoparticles. Nowaczyk et al.<sup>325</sup> also observed a similar effect when loading doxorubicin onto the PNIPAM copolymerized system, further validating the effect of drug loading on particle size. However, the unlikely situation of KAM1-loaded nanoparticles where the number of particles and nanoparticles decreased significantly, could be attributed to several factors such as the overall charge of blank nanoparticles versus the charge of compound as well as drug encapsulation can have an effect on the number of particles.

During drug encapsulation, as the drug molecules are loaded onto the nanoparticles, they occupy space within the particle matrix. This then could result in an overall decrease in the number of available nanoparticle units, thereby decreasing the particles number. Xu et al.<sup>340</sup> reported similar findings whereby they formulated a system for controllable microfluidic production of drug-loaded PLGA nanoparticles using partial water-miscible mixed solvent

microdroplets as a precursor. In their system, DCM and DMSO are used to make the microdroplets that produce PLGA nanoparticles in their core system. The few particles obtained in their system were attributed to the concentration of the polymer chains inside the droplets that did not reach the supersaturation threshold thus forming fewer nuclei <sup>340</sup>. In the same study by Xu and co-workers, they also showed that particle size was not affected by the loading of a drug because even after DOX (doxorubicin) loading, the sizes remained small and the polydispersity was low. According to Xu and co-workers, the size variation of DOX-loaded NPs was directly related to the flow rate ratios and solvent compositions ( $V_{\text{DCM}}/V_{\text{DMSO}}$ ) <sup>340</sup>.

Therefore, the deduction from these referenced studies is that the issue of size and shape of nanoparticles can be quite complex. This study showed that size can decrease upon loading even though this happens in rare cases. The DLS results can shed light to some of the contributing factors to these rare phenomena through studies focussing on the surface properties of these materials.

#### **6.4.3 Dynamic light scattering (DLS)**

Particle size, surface charge, and polydispersity index (PDI) are known to be important parameters that greatly impact the physical stability of the nanoparticles as well as their cellular uptake, interaction with biological membranes, absorption rate, and biodistribution throughout the body <sup>339,341</sup>. Particle size is important in terms of cellular uptake, circulation, biodistribution and penetration into the tumor leaky vasculatures in cancer therapeutics applications. Along the same line, surface properties have an impact on the uptake, biodistribution, stability, and cell interactions. A low phagocytic uptake is exhibited by negatively charged nanoparticles leading to prolonged blood circulation of nanoparticles <sup>341</sup>. Positively charged nanoparticles, on the other hand, will likely result in increased phagocytosis due to improved nanoparticle-anionic cell membrane interaction <sup>341</sup>. Furthermore, the nanomaterial polydispersity index (PDI) is very important since the PDI measures the quality of the nanomaterials in relation to the size distribution <sup>342</sup>. Therefore, the preparation of homogenous (monodispersed) populations of nanocarriers of a specific size is necessary for the successful formulation of safe, stable, and efficient nanocarriers.

In this study, the particle size diameter of the prepared nanomaterials was found to be higher for DLS in comparison to the TEM obtained particles sizes. This is the case because in DLS the hydrodynamic diameter of an equivalent sphere (Z-average) of the nanoparticles is measured. While TEM does not only view each particle as a spherical particle, it also shows

different morphologies (X & Y plane of view dimensions) of particles. In addition, Inam et al. attributed the difference in particle size of TEM and DLS for their PLGA nanoparticles to a cloud of ionic species that enveloped the particle, which led to an increase in the hydrodynamic size of the particles<sup>343</sup>. Therefore, sizes obtained from a DLS instrument are normally higher (larger) in comparison to TEM particle sizes.

Blank nanoparticles gave an average diameter size of 40.59 nm, but upon loading with compounds a similar trend to TEM results was observed wherein C1-loaded nanoparticles size increased, while KAM1-loaded particle sizes decreased. Therefore, DLS results were in agreement with those for TEM further confirming the formation of nanoparticles and their ability to load the selected compounds. However, the main difference between TEM and DLS results proved to be based on nanoparticle size distribution (as seen in Appendix F1, particle distribution graphs). The PDI for blank and C1-loaded nanoparticles showed that the nanoparticles were monodispersed since they showed a particle size distribution  $\leq 0.5$ , while the TEM histograms (See Fig. 6.2 (e) and Fig. 6.3 (c-d)) showed broad particle distribution. KAM1-loaded on the other hand showed a PDI value that is  $> 0.5$  indicating broad and non-uniform particle distribution, and this was also the case in TEM results that also showed high particle distribution. Wilson and Prud'homme<sup>344</sup> reported different size distributions of TEM and DLS attributing the difference to the hydrated polymer corona that impacted the particle hydrodynamics, while for TEM the nanoparticles appeared as collapsed and desiccated shells<sup>344</sup>. Their results show that different surface morphologies could play a huge role in the overall particle distribution of nanoparticles, especially polymer nanoparticles that have swelling and coiling abilities.

In regards to the surface properties of the nanoparticles. The blank nanoparticles gave a negative surface charge and this could be attributed to the pendant carboxyl group on the trans-cinnamic acid that is ionisable. The loaded nanoparticles on the other, gave a positive surface charge, with C1-loaded giving a high positive charge of +16.4 mV and KAM1-loaded gave a lower positive charge surface of +7.33 mV. The high negative charge in polymer nanoparticles allowed electrostatic interaction with the compounds proving to be of value and importance in preventing agglomeration of particles. In addition to preventing coalescence and aggregation in aqueous media, the negatively charged surface of the NPs keeps them colloidally stable due to electrostatic repulsion between the negative charges, both of which are critical for biomedical uses<sup>345</sup>. The difference in the positively charged particles of C1-loaded and KAM1-loaded could be attributed to difference in the structural conjugation.

## 6.5 Conclusion

The FTIR results confirmed successful polymerization of the monomers since there was absence of characteristic monomeric peaks in the polymer spectra. However, a phenomenal interaction for the polymerization reaction was observed between the amide moieties of the NIPAM and carbonyl group of TCA. Loading the compounds onto nanoparticles was found to be facilitated by hydrogen bonding rather than ionic interactions since new vibrational peaks were present in both the loaded compounds. TEM on the other hand, suggested that the synthesized nanoparticles were in the nano range and this was corroborated by DLS results that further proved nano sized particles. The monomer ratio had an effect on both the size and shape of the nanoparticles since low NIPAM content with high TCA content led to the formation of small but aggregated particles, while equal content (ratio) of NIPAM and TCA gave small and well dispersed nanoparticles as demonstrated in results for DLS hydrodynamic size and PDI less than 0.5. High NIPAM content (70 %) and low TCA (30%) content showed anisotropic flower-like nanoparticles with large particle size. Loading compounds to nanoparticles led to larger particle sizes. Furthermore DLS exhibited that the blank nanoparticles had a high negative surface charge while loaded nanoparticles showed a positive surface charge.

The findings deliberated on in this chapter leave room for further exploration of these nanoparticles toward use in *in-vitro* biological assays because of their small sizes and variation in surface properties (which is required for bio-assays).



## Chapter 7: Antimicrobial assay and activity

---

### 7.1. Introduction

Antimicrobial agents play a critical role in combating microbial infectious diseases, which have plagued humanity throughout history and continue to pose significant health challenges today. However, the effectiveness of existing antimicrobial agents is being challenged by factors such as the emergence of drug-resistant pathogens and the discovery of previously unknown bacterial strains. Consequently, there is a pressing need for the development of new antibiotics and the modification of existing ones to address these challenges<sup>346</sup>.

To meet this demand, researchers have explored a wide range of organic and synthetic antibiotics with diverse structures, modes of action, and functional spectra. These efforts have led to the design and utilization of numerous antimicrobial agents sourced from various origins<sup>346</sup>.

In the context of this study, the focus was on the design, synthesis, and evaluation of amide and ester derivatives of hydroxybenzoic acid, along with their polymer nanoparticle conjugates, as potential antimicrobial agents. These compounds were subjected to screening against a panel of microbial strains, including two Gram-positive bacteria, two Gram-negative bacteria, and four fungal strains. The selection of these specific strains was based on their relevance to hospital settings, where they are frequently encountered and pose a significant risk to immunocompromised individuals, such as cancer patients.

By targeting strains commonly found in hospital environments and known to affect vulnerable patient populations, such as those undergoing cancer treatment, this study aimed to contribute to the development of antimicrobial agents that could address critical medical needs and potentially improve patient outcomes.

### 7.2. Methods and materials

#### 7.2.1. Test compounds

Figure 7.1 the 2D structures of the designed and synthesized amide compounds KAM 1 to KAM 12. On the other hand, ester compounds are presented in Figure 7.2 (C1 – C15).

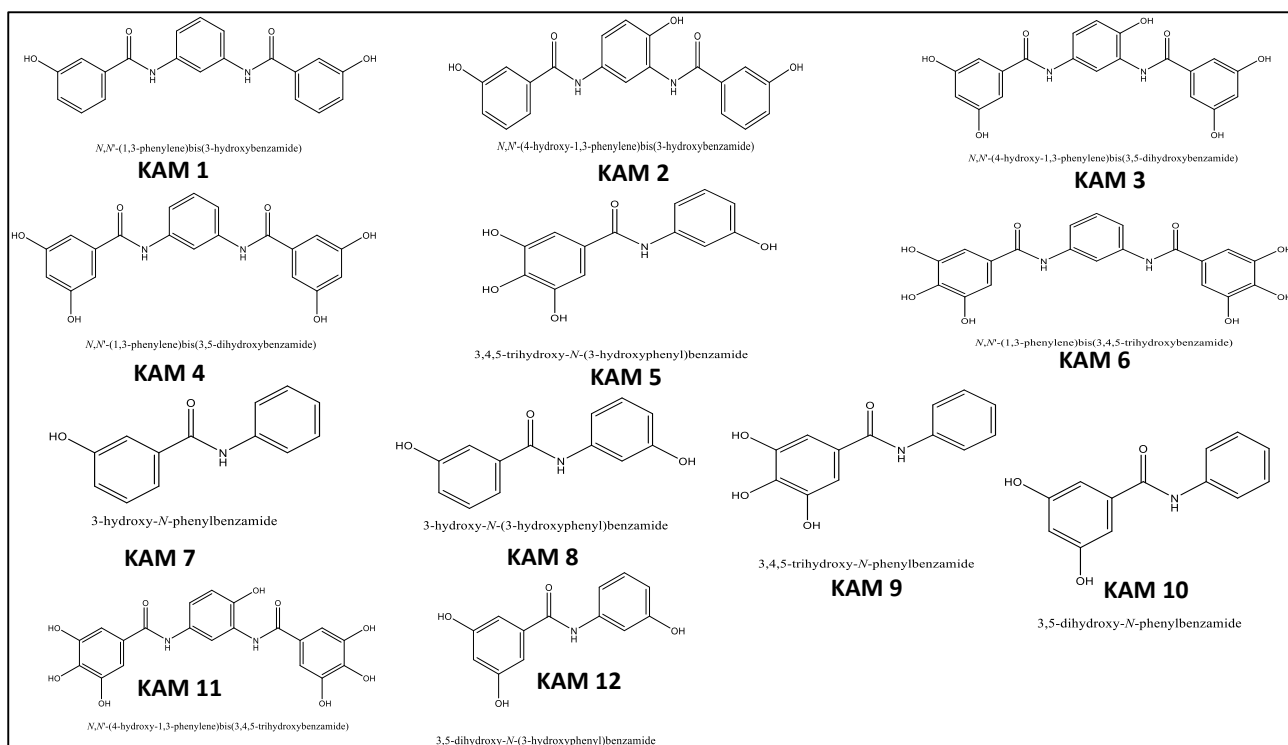


**Figure 7. 1:** 2D structures of the designed hydroxybenzoic acid amide derivative compounds.

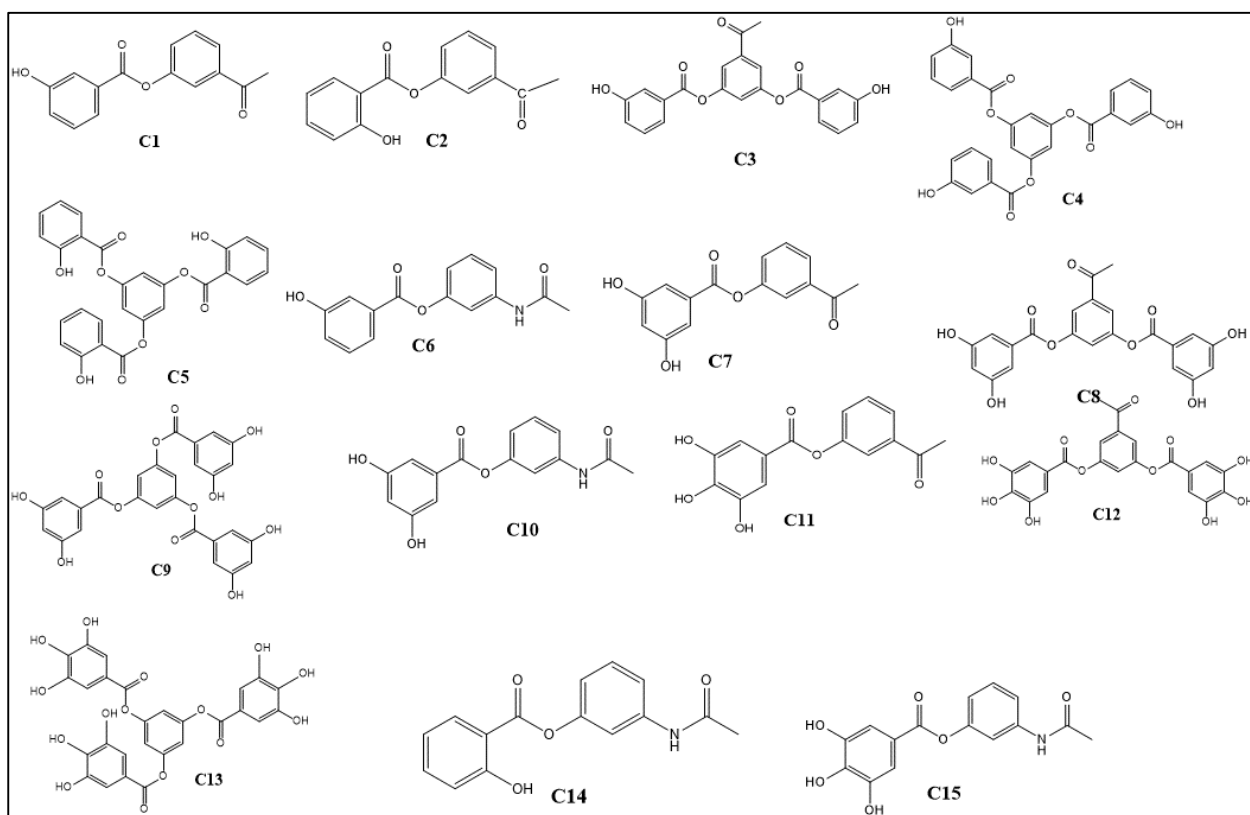


**Figure 7. 2:** 2D structures of the designed hydroxybenzoic acid amide derivative compounds.

### 7.2.2. Test organisms

For this study, *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC 8665), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 2850), *Candida parapsilosis* (ATCC 22019) and *Candida tropicalis* (ATCC 750) were used in the below-described assays. The strains were obtained from the Oral microbiology laboratory. An ethical clearance waiver was obtained from the Human Research Ethical Committee (HREC), Wits. (Certificate number: W-CBP-190621-1)

### 7.2.3. MIC and MBC/MFC assay

A freshly grown culture was used for each experiment. Inoculum that was used throughout the tests was prepared as follows; colonies were suspended in Phosphate-Buffered Saline (PBS) to obtain approximately  $10^7$  cfu/mL (0.5 MacFarland standard). Using a modified version of Eloff's stated microtitre twofold dilution approach, the minimum inhibitory concentration (MIC) was determined<sup>347</sup>. In a 96-well microtitre plate, 50  $\mu$ L of the test compounds (amides, esters, blank polymer nanoparticles, and selected loaded-polymer nanoparticles) diluted in Sabouraud Dextrose Broth for bacteria and Muller-Hinton Broth for fungi, was added to 50  $\mu$ L inoculum and serially diluted to obtain a 50  $\mu$ L mixture inoculum plus test compound in each well. Chlorhexidine gluconate was used as a positive control and the broth was used as a negative control. The prepared microtitre plates were then incubated at 37 °C for 24 hours for bacteria and 37 °C for 48 hours for fungi. The minimum inhibitory concentration (MIC) was determined to be the lowest concentration that showed no visible growth with the naked eye. After incubation, each well was cultured (5  $\mu$ L) on agar plates and examined for the presence of viable organisms for the determination of the minimum bactericidal concentration (MBC)/minimum fungicidal concentration (MFC). The MBC and MFC were determined by the lowest concentration that did not show any visible growth on the agar plate. Each test compound and the controls were tested in triplicate for reproducibility purposes.

## 7.3. Results

### 7.3.1. MIC/MBC results of Amides

Twelve amides were developed and their antibacterial properties against gram-positive and gram-negative bacterial pathogens. The results are presented in Table 7.1 and the summary is

provided in Table 7.2. The MIC ranged between 0.04 and 1.25 mg/mL whereas the MBC range was between 0.31 to 2.5 mg/mL. *Streptococcus pyogenes* was the most sensitive bacteria and *S. aureus* was the most resistant bacteria producing high MIC and MBC results. KAM1 and KAM11 were the best or more effective compounds producing an average MIC across the test organisms of 0.4 and 0.42 mg/mL respectively.

**Table 7. 1:** Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentrations (MBC) of Amide derivatives against gram-positive and gram-negative bacteria.

| Compound | Repeats     | MIC/MBC values for test bacteria (mg/mL) |             |                           |             |                               |             |                             |             |
|----------|-------------|------------------------------------------|-------------|---------------------------|-------------|-------------------------------|-------------|-----------------------------|-------------|
| Amides   |             | <i>E. coli</i><br>(GNB)                  |             | <i>S. aureus</i><br>(GPB) |             | <i>P. aeruginosa</i><br>(GNB) |             | <i>S. pyogenes</i><br>(GPB) |             |
|          |             | MIC                                      | MBC         | MIC                       | MBC         | MIC                           | MBC         | MIC                         | MBC         |
| KAM 1    | 1           | 0.63                                     | 1.25        | 0.16                      | 1.25        | 0.63                          | 1.25        | 0.16                        | 0.31        |
|          | 2           | 0.63                                     | 1.25        | 0.16                      | 1.25        | 0.63                          | 1.25        | 0.16                        | 0.31        |
|          | 3           | 0.63                                     | 1.25        | 0.31                      | 1.25        | 0.63                          | 1.25        | 0.16                        | 0.31        |
|          | <b>Mean</b> | <b>0.63</b>                              | <b>1.25</b> | <b>0.21</b>               | <b>1.25</b> | <b>0.63</b>                   | <b>1.25</b> | <b>0.16</b>                 | <b>0.31</b> |
| KAM 2    | 1           | 0.31                                     | 1.25        | 0.63                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | 2           | 0.31                                     | 1.25        | 0.63                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | 3           | 0.31                                     | 1.25        | 0.63                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | <b>Mean</b> | <b>0.31</b>                              | <b>1.25</b> | <b>0.63</b>               | <b>2.5</b>  | <b>0.63</b>                   | <b>1.25</b> | <b>0.31</b>                 | <b>0.63</b> |
| KAM 3    | 1           | 0.31                                     | 1.25        | 0.63                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | 2           | 0.31                                     | 1.25        | 0.63                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | 3           | 0.31                                     | 1.25        | 0.63                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | <b>Mean</b> | <b>0.31</b>                              | <b>1.25</b> | <b>0.63</b>               | <b>2.5</b>  | <b>0.63</b>                   | <b>1.25</b> | <b>0.31</b>                 | <b>0.63</b> |
| KAM 4    | 1           | 0.63                                     | 1.25        | 1.25                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | 2           | 0.63                                     | 1.25        | 1.25                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | 3           | 0.63                                     | 2.5         | 1.25                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | <b>Mean</b> | <b>0.63</b>                              | <b>2.08</b> | <b>1.25</b>               | <b>2.5</b>  | <b>0.63</b>                   | <b>1.25</b> | <b>0.31</b>                 | <b>0.63</b> |
| KAM 5    | 1           | 0.63                                     | 1.25        | 0.63                      | 2.5         | 0.63                          | 1.25        | 0.63                        | 1.25        |
|          | 2           | 0.63                                     | 1.25        | 0.63                      | 2.5         | 0.63                          | 1.25        | 0.63                        | 1.25        |
|          | 3           | 0.63                                     | 1.25        | 0.63                      | 2.5         | 0.63                          | 1.25        | 0.63                        | 1.25        |
|          | <b>Mean</b> | <b>0.63</b>                              | <b>1.25</b> | <b>0.63</b>               | <b>2.5</b>  | <b>0.63</b>                   | <b>1.25</b> | <b>0.63</b>                 | <b>1.25</b> |
| KAM 6    | 1           | 0.31                                     | 0.63        | 1.25                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | 2           | 0.31                                     | 0.63        | 1.25                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 1.25        |
|          | 3           | 0.31                                     | 0.63        | 1.25                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 1.25        |
|          | <b>Mean</b> | <b>0.31</b>                              | <b>0.63</b> | <b>1.25</b>               | <b>2.5</b>  | <b>0.63</b>                   | <b>1.25</b> | <b>0.31</b>                 | <b>1.04</b> |
| KAM 7    | 1           | 0.16                                     | 0.63        | 1.25                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 1.25        |
|          | 2           | 0.16                                     | 0.63        | 1.25                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 1.25        |
|          | 3           | 0.16                                     | 0.63        | 1.25                      | 2.5         | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | <b>Mean</b> | <b>0.16</b>                              | <b>0.63</b> | <b>1.25</b>               | <b>2.5</b>  | <b>0.63</b>                   | <b>1.25</b> | <b>0.31</b>                 | <b>1.04</b> |
| KAM 8    | 1           | 0.16                                     | 2.5         | 1.25                      | 2.5         | 0.16                          | 1.25        | 0.31                        | 0.63        |
|          | 2           | 0.16                                     | 1.25        | 1.25                      | 2.5         | 0.16                          | 1.25        | 0.31                        | 1.25        |
|          | 3           | 0.16                                     | 1.25        | 1.25                      | 2.5         | 0.16                          | 1.25        | 0.31                        | 1.25        |

|                      |             |             |             |             |            |             |             |             |             |
|----------------------|-------------|-------------|-------------|-------------|------------|-------------|-------------|-------------|-------------|
|                      | <b>Mean</b> | <b>0.16</b> | <b>1.67</b> | <b>1.25</b> | <b>2.5</b> | <b>0.16</b> | <b>1.25</b> | <b>0.31</b> | <b>1.04</b> |
| KAM 9                | 1           | 0.31        | 1.25        | 1.25        | 2.5        | 0.63        | 1.25        | 0.63        | 1.25        |
|                      | 2           | 0.31        | 1.25        | 1.25        | 2.5        | 0.63        | 1.25        | 0.63        | 1.25        |
|                      | 3           | 0.31        | 1.25        | 1.25        | 2.5        | 0.63        | 1.25        | 0.63        | 1.25        |
|                      | <b>Mean</b> | <b>0.31</b> | <b>1.25</b> | <b>1.25</b> | <b>2.5</b> | <b>0.63</b> | <b>1.25</b> | <b>0.63</b> | <b>1.25</b> |
| KAM 10               | 1           | 0.63        | 2.5         | 1.25        | 2.5        | 0.63        | 1.25        | 0.63        | 1.25        |
|                      | 2           | 0.63        | 2.5         | 1.25        | 2.5        | 0.63        | 1.25        | 0.63        | 1.25        |
|                      | 3           | 0.63        | 2.5         | 1.25        | 2.5        | 0.63        | 1.25        | 0.63        | 1.25        |
|                      | <b>Mean</b> | <b>0.63</b> | <b>2.5</b>  | <b>1.25</b> | <b>2.5</b> | <b>0.63</b> | <b>1.25</b> | <b>0.63</b> | <b>1.25</b> |
| KAM 11               | 1           | 0.04        | 0.63        | 1.25        | 2.5        | 0.08        | 0.63        | 0.31        | 0.63        |
|                      | 2           | 0.04        | 0.63        | 1.25        | 2.5        | 0.08        | 0.63        | 0.31        | 0.63        |
|                      | 3           | 0.04        | 0.63        | 1.25        | 2.5        | 0.08        | 0.63        | 0.31        | 0.63        |
|                      | <b>Mean</b> | <b>0.04</b> | <b>0.63</b> | <b>1.25</b> | <b>2.5</b> | <b>0.08</b> | <b>0.63</b> | <b>0.31</b> | <b>0.63</b> |
| KAM 12               | 1           | 0.63        | 2.5         | 1.25        | 2.5        | 0.63        | 2.5         | 0.31        | 1.25        |
|                      | 2           | 0.63        | 2.5         | 1.25        | 2.5        | 0.63        | 2.5         | 0.31        | 1.25        |
|                      | 3           | 0.63        | 2.5         | 1.25        | 2.5        | 0.63        | 2.5         | 0.31        | 1.25        |
|                      | <b>Mean</b> | <b>0.63</b> | <b>2.5</b>  | <b>1.25</b> | <b>2.5</b> | <b>0.63</b> | <b>2.5</b>  | <b>0.31</b> | <b>1.25</b> |
| CHX<br>(+ Control)   |             | No growth   |             | No growth   |            | No growth   |             | No growth   |             |
| Broth<br>(- Control) |             | Growth      |             | Growth      |            | Growth      |             | Growth      |             |

\*\*GNB: Gram-negative bacteria

GNP: Gram-positive bacteria

**Table 7. 2:** Summary of MIC/MBC results of Amide derivatives against Gram-negative and Gram-positive bacteria.

| Compound      | Mean of MIC/MBC values for test bacteria (mg/mL) n=3 |             |                  |             |                      |             |                    |             | Mean $\pm$ SD MIC                 |
|---------------|------------------------------------------------------|-------------|------------------|-------------|----------------------|-------------|--------------------|-------------|-----------------------------------|
|               | <i>E. coli</i>                                       |             | <i>S. aureus</i> |             | <i>P. aeruginosa</i> |             | <i>S. pyogenes</i> |             | mg/mL                             |
|               | MIC                                                  | MBC         | MIC              | MBC         | MIC                  | MBC         | MIC                | MBC         |                                   |
| <b>KAM 1</b>  | <b>0.63</b>                                          | <b>1.25</b> | <b>0.21</b>      | <b>1.25</b> | <b>0.63</b>          | <b>1.25</b> | <b>0.16</b>        | <b>0.31</b> | <b>0.40 <math>\pm</math> 0.22</b> |
| KAM 2         | 0.31                                                 | 1.25        | 0.63             | 2.5         | 0.63                 | 1.25        | 0.31               | 0.63        | 0.47 $\pm$ 0.18                   |
| KAM 3         | 0.31                                                 | 1.25        | 0.63             | 2.5         | 0.63                 | 1.25        | 0.31               | 0.63        | 0.47 $\pm$ 0.18                   |
| KAM 4         | 0.63                                                 | 2.08        | 1.25             | 2.5         | 0.63                 | 1.25        | 0.31               | 0.63        | 0.71 $\pm$ 0.39                   |
| KAM 5         | 0.63                                                 | 1.25        | 0.63             | 2.5         | 0.63                 | 1.25        | 0.63               | 1.25        | 0.63 $\pm$ 0.00                   |
| KAM 6         | 0.31                                                 | 0.63        | 1.25             | 2.5         | 0.63                 | 1.25        | 0.31               | 1.04        | 0.63 $\pm$ 0.44                   |
| KAM 7         | 0.16                                                 | 0.63        | 1.25             | 2.5         | 0.63                 | 1.25        | 0.31               | 1.04        | 0.59 $\pm$ 0.48                   |
| KAM 8         | 0.16                                                 | 1.67        | 1.25             | 2.5         | 0.16                 | 1.25        | 0.31               | 1.04        | 0.47 $\pm$ 0.52                   |
| KAM 9         | 0.31                                                 | 1.25        | 1.25             | 2.5         | 0.63                 | 1.25        | 0.63               | 1.25        | 0.71 $\pm$ 0.39                   |
| KAM 10        | 0.63                                                 | 2.5         | 1.25             | 2.5         | 0.63                 | 1.25        | 0.63               | 1.25        | 0.79 $\pm$ 0.31                   |
| <b>KAM 11</b> | <b>0.04</b>                                          | <b>0.63</b> | <b>1.25</b>      | <b>2.5</b>  | <b>0.08</b>          | <b>0.63</b> | <b>0.31</b>        | <b>0.63</b> | <b>0.42 <math>\pm</math> 0.57</b> |
| KAM 12        | 0.63                                                 | 2.5         | 1.25             | 2.5         | 0.63                 | 2.5         | 0.31               | 1.25        | 0.71 $\pm$ 0.39                   |

### 7.3.2. MIC/MFCs results of Amides

Antifungal properties of the amide compounds were also explored and the results are illustrated in Table 7.3 and the summary in Table 7.4. The MIC ranged between 0.31 and 1.25 mg/mL, while the MFC ranged between 0.31 and 1.25 mg/mL. Amongst the studied fungal species, *Candida tropicalis* was found to be a more sensitive fungi while other *Candida* species were less sensitive to amide compounds. Compound KAM 12 was the least active compound against *C. tropicalis* with a mean MIC of  $0.71 \pm 0.39$  mg/mL. *Candida albicans*, *Candida glabrata*, and *Candida parapsilosis* showed similar MIC and MFC values. However, compound KAM 12 was inactive against *C. tropicalis* showing a MIC value of 1.25 mg/mL. Across a broad spectrum of fungal strains, compound KAM 5 performed better in comparison to other amide compounds, with an average MIC of  $0.47 \pm 0.18$  mg/mL.

**Table 7. 3:** Minimum inhibitory concentrations (MIC) and Minimum fungicidal concentrations (MFC) of Amide derivatives against *Candida* species.

| Compound | Repeats     | MIC/MFC values for test <i>Candida</i> species (mg/mL) n=3 |             |                    |             |                      |             |                        |             |
|----------|-------------|------------------------------------------------------------|-------------|--------------------|-------------|----------------------|-------------|------------------------|-------------|
|          |             | <i>C. albicans</i>                                         |             | <i>C. glabrata</i> |             | <i>C. tropicalis</i> |             | <i>C. parapsilosis</i> |             |
|          |             | MIC                                                        | MFC         | MIC                | MFC         | MIC                  | MFC         | MIC                    | MFC         |
| KAM 1    | 1           | 0.63                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.31        | 0.63                   | 0.63        |
|          | 2           | 0.63                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.31        | 0.63                   | 0.63        |
|          | 3           | 0.63                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.31        | 0.63                   | 0.63        |
|          | <b>Mean</b> | <b>0.63</b>                                                | <b>0.63</b> | <b>0.63</b>        | <b>0.63</b> | <b>0.31</b>          | <b>0.31</b> | <b>0.63</b>            | <b>0.63</b> |
| KAM 2    | 1           | 0.63                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.63        | 0.63                   | 0.63        |
|          | 2           | 0.63                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.31        | 0.63                   | 0.63        |
|          | 3           | 0.63                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.31        | 0.63                   | 0.63        |
|          | <b>Mean</b> | <b>0.63</b>                                                | <b>0.63</b> | <b>0.63</b>        | <b>0.63</b> | <b>0.31</b>          | <b>0.42</b> | <b>0.63</b>            | <b>0.63</b> |
| KAM 3    | 1           | 0.63                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.63        | 0.63                   | 0.63        |
|          | 2           | 0.63                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.63        | 0.63                   | 0.63        |
|          | 3           | 0.63                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.63        | 0.63                   | 0.63        |
|          | <b>Mean</b> | <b>0.63</b>                                                | <b>0.63</b> | <b>0.63</b>        | <b>0.63</b> | <b>0.31</b>          | <b>0.63</b> | <b>0.63</b>            | <b>0.63</b> |
| KAM 4    | 1           | 0.63                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.63        | 0.63                   | 0.63        |
|          | 2           | 0.63                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.63        | 0.63                   | 0.63        |
|          | 3           | 0.63                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.63        | 0.63                   | 0.63        |
|          | <b>Mean</b> | <b>0.63</b>                                                | <b>0.63</b> | <b>0.63</b>        | <b>0.63</b> | <b>0.31</b>          | <b>0.63</b> | <b>0.63</b>            | <b>0.63</b> |
| KAM 5    | 1           | 0.31                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.63        | 0.63                   | 0.63        |
|          | 2           | 0.31                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.63        | 0.63                   | 0.63        |
|          | 3           | 0.31                                                       | 0.63        | 0.63               | 0.63        | 0.31                 | 0.63        | 0.63                   | 0.63        |



|        |             |             |             |             |             |             |             |             |             |
|--------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
|        |             | <b>0.31</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.31</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> |
| KAM 6  | 1           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.63        | 0.63        | 0.63        |
|        | 2           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.63        | 0.63        | 0.63        |
|        | 3           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.63        | 0.63        | 0.63        |
|        | <b>Mean</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.31</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> |
| KAM 7  | 1           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.63        | 0.63        | 0.63        |
|        | 2           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.63        | 0.63        | 0.63        |
|        | 3           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.63        | 0.63        | 0.63        |
|        | <b>Mean</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.31</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> |
| KAM 8  | 1           | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        |
|        | 2           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.31        | 0.63        | 0.63        |
|        | 3           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.31        | 0.63        | 0.63        |
|        | <b>Mean</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.42</b> | <b>0.42</b> | <b>0.63</b> | <b>0.63</b> |
| KAM 9  | 1           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.63        | 0.63        | 0.63        |
|        | 2           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.63        | 0.63        | 0.63        |
|        | 3           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.63        | 0.63        | 0.63        |
|        | <b>Mean</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.31</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> |
| KAM 10 | 1           | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        |
|        | 2           | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        |
|        | 3           | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        |
|        | <b>Mean</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> |
| KAM 11 | 1           | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        | 0.63        |
|        | 2           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.63        | 0.63        | 0.63        |
|        | 3           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.63        | 0.63        | 0.63        |
|        | <b>Mean</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.42</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> |
| KAM 12 | 1           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.63        | 1.25        | 0.63        |
|        | 2           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.63        | 1.25        | 0.63        |
|        | 3           | 0.63        | 0.63        | 0.63        | 0.63        | 0.31        | 0.63        | 1.25        | 0.63        |

|                      |             |             |             |             |             |             |             |             |             |
|----------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
|                      | <b>Mean</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.31</b> | <b>0.63</b> | <b>1.25</b> | <b>0.63</b> |
| CHLX<br>(+ Control)  |             | No growth   |             | No growth   |             | No growth   |             | No growth   |             |
| Broth<br>(- Control) |             | Growth      |             | Growth      |             | Growth      |             | Growth      |             |

**Table 7. 4:** Summary of MIC/MFC results of Amide derivatives.

| <b>Compound</b> | <b>Mean MIC/MFC values for test <i>Candida</i> species (mg/mL) n=3</b> |             |                           |             |                             |             |                               |             | <b>Mean <math>\pm</math> SD MIC</b> |
|-----------------|------------------------------------------------------------------------|-------------|---------------------------|-------------|-----------------------------|-------------|-------------------------------|-------------|-------------------------------------|
| <b>Amides</b>   | <b><i>C. albicans</i></b>                                              |             | <b><i>C. glabrata</i></b> |             | <b><i>C. tropicalis</i></b> |             | <b><i>C. parapsilosis</i></b> |             | <b>mg/mL</b>                        |
|                 | <b>MIC</b>                                                             | <b>MFC</b>  | <b>MIC</b>                | <b>MFC</b>  | <b>MIC</b>                  | <b>MFC</b>  | <b>MIC</b>                    | <b>MFC</b>  |                                     |
| KAM 1           | 0.63                                                                   | 0.63        | 0.63                      | 0.63        | 0.31                        | 0.31        | 0.63                          | 0.63        | 0.55 $\pm$ 0.16                     |
| KAM 2           | 0.63                                                                   | 0.63        | 0.63                      | 0.63        | 0.31                        | 0.42        | 0.63                          | 0.63        | 0.55 $\pm$ 0.16                     |
| KAM 3           | 0.63                                                                   | 0.63        | 0.63                      | 0.63        | 0.31                        | 0.63        | 0.63                          | 0.63        | 0.55 $\pm$ 0.16                     |
| KAM 4           | 0.63                                                                   | 0.63        | 0.63                      | 0.63        | 0.31                        | 0.63        | 0.63                          | 0.63        | 0.55 $\pm$ 0.16                     |
| KAM 5           | <b>0.31</b>                                                            | <b>0.63</b> | <b>0.63</b>               | <b>0.63</b> | <b>0.31</b>                 | <b>0.63</b> | <b>0.63</b>                   | <b>0.63</b> | <b>0.47 <math>\pm</math> 0.18</b>   |
| KAM 6           | 0.63                                                                   | 0.63        | 0.63                      | 0.63        | 0.31                        | 0.63        | 0.63                          | 0.63        | 0.55 $\pm$ 0.16                     |
| KAM 7           | 0.63                                                                   | 0.63        | 0.63                      | 0.63        | 0.31                        | 0.63        | 0.63                          | 0.63        | 0.55 $\pm$ 0.16                     |
| KAM 8           | 0.63                                                                   | 0.63        | 0.63                      | 0.63        | 0.42                        | 0.42        | 0.63                          | 0.63        | 0.58 $\pm$ 0.11                     |
| KAM 9           | 0.63                                                                   | 0.63        | 0.63                      | 0.63        | 0.31                        | 0.63        | 0.63                          | 0.63        | 0.55 $\pm$ 0.16                     |
| KAM 10          | 0.63                                                                   | 0.63        | 0.63                      | 0.63        | 0.63                        | 0.63        | 0.63                          | 0.63        | 0.63 $\pm$ 0.00                     |
| KAM 11          | 0.63                                                                   | 0.63        | 0.63                      | 0.63        | 0.42                        | 0.63        | 0.63                          | 0.63        | 0.58 $\pm$ 0.11                     |
| KAM 12          | 0.63                                                                   | 0.63        | 0.63                      | 0.63        | 0.31                        | 0.63        | 1.25                          | 0.63        | 0.71 $\pm$ 0.39                     |

### 7.3.3. MIC/MBC of Ester derivatives

The antibacterial properties of the ester compounds were investigated, with the results shown in Table 7.5 and a summary in Table 7.6. The MIC of all the synthesized compounds ranged from 0.02 to 1.25 mg/mL, whereas the MBC ranged from 0.04 to 2.5 mg/mL. Compound C1 showed the best mean MIC with a value of  $0.28 \pm 0.26$  mg/mL, followed by compound C9 which showed an MIC value of  $0.38 \pm 0.30$  mg/mL across all bacteria. *Streptococcus pyogenes* was the most sensitive strain, while *E. coli* and *S. aureus* are less sensitive to some of the compounds. Only compounds C1 and C9 showed the best MIC value of 0.16 mg/mL against *S. aureus*.

**Table 7.5:** Minimum inhibitory concentrations (MIC) and Minimum Bactericidal concentrations (MBC) of Ester derivates against Gram-negative and Gram-positive bacteria.

| Compound | Repeats     | MIC/MBC values for test bacteria (mg/mL) n=3 |             |                           |             |                               |             |                             |             |
|----------|-------------|----------------------------------------------|-------------|---------------------------|-------------|-------------------------------|-------------|-----------------------------|-------------|
| Esters   |             | <i>E. coli</i><br>(GNB)                      |             | <i>S. aureus</i><br>(GPB) |             | <i>P. aeruginosa</i><br>(GNB) |             | <i>S. pyogenes</i><br>(GPB) |             |
|          |             | MIC                                          | MBC         | MIC                       | MBC         | MIC                           | MBC         | MIC                         | MBC         |
| C1       | 1           | 0.63                                         | 1.25        | 0.16                      | 0.31        | 0.31                          | 1.25        | 0.02                        | 0.04        |
|          | 2           | 0.63                                         | 1.25        | 0.16                      | 0.31        | 0.31                          | 1.25        | 0.02                        | 0.04        |
|          | 3           | 0.63                                         | 1.25        | 0.16                      | 0.31        | 0.31                          | 1.25        | 0.02                        | 0.04        |
|          | <b>Mean</b> | <b>0.63</b>                                  | <b>1.25</b> | <b>0.16</b>               | <b>0.31</b> | <b>0.31</b>                   | <b>1.25</b> | <b>0.02</b>                 | <b>0.04</b> |
| C2       | 1           | 0.63                                         | 2.5         | 1.25                      | 1.25        | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | 2           | 0.63                                         | 2.5         | 1.25                      | 1.25        | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | 3           | 0.63                                         | 2.5         | 1.25                      | 1.25        | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | <b>Mean</b> | <b>0.63</b>                                  | <b>2.5</b>  | <b>1.25</b>               | <b>1.25</b> | <b>0.63</b>                   | <b>1.25</b> | <b>0.31</b>                 | <b>0.63</b> |
| C3       | 1           | 0.63                                         | 1.25        | 1.25                      | 1.25        | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | 2           | 0.63                                         | 1.25        | 1.25                      | 1.25        | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | 3           | 0.63                                         | 2.5         | 1.25                      | 1.25        | 0.63                          | 1.25        | 0.31                        | 0.53        |
|          | <b>Mean</b> | <b>0.63</b>                                  | <b>1.67</b> | <b>1.25</b>               | <b>1.25</b> | <b>0.63</b>                   | <b>1.25</b> | <b>0.31</b>                 | <b>0.63</b> |
| C4       | 1           | 0.63                                         | 1.25        | 1.25                      | 1.25        | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | 2           | 0.63                                         | 1.25        | 1.25                      | 1.25        | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | 3           | 0.63                                         | 1.25        | 1.25                      | 1.25        | 0.63                          | 1.25        | 0.31                        | 0.63        |
|          | <b>Mean</b> | <b>0.63</b>                                  | <b>1.25</b> | <b>1.25</b>               | <b>1.25</b> | <b>0.63</b>                   | <b>1.25</b> | <b>0.31</b>                 | <b>0.63</b> |
| C5       | 1           | 1.25                                         | 2.5         | 0.63                      | 1.25        | 0.63                          | 2.5         | 0.04                        | 0.04        |
|          | 2           | 1.25                                         | 2.5         | 0.63                      | 1.25        | 0.63                          | 2.5         | 0.04                        | 0.04        |
|          | 3           | 1.25                                         | 2.5         | 0.63                      | 1.25        | 0.63                          | 2.5         | 0.04                        | 0.04        |
|          | <b>Mean</b> | <b>1.25</b>                                  | <b>2.5</b>  | <b>0.63</b>               | <b>1.25</b> | <b>0.63</b>                   | <b>2.5</b>  | <b>0.04</b>                 | <b>0.04</b> |

|     |             |             |            |             |             |             |             |             |             |
|-----|-------------|-------------|------------|-------------|-------------|-------------|-------------|-------------|-------------|
| C6  | 1           | 0.63        | 2.5        | 0.63        | 1.25        | 0.63        | 2.5         | 0.08        | 0.16        |
|     | 2           | 0.63        | 2.5        | 0.63        | 1.25        | 0.63        | 2.5         | 0.08        | 0.16        |
|     | 3           | 0.63        | 2.5        | 0.63        | 1.25        | 0.63        | 2.5         | 0.08        | 0.16        |
|     | <b>Mean</b> | <b>0.63</b> | <b>2.5</b> | <b>0.63</b> | <b>1.25</b> | <b>0.63</b> | <b>2.5</b>  | <b>0.08</b> | <b>0.16</b> |
| C7  | 1           | 0.63        | 2.5        | 0.63        | 2.5         | 0.63        | 2.5         | 0.16        | 0.31        |
|     | 2           | 0.63        | 2.5        | 0.63        | 2.5         | 0.63        | 2.5         | 0.16        | 0.31        |
|     | 3           | 0.63        | 2.5        | 0.63        | 2.5         | 0.63        | 2.5         | 0.16        | 0.31        |
|     | <b>Mean</b> | <b>0.63</b> | <b>2.5</b> | <b>0.63</b> | <b>2.5</b>  | <b>0.63</b> | <b>2.5</b>  | <b>0.16</b> | <b>0.31</b> |
| C8  | 1           | 0.63        | 2.5        | 0.63        | 2.5         | 0.63        | 2.5         | 0.16        | 0.31        |
|     | 2           | 0.63        | 2.5        | 0.63        | 2.5         | 0.63        | 2.5         | 0.16        | 0.31        |
|     | 3           | 0.63        | 2.5        | 0.63        | 2.5         | 0.63        | 2.5         | 0.16        | 0.31        |
|     | <b>Mean</b> | <b>0.63</b> | <b>2.5</b> | <b>0.63</b> | <b>2.5</b>  | <b>0.63</b> | <b>2.5</b>  | <b>0.16</b> | <b>0.31</b> |
| C9  | 1           | 0.63        | 2.5        | 0.16        | 2.5         | 0.63        | 2.5         | 0.08        | 0.16        |
|     | 2           | 0.63        | 2.5        | 0.16        | 2.5         | 0.63        | 2.5         | 0.08        | 0.16        |
|     | 3           | 0.63        | 2.5        | 0.16        | 2.5         | 0.63        | 2.5         | 0.08        | 0.16        |
|     | <b>Mean</b> | <b>0.63</b> | <b>2.5</b> | <b>0.16</b> | <b>2.5</b>  | <b>0.63</b> | <b>2.5</b>  | <b>0.08</b> | <b>0.16</b> |
| C10 | 1           | 0.63        | 2.5        | 0.63        | 2.5         | 0.63        | 2.5         | 0.16        | 0.31        |
|     | 2           | 0.63        | 2.5        | 0.63        | 2.5         | 0.63        | 2.5         | 0.16        | 0.31        |
|     | 3           | 0.63        | 2.5        | 0.63        | 2.5         | 0.63        | 2.5         | 0.16        | 0.31        |
|     | <b>Mean</b> | <b>0.63</b> | <b>2.5</b> | <b>0.63</b> | <b>2.5</b>  | <b>0.63</b> | <b>2.5</b>  | <b>0.16</b> | <b>0.31</b> |
| C11 | 1           | 0.63        | 2.5        | 0.63        | 1.25        | 0.63        | 1.25        | 0.31        | 0.63        |
|     | 2           | 0.63        | 2.5        | 0.63        | 1.25        | 0.63        | 1.25        | 0.31        | 0.63        |
|     | 3           | 0.63        | 2.5        | 0.63        | 1.25        | 0.63        | 2.5         | 0.31        | 0.63        |
|     | <b>Mean</b> | <b>0.63</b> | <b>2.5</b> | <b>0.63</b> | <b>1.25</b> | <b>0.63</b> | <b>1.67</b> | <b>0.31</b> | <b>0.63</b> |
| C12 | 1           | 0.63        | 2.5        | 0.63        | 2.5         | 0.63        | 2.5         | 0.31        | 0.63        |
|     | 2           | 0.63        | 2.5        | 0.63        | 2.5         | 0.63        | 2.5         | 0.31        | 0.63        |
|     | 3           | 0.63        | 2.5        | 0.63        | 2.5         | 0.63        | 1.25        | 0.31        | 0.63        |
|     | <b>Mean</b> | <b>0.63</b> | <b>2.5</b> | <b>0.63</b> | <b>1.25</b> | <b>0.63</b> | <b>2.08</b> | <b>0.31</b> | <b>0.63</b> |

|                      |             |             |            |             |             |             |             |             |             |
|----------------------|-------------|-------------|------------|-------------|-------------|-------------|-------------|-------------|-------------|
| C13                  | 1           | 0.63        | 2.5        | 0.31        | 1.25        | 0.63        | 1.25        | 0.31        | 0.63        |
|                      | 2           | 0.63        | 2.5        | 0.31        | 1.25        | 0.63        | 1.25        | 0.16        | 0.31        |
|                      | 3           | 0.63        | 2.5        | 0.31        | 1.25        | 0.63        | 1.25        | 0.16        | 0.31        |
|                      | <b>Mean</b> | <b>0.63</b> | <b>2.5</b> | <b>0.31</b> | <b>1.25</b> | <b>0.63</b> | <b>1.25</b> | <b>0.21</b> | <b>0.42</b> |
| C14                  | 1           | 0.63        | 2.5        | 1.25        | 2.5         | 0.63        | 1.25        | 0.31        | 0.63        |
|                      | 2           | 0.63        | 2.5        | 1.25        | 2.5         | 0.63        | 1.25        | 0.31        | 0.63        |
|                      | 3           | 0.63        | 2.5        | 1.25        | 2.5         | 1.25        | 2.5         | 0.31        | 0.63        |
|                      | <b>Mean</b> | <b>0.63</b> | <b>2.5</b> | <b>1.25</b> | <b>2.5</b>  | <b>0.84</b> | <b>1.67</b> | <b>0.31</b> | <b>0.63</b> |
| C15                  | 1           | 0.63        | 2.5        | 1.25        | 2.5         | 0.63        | 1.25        | 0.31        | 0.63        |
|                      | 2           | 0.63        | 2.5        | 1.25        | 2.5         | 0.63        | 1.25        | 0.31        | 0.63        |
|                      | 3           | 0.63        | 2.5        | 1.25        | 2.5         | 0.63        | 1.25        | 0.31        | 0.63        |
|                      | <b>Mean</b> | <b>0.63</b> | <b>2.5</b> | <b>1.25</b> | <b>2.5</b>  | <b>0.63</b> | <b>1.25</b> | <b>0.31</b> | <b>0.63</b> |
| CHX<br>(+ Control)   |             | No growth   |            | No growth   |             | No growth   |             | No growth   |             |
| Broth<br>(- Control) |             | Growth      |            | Growth      |             | Growth      |             | Growth      |             |

\*\*GNB: Gram-negative bacteria

GPB: Gram-positive bacteria

**Table 7. 6:** Summary of MIC/MBC results of esters derivatives.

| Compound | Mean of MIC/MBC values for test bacteria (mg/mL) n=3 |             |                  |             |                      |             |                    |             | Mean ± SD MIC      |
|----------|------------------------------------------------------|-------------|------------------|-------------|----------------------|-------------|--------------------|-------------|--------------------|
|          | <i>E. coli</i>                                       |             | <i>S. aureus</i> |             | <i>P. aeruginosa</i> |             | <i>S. pyogenes</i> |             | mg/mL              |
|          | MIC                                                  | MBC         | MIC              | MBC         | MIC                  | MBC         | MIC                | MBC         |                    |
| C1       | <b>0.63</b>                                          | <b>1.25</b> | <b>0.16</b>      | <b>0.31</b> | <b>0.31</b>          | <b>1.25</b> | <b>0.02</b>        | <b>0.04</b> | <b>0.28 ± 0.26</b> |
| C2       | 0.63                                                 | 2.5         | 1.25             | 1.25        | 0.63                 | 1.25        | 0.31               | 0.62        | 0.71 ± 0.39        |
| C3       | 0.63                                                 | 1.67        | 1.25             | 1.25        | 0.63                 | 1.25        | 0.31               | 0.63        | 0.71 ± 0.39        |

|     |             |            |             |            |             |            |             |             |                                   |
|-----|-------------|------------|-------------|------------|-------------|------------|-------------|-------------|-----------------------------------|
| C4  | 0.63        | 1.25       | 1.25        | 1.25       | 0.63        | 1.25       | 0.31        | 0.63        | $0.71 \pm 0.39$                   |
| C5  | 1.25        | 2.5        | 0.63        | 1.25       | 0.63        | 2.5        | 0.04        | 0.04        | $0.64 \pm 0.49$                   |
| C6  | 0.63        | 2.5        | 0.63        | 1.25       | 0.63        | 2.5        | 0.08        | 0.16        | $0.49 \pm 0.28$                   |
| C7  | 0.63        | 2.5        | 0.63        | 2.5        | 0.63        | 2.5        | 0.16        | 0.31        | $0.51 \pm 0.24$                   |
| C8  | 0.63        | 2.5        | 0.63        | 2.5        | 0.63        | 2.5        | 0.16        | 0.31        | $0.51 \pm 0.24$                   |
| C9  | <b>0.63</b> | <b>2.5</b> | <b>0.16</b> | <b>2.5</b> | <b>0.63</b> | <b>2.5</b> | <b>0.08</b> | <b>0.16</b> | <b><math>0.38 \pm 0.30</math></b> |
| C10 | 0.63        | 2.5        | 0.63        | 2.5        | 0.63        | 2.5        | 0.16        | 0.31        | $0.51 \pm 0.24$                   |
| C11 | 0.63        | 2.5        | 0.63        | 1.25       | 0.63        | 1.67       | 0.31        | 0.63        | $0.55 \pm 0.16$                   |
| C12 | 0.63        | 2.5        | 0.63        | 1.25       | 0.63        | 2.08       | 0.31        | 0.63        | $0.55 \pm 0.16$                   |
| C13 | 0.63        | 2.5        | 0.31        | 1.25       | 0.63        | 1.25       | 0.21        | 0.42        | $0.45 \pm 0.22$                   |
| C14 | 0.63        | 2.5        | 1.25        | 2.5        | 0.84        | 1.67       | 0.31        | 0.63        | $0.76 \pm 0.39$                   |
| C15 | 0.63        | 2.5        | 1.25        | 2.5        | 0.63        | 1.25       | 0.31        | 0.63        | $0.71 \pm 0.39$                   |

#### 7.3.4. MIC/MFC of Ester derivatives

The antifungal properties of the ester compounds were also investigated, the results are presented in Table 7.7, and the summary is in Table 7.8. From the tested compounds, compounds C1 and C4 showed a broad spectrum activity against all fungal species with mean MIC values of  $0.43 \pm 0.24$  and  $0.39 \pm 0.16$  mg/mL respectively. *C. parapsilosis* was sensitive to C1 with a MIC value of 0.16 mg/mL. While compounds C2 and C3 were also active against all tested fungi with a mean MIC value of  $0.47 \pm 0.18$  mg/mL. The MFC results of the tested fungi ranged from 0.16 to 2.5 mg/mL. The 2.5 mg/mL MFC was observed only in *C. glabrata* suggesting reduced sensitivity for compounds C5-C15.



**Table 7. 7:** Minimum inhibitory concentrations and Minimum fungicidal concentrations of Ester derivates.

| Compound | Repeats     | MIC/MFC values for test Candida (mg/mL) n=3 |             |                    |             |                      |             |                        |             |
|----------|-------------|---------------------------------------------|-------------|--------------------|-------------|----------------------|-------------|------------------------|-------------|
|          |             | <i>C. albicans</i>                          |             | <i>C. glabrata</i> |             | <i>C. tropicalis</i> |             | <i>C. parapsilosis</i> |             |
|          |             | MIC                                         | MFC         | MIC                | MFC         | MIC                  | MFC         | MIC                    | MFC         |
| C1       | 1           | 0.63                                        | 0.63        | 0.63               | 1.25        | 0.31                 | 0.63        | 0.16                   | 0.31        |
|          | 2           | 0.63                                        | 0.63        | 0.63               | 1.25        | 0.31                 | 0.63        | 0.16                   | 0.31        |
|          | 3           | 0.63                                        | 0.63        | 0.63               | 1.25        | 0.31                 | 0.63        | 0.16                   | 0.31        |
|          | <b>Mean</b> | <b>0.63</b>                                 | <b>0.63</b> | <b>0.63</b>        | <b>1.25</b> | <b>0.31</b>          | <b>0.63</b> | <b>0.16</b>            | <b>0.31</b> |
| C2       | 1           | 0.63                                        | 0.63        | 0.63               | 1.25        | 0.31                 | 0.63        | 0.31                   | 0.63        |
|          | 2           | 0.63                                        | 0.63        | 0.63               | 1.25        | 0.31                 | 0.63        | 0.31                   | 0.63        |
|          | 3           | 0.63                                        | 0.63        | 0.63               | 1.25        | 0.31                 | 0.63        | 0.31                   | 0.63        |
|          | <b>Mean</b> | <b>0.63</b>                                 | <b>0.63</b> | <b>0.63</b>        | <b>1.25</b> | <b>0.31</b>          | <b>0.63</b> | <b>0.31</b>            | <b>0.63</b> |
| C3       | 1           | 0.63                                        | 0.63        | 0.63               | 1.25        | 0.31                 | 0.63        | 0.31                   | 0.63        |
|          | 2           | 0.63                                        | 0.63        | 0.63               | 1.25        | 0.31                 | 0.63        | 0.31                   | 0.63        |
|          | 3           | 0.63                                        | 0.31        | 0.63               | 1.25        | 0.31                 | 0.63        | 0.31                   | 0.63        |
|          | <b>Mean</b> | <b>0.63</b>                                 | <b>0.52</b> | <b>0.63</b>        | <b>1.25</b> | <b>0.31</b>          | <b>0.63</b> | <b>0.31</b>            | <b>0.63</b> |
| C4       | 1           | 0.31                                        | 0.63        | 0.63               | 1.25        | 0.31                 | 0.63        | 0.31                   | 0.63        |
|          | 2           | 0.31                                        | 0.63        | 0.63               | 1.25        | 0.31                 | 0.63        | 0.31                   | 0.63        |
|          | 3           | 0.31                                        | 0.63        | 0.63               | 1.25        | 0.31                 | 0.63        | 0.31                   | 0.63        |
|          | <b>Mean</b> | <b>0.31</b>                                 | <b>0.63</b> | <b>0.63</b>        | <b>1.25</b> | <b>0.31</b>          | <b>0.63</b> | <b>0.31</b>            | <b>0.63</b> |
| C5       | 1           | 0.63                                        | 0.63        | 0.63               | 2.5         | 0.63                 | 0.63        | 0.31                   | 0.63        |
|          | 2           | 0.63                                        | 0.63        | 0.63               | 2.5         | 0.63                 | 0.63        | 0.31                   | 0.63        |
|          | 3           | 0.63                                        | 0.63        | 0.63               | 2.5         | 0.63                 | 0.63        | 0.31                   | 0.63        |
|          | <b>Mean</b> | <b>0.63</b>                                 | <b>0.63</b> | <b>0.63</b>        | <b>2.5</b>  | <b>0.63</b>          | <b>0.63</b> | <b>0.31</b>            | <b>0.63</b> |
| C6       | 1           | 0.63                                        | 0.63        | 0.63               | 2.5         | 0.63                 | 0.63        | 0.31                   | 0.63        |
|          | 2           | 0.63                                        | 0.63        | 0.63               | 2.5         | 0.63                 | 0.63        | 0.31                   | 0.63        |

|     |             |             |             |             |            |             |             |             |             |
|-----|-------------|-------------|-------------|-------------|------------|-------------|-------------|-------------|-------------|
|     | 3           | 0.63        | 0.63        | 0.63        | 2.5        | 0.63        | 0.63        | 0.31        | 0.63        |
|     | <b>Mean</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>2.5</b> | <b>0.63</b> | <b>0.63</b> | <b>0.31</b> | <b>0.63</b> |
| C7  | 1           | 0.63        | 0.63        | 0.63        | 2.5        | 0.63        | 0.63        | 0.31        | 0.63        |
|     | 2           | 0.63        | 0.63        | 0.63        | 2.5        | 0.63        | 0.63        | 0.31        | 0.63        |
|     | 3           | 0.63        | 0.63        | 0.63        | 2.5        | 0.63        | 0.63        | 0.31        | 0.63        |
|     | <b>Mean</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>2.5</b> | <b>0.63</b> | <b>0.63</b> | <b>0.31</b> | <b>0.63</b> |
| C8  | 1           | 0.63        | 1.25        | 0.63        | 2.5        | 0.63        | 0.63        | 0.31        | 0.63        |
|     | 2           | 0.63        | 1.25        | 0.63        | 2.5        | 0.63        | 0.63        | 0.31        | 0.63        |
|     | 3           | 0.63        | 1.25        | 0.63        | 2.5        | 0.63        | 0.63        | 0.31        | 0.63        |
|     | <b>Mean</b> | <b>0.63</b> | <b>1.25</b> | <b>0.63</b> | <b>2.5</b> | <b>0.63</b> | <b>0.63</b> | <b>0.31</b> | <b>0.63</b> |
| C9  | 1           | 0.63        | 0.63        | 0.63        | 2.5        | 0.63        | 0.63        | 0.31        | 0.63        |
|     | 2           | 0.63        | 0.63        | 0.63        | 2.5        | 0.63        | 0.63        | 0.31        | 0.63        |
|     | 3           | 0.63        | 0.63        | 0.63        | 2.5        | 0.63        | 0.63        | 0.31        | 0.63        |
|     | <b>Mean</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>2.5</b> | <b>0.63</b> | <b>0.63</b> | <b>0.31</b> | <b>0.63</b> |
| C10 | 1           | 0.63        | 0.63        | 0.63        | 2.5        | 0.31        | 0.63        | 0.63        | 0.63        |
|     | 2           | 0.63        | 0.63        | 0.63        | 2.5        | 0.31        | 0.63        | 0.63        | 0.63        |
|     | 3           | 0.63        | 0.63        | 0.63        | 2.5        | 0.31        | 0.63        | 0.63        | 0.63        |
|     | <b>Mean</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>2.5</b> | <b>0.31</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> |
| C11 | 1           | 0.63        | 0.63        | 0.63        | 2.5        | 0.63        | 0.63        | 0.63        | 0.63        |
|     | 2           | 0.63        | 0.63        | 0.63        | 2.5        | 0.63        | 0.63        | 0.63        | 0.63        |
|     | 3           | 0.63        | 0.63        | 0.63        | 2.5        | 0.63        | 0.63        | 0.63        | 0.63        |
|     | <b>Mean</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>2.5</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> |
| C12 | 1           | 0.63        | 1.25        | 0.63        | 2.5        | 0.63        | 0.63        | 0.63        | 0.63        |
|     | 2           | 0.63        | 1.25        | 0.63        | 2.5        | 0.63        | 0.63        | 0.63        | 0.63        |
|     | 3           | 0.63        | 1.25        | 0.63        | 2.5        | 0.63        | 0.63        | 0.63        | 0.63        |
|     | <b>Mean</b> | <b>0.63</b> | <b>1.25</b> | <b>0.63</b> | <b>2.5</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> |
| C13 | 1           | 0.63        | 1.25        | 0.63        | 1.25       | 0.63        | 0.63        | 0.63        | 0.63        |
|     | 2           | 0.63        | 1.25        | 0.63        | 1.25       | 0.63        | 0.63        | 0.63        | 0.63        |

|                      |             |             |             |             |             |             |             |             |             |
|----------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
|                      | 3           | 0.63        | 1.25        | 0.63        | 1.25        | 0.63        | 0.63        | 0.63        | 0.63        |
|                      | <b>Mean</b> | <b>0.63</b> | <b>1.25</b> | <b>0.63</b> | <b>1.25</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> |
| C14                  | 1           | 0.63        | 1.25        | 0.63        | 2.5         | 0.63        | 0.63        | 0.63        | 0.63        |
|                      | 2           | 0.63        | 1.25        | 0.63        | 2.5         | 0.63        | 0.63        | 0.63        | 0.63        |
|                      | 3           | 0.63        | 1.25        | 0.63        | 2.5         | 0.63        | 0.63        | 0.63        | 0.63        |
|                      | <b>Mean</b> | <b>0.63</b> | <b>1.25</b> | <b>0.63</b> | <b>2.5</b>  | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> |
| C15                  | 1           | 0.63        | 1.25        | 0.63        | 2.5         | 0.63        | 0.63        | 0.63        | 0.63        |
|                      | 2           | 0.63        | 1.25        | 0.63        | 2.5         | 0.63        | 0.63        | 0.63        | 0.63        |
|                      | 3           | 0.63        | 1.25        | 0.63        | 2.5         | 0.63        | 0.63        | 0.63        | 0.63        |
|                      | <b>Mean</b> | <b>0.63</b> | <b>1.25</b> | <b>0.63</b> | <b>2.5</b>  | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> | <b>0.63</b> |
| CHX<br>(+ Control)   |             | No growth   |             | No growth   |             | No growth   |             | No growth   |             |
| Broth<br>(- Control) |             | Growth      |             | Growth      |             | Growth      |             | Growth      |             |

**Table 7. 8:** Summary of MIC/MFC results of ester derivates.

| Compound | Mean of MIC/MFC values for test <i>Candida</i> species (mg/mL) |             |                    |             |                      |             |                        |             | Mean ± SD MIC      |
|----------|----------------------------------------------------------------|-------------|--------------------|-------------|----------------------|-------------|------------------------|-------------|--------------------|
|          | <i>C. albicans</i>                                             |             | <i>C. glabrata</i> |             | <i>C. tropicalis</i> |             | <i>C. parapsilosis</i> |             | mg/mL              |
|          | MIC                                                            | MFC         | MIC                | MFC         | MIC                  | MFC         | MIC                    | MFC         |                    |
| C1       | <b>0.63</b>                                                    | <b>0.63</b> | <b>0.63</b>        | <b>1.25</b> | <b>0.31</b>          | <b>0.63</b> | <b>0.16</b>            | <b>0.31</b> | <b>0.43 ± 0.24</b> |
| C2       | 0.63                                                           | 0.63        | 0.63               | 1.25        | 0.31                 | 0.63        | 0.31                   | 0.63        | 0.47 ± 0.18        |
| C3       | 0.63                                                           | 0.52        | 0.63               | 1.25        | 0.31                 | 0.63        | 0.31                   | 0.63        | 0.47 ± 0.18        |
| C4       | <b>0.31</b>                                                    | <b>0.63</b> | <b>0.63</b>        | <b>1.25</b> | <b>0.31</b>          | <b>0.63</b> | <b>0.31</b>            | <b>0.63</b> | <b>0.39 ± 0.16</b> |
| C5       | 0.63                                                           | 0.63        | 0.63               | 2.5         | 0.63                 | 0.63        | 0.31                   | 0.63        | 0.55 ± 0.16        |
| C6       | 0.63                                                           | 0.63        | 0.63               | 2.5         | 0.63                 | 0.63        | 0.31                   | 0.63        | 0.55 ± 0.16        |
| C7       | 0.63                                                           | 1.25        | 0.63               | 2.5         | 0.63                 | 0.63        | 0.31                   | 0.63        | 0.55 ± 0.16        |
| C8       | 0.63                                                           | 1.25        | 0.63               | 2.5         | 0.63                 | 0.63        | 0.31                   | 0.63        | 0.55 ± 0.16        |

|     |      |      |      |      |      |      |      |      |                 |
|-----|------|------|------|------|------|------|------|------|-----------------|
| C9  | 0.63 | 0.63 | 0.63 | 2.5  | 0.63 | 0.63 | 0.31 | 0.63 | $0.55 \pm 0.16$ |
| C10 | 0.63 | 0.63 | 0.63 | 2.5  | 0.31 | 0.63 | 0.63 | 0.63 | $0.55 \pm 0.16$ |
| C11 | 0.63 | 0.63 | 0.63 | 2.5  | 0.63 | 0.63 | 0.63 | 0.63 | $0.63 \pm 0.00$ |
| C12 | 0.63 | 1.25 | 0.63 | 2.5  | 0.63 | 0.63 | 0.63 | 0.63 | $0.63 \pm 0.00$ |
| C13 | 0.63 | 1.25 | 0.63 | 1.25 | 0.63 | 0.63 | 0.63 | 0.63 | $0.63 \pm 0.00$ |
| C14 | 0.63 | 1.25 | 0.63 | 2.5  | 0.63 | 0.63 | 0.63 | 0.63 | $0.63 \pm 0.00$ |
| C15 | 0.63 | 1.25 | 0.63 | 2.5  | 0.63 | 0.63 | 0.63 | 0.63 | $0.63 \pm 0.00$ |

### 7.3.5. MIC/MBC of nanoparticles loaded with Ester and Amide derivatives

The nanoparticles were tested for their antimicrobial activity and their results are presented in Table 7.9 and summary in Table 7.10. The range of activity for loaded nanoparticles was similar, the MIC activity ranged from 0.0625 to 0.25 mg/mL for both C1-loaded (ester) and KAM1-loaded (amide) nanoparticles. A similar range was also observed for blank polymer nanoparticles (PNPs). The MBC also ranged from 0.0625 to 0.25 mg/mL. The most susceptible strain was found to be *S. pyogenes* with the lowest mean MIC value of 0.0625 mg/mL for blank PNPs and 0.0625 mg/mL for both C1-loaded and KAM1-loaded and the MBC also ranged from 0.0625 to 0.125 mg/mL. The resistant strain was *S. aureus* which showed 0.25 mg/mL MIC and MBC for all the tested compounds.

**Table 7. 9:** Minimum inhibitory concentrations and Minimum bactericidal concentrations of nanoparticles loaded with Amide and Ester derivatives.

| Compound    | Repeats     | MIC/MBC values for test bacteria (mg/mL) |              |                  |             |                      |              |                    |               |
|-------------|-------------|------------------------------------------|--------------|------------------|-------------|----------------------|--------------|--------------------|---------------|
|             |             | <i>E. coli</i>                           |              | <i>S. aureus</i> |             | <i>P. aeruginosa</i> |              | <i>S. pyogenes</i> |               |
|             |             | MIC                                      | MBC          | MIC              | MBC         | MIC                  | MBC          | MIC                | MBC           |
| PNP Blank   | 1           | 0.125                                    | 0.25         | 0.25             | 0.25        | 0.125                | 0.125        | 0.0625             | 0.0625        |
|             | 2           | 0.125                                    | 0.25         | 0.25             | 0.25        | 0.125                | 0.125        | 0.0625             | 0.0625        |
|             | 3           | 0.125                                    | 0.25         | 0.25             | 0.25        | 0.125                | 0.125        | 0.0625             | 0.0625        |
|             | <b>Mean</b> | <b>0.125</b>                             | <b>0.25</b>  | <b>0.25</b>      | <b>0.25</b> | <b>0.125</b>         | <b>0.125</b> | <b>0.0625</b>      | <b>0.0625</b> |
| C1-Loaded   | 1           | 0.125                                    | 0.125        | 0.25             | 0.25        | 0.125                | 0.25         | 0.0625             | 0.125         |
|             | 2           | 0.125                                    | 0.125        | 0.25             | 0.25        | 0.125                | 0.25         | 0.0625             | 0.125         |
|             | 3           | 0.125                                    | 0.125        | 0.25             | 0.25        | 0.25                 | 0.125        | 0.0625             | 0.125         |
|             | <b>Mean</b> | <b>0.125</b>                             | <b>0.125</b> | <b>0.25</b>      | <b>0.25</b> | <b>0.167</b>         | <b>0.208</b> | <b>0.0625</b>      | <b>0.125</b>  |
| KAM1-Loaded | 1           | 0.125                                    | 0.125        | 0.25             | 0.25        | 0.25                 | 0.125        | 0.0625             | 0.0625        |
|             | 2           | 0.125                                    | 0.125        | 0.25             | 0.25        | 0.125                | 0.125        | 0.0625             | 0.0625        |
|             | 3           | 0.125                                    | 0.125        | 0.25             | 0.25        | 0.125                | 0.125        | 0.0625             | 0.0625        |
|             | <b>Mean</b> | <b>0.125</b>                             | <b>0.125</b> | <b>0.25</b>      | <b>0.25</b> | <b>0.167</b>         | <b>0.125</b> | <b>0.0625</b>      | <b>0.125</b>  |

**Table 7. 10:** Summary of MIC/MBC results of nanoparticles loaded with Amide and Ester derivates.

| Compound    | Mean $\pm$ SD of MIC/MBC values for test bacteria (mg/mL) n=3 |       |                  |      |                      |       |                    |        | Mean $\pm$ SD MIC |
|-------------|---------------------------------------------------------------|-------|------------------|------|----------------------|-------|--------------------|--------|-------------------|
|             | <i>E. coli</i>                                                |       | <i>S. aureus</i> |      | <i>P. aeruginosa</i> |       | <i>S. pyogenes</i> |        | mg/mL             |
|             | MIC                                                           | MBC   | MIC              | MBC  | MIC                  | MBC   | MIC                | MBC    |                   |
| PNP Blank   | 0.125                                                         | 0.25  | 0.25             | 0.25 | 0.125                | 0.125 | 0.0625             | 0.0625 | 0.14 $\pm$ 0.079  |
| C1-Loaded   | 0.125                                                         | 0.125 | 0.25             | 0.25 | 0.167                | 0.208 | 0.0625             | 0.125  | 0.15 $\pm$ 0.079  |
| KAM1-Loaded | 0.125                                                         | 0.125 | 0.25             | 0.25 | 0.167                | 0.125 | 0.0625             | 0.125  | 0.15 $\pm$ 0.079  |

### 7.3.6. MIC/MFC of nanoparticles loaded with Ester and Amide derivatives

The antifungal activity of the prepared nanoparticles was also investigated and the results are shown in Table 7.11 while the summary is depicted in Table 7.12. The MIC of the tested compounds ranged from 0.0625 to 0.25 mg/mL and the MFC ranged from 0.125 to 0.5 mg/mL. The most sensitive fungal strain was found to be *C.tropicalis* with a mean MIC of 0.0625 mg/mL for all the compounds. The resistant strain was found to be *C. parapsilosis*. Compound KAM1-loaded nanoparticles showed the best MFC mean value of 0.25 mg/mL in comparison to PNP Blank and C1-loaded which both showed a mean MFC value of 0.5 mg/mL. All the compounds showed a broad spectrum of antifungal activity with all three of them showing a mean MIC value of  $0.11 \pm 0.031$  mg/mL against all the tested fungal species.



**Table 7. 11:** Minimum inhibitory concentrations and Minimum fungicidal concentrations of nanoparticles loaded with Amide and Ester derivatives.

| Compound    | Repeats     | MIC/MFC values for test <i>Candida species</i> (mg/mL) |              |                    |              |                      |              |                        |             |
|-------------|-------------|--------------------------------------------------------|--------------|--------------------|--------------|----------------------|--------------|------------------------|-------------|
|             |             | <i>C. albicans</i>                                     |              | <i>C. glabrata</i> |              | <i>C. tropicalis</i> |              | <i>C. parapsilosis</i> |             |
|             |             | MIC                                                    | MFC          | MIC                | MFC          | MIC                  | MFC          | MIC                    | MFC         |
| PNP Blank   | 1           | 0.125                                                  | 0.125        | 0.125              | 0.25         | 0.0625               | 0.125        | 0.125                  | 0.5         |
|             | 2           | 0.125                                                  | 0.125        | 0.125              | 0.125        | 0.0625               | 0.125        | 0.125                  | 0.5         |
|             | 3           | 0.125                                                  | 0.125        | 0.125              | 0.25         | 0.0625               | 0.125        | 0.125                  | 0.5         |
|             | <b>Mean</b> | <b>0.125</b>                                           | <b>0.125</b> | <b>0.125</b>       | <b>0.208</b> | <b>0.0625</b>        | <b>0.125</b> | <b>0.125</b>           | <b>0.5</b>  |
| C1-Loaded   | 1           | 0.125                                                  | 0.125        | 0.125              | 0.25         | 0.0625               | 0.25         | 0.125                  | 0.5         |
|             | 2           | 0.125                                                  | 0.125        | 0.125              | 0.25         | 0.0625               | 0.125        | 0.125                  | 0.5         |
|             | 3           | 0.125                                                  | 0.125        | 0.125              | 0.25         | 0.0625               | 0.125        | 0.125                  | 0.5         |
|             | <b>Mean</b> | <b>0.125</b>                                           | <b>0.125</b> | <b>0.125</b>       | <b>0.25</b>  | <b>0.0625</b>        | <b>0.167</b> | <b>0.125</b>           | <b>0.5</b>  |
| KAM1-Loaded | 1           | 0.125                                                  | 0.125        | 0.125              | 0.125        | 0.0625               | 0.125        | 0.125                  | 0.125       |
|             | 2           | 0.125                                                  | 0.125        | 0.125              | 0.25         | 0.0625               | 0.125        | 0.125                  | 0.125       |
|             | 3           | 0.125                                                  | 0.125        | 0.125              | 0.125        | 0.0625               | 0.125        | 0.125                  | 0.5         |
|             | <b>Mean</b> | <b>0.125</b>                                           | <b>0.125</b> | <b>0.125</b>       | <b>0.167</b> | <b>0.0625</b>        | <b>0.125</b> | <b>0.125</b>           | <b>0.25</b> |

**Table 7. 12:** Summary of MIC/MFC results of nanoparticles loaded with Amide and Ester derivatives.

| Compound    | Mean $\pm$ SD MIC/MFC values for test <i>Candida</i> (mg/mL) n=3 |       |                    |       |                      |       |                        |      | Mean $\pm$ SD MIC |
|-------------|------------------------------------------------------------------|-------|--------------------|-------|----------------------|-------|------------------------|------|-------------------|
|             | <i>C. albicans</i>                                               |       | <i>C. glabrata</i> |       | <i>C. tropicalis</i> |       | <i>C. parapsilosis</i> |      | mg/mL             |
|             | MIC                                                              | MFC   | MIC                | MFC   | MIC                  | MFC   | MIC                    | MFC  |                   |
| PNP Blank   | 0.125                                                            | 0.125 | 0.125              | 0.208 | 0.0625               | 0.125 | 0.125                  | 0.5  | 0.11 $\pm$ 0.031  |
| C1-Loaded   | 0.125                                                            | 0.125 | 0.125              | 0.25  | 0.0625               | 0.167 | 0.125                  | 0.5  | 0.11 $\pm$ 0.031  |
| KAM1-Loaded | 0.125                                                            | 0.125 | 0.125              | 0.167 | 0.0625               | 0.125 | 0.125                  | 0.25 | 0.11 $\pm$ 0.031  |

### 7.3.7. MIC/MBC/MFC of Combination of Amide and Ester derivatives

$$CI = \frac{D_1}{(D_x)_1} + \frac{D_2}{(D_x)_2}$$

Where CI is the combination index,  $D_1$  and  $D_2$  are doses of drug 1 and drug 2.  $(D_x)_1$  and  $(D_x)_2$  are doses of drug 1 and drug 2 required to achieve a certain effect when used alone, respectively.

**CI < 1:** Synergistic effect (the combined effect of the drugs is greater than the sum of their individual effects)

**CI = 1:** Additive effect (the combined effect of the drugs is equal to the sum of their individual effects)

**CI > 1:** Antagonistic effect (the combined effect of the drugs is less than the sum of their individual effects)

The combination of amide (KAM1) and ester (C1) in a 1:1 ratio was also explored against the previously tested bacteria and the results are shown in Table 7.13 below. The combination of these compounds showed an antagonistic effect for bacterial strains such as *E. coli*, *P. aeruginosa* and *S. pyogenes* since they gave value greater than 1. However, for *S. aureus* the compounds worked synergistically showing CI value of 0.295.

**Table 7. 13:** Minimum inhibitory concentrations and Minimum bactericidal concentrations of the combination of Amide and Ester derivates.

| Compound  | Repeats          | MIC/MBC values for test bacteria (mg/mL), n=3 |                    |                    |                    |                      |                    |                    |                    |
|-----------|------------------|-----------------------------------------------|--------------------|--------------------|--------------------|----------------------|--------------------|--------------------|--------------------|
|           |                  | <i>E. coli</i>                                |                    | <i>S. aureus</i>   |                    | <i>P. aeruginosa</i> |                    | <i>S. pyogenes</i> |                    |
|           |                  | MIC                                           | MBC                | MIC                | MBC                | MIC                  | MBC                | MIC                | MBC                |
| KAM1+C1   | 1                | 0.63                                          | 0.63               | 1.25               | 1.25               | 0.63                 | 0.63               | 0.02               | 0.02               |
|           | 2                | 0.63                                          | 0.63               | 1.25               | 1.25               | 0.63                 | 0.63               | 0.02               | 0.02               |
|           | 3                | 0.63                                          | 0.63               | 1.25               | 1.25               | 0.63                 | 0.63               | 0.02               | 0.02               |
|           | <b>Mean ± SD</b> | <b>0.63 ± 0.00</b>                            | <b>0.63 ± 0.00</b> | <b>1.25 ± 0.00</b> | <b>1.25 ± 0.00</b> | <b>0.63 ± 0.00</b>   | <b>0.63 ± 0.00</b> | <b>0.02 ± 0.00</b> | <b>0.02 ± 0.00</b> |
| <b>CI</b> |                  | 1.60                                          |                    | 0.296              |                    | 1.49                 |                    | 9                  |                    |

MIC and MFC results of the combination of KAM1 and C1 are presented in Table 7.14. The mean MIC ranged from 0.31 to 0.63 mg/mL. The results were not different from the individual compounds except for the results of *C.parapsilosis* which previously shown sensitivity towards compound C1. The mean MIC of the combination was found to be  $0.31 \pm 0.00$  mg/mL and the MFC was found to be  $0.63 \pm 0.00$  mg/mL which was still similar for compound KAM1 and higher for compound C1.

**Table 7. 14:** Minimum inhibitory concentrations and Minimum fungicidal concentrations of the combination of Amide and Ester derivatives.

| Compound | Repeats   | MIC/MFC values for test <i>Candida species</i> (mg/mL) n=3 |             |                    |             |                      |             |                        |             |
|----------|-----------|------------------------------------------------------------|-------------|--------------------|-------------|----------------------|-------------|------------------------|-------------|
|          |           | <i>C. albicans</i>                                         |             | <i>C. glabrata</i> |             | <i>C. tropicalis</i> |             | <i>C. parapsilosis</i> |             |
|          |           | MIC                                                        | MFC         | MIC                | MFC         | MIC                  | MFC         | MIC                    | MFC         |
| KAM1+C1  | 1         | 0.63                                                       | 0.63        | 0.31               | 1.25        | 0.31                 | 0.31        | 0.31                   | 0.63        |
| 1:1      | 2         | 0.63                                                       | 0.63        | 0.31               | 1.25        | 0.31                 | 0.31        | 0.31                   | 0.63        |
|          | 3         | 0.63                                                       | 0.63        | 0.31               | 0.63        | 0.31                 | 0.63        | 0.31                   | 0.63        |
|          | Mean ± SD | 0.63 ± 0.00                                                | 0.63 ± 0.00 | 0.31 ± 0.00        | 1.04 ± 0.36 | 0.31 ± 0.00          | 0.42 ± 0.18 | 0.31 ± 0.00            | 0.63 ± 0.00 |
| CI       |           | 2                                                          |             | 4.06               |             | 1.52                 |             | 2.54                   |             |

## 7.4. Discussion

### 7.4.1. Antibacterial activity of Amides and Ester derivatives

The synthesized amides (KAM 1-KAM 12) and esters (C1-C15) were tested for their antibacterial activity against four bacterial strains: two Gram-positive namely *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC 8665), and two Gram-negative, *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853). Chlorhexidine Gluconate, a known antiseptic and disinfectant was used as a positive control. Chlorhexidine was used in the study because it is the main disinfectant used in hospital settings. The samples were prepared by dissolving them in dimethyl sulfoxide (DMSO) before using them in the bioassay.

The antibacterial activity of the designed and synthesized compounds was determined using a micro-dilution assay. The activity of the synthesized compounds is mostly influenced by the moieties that are present in the structure and the type of bond that constitutes the overall structure. Herein, is a report on the amide and ester derivatives of hydroxybenzoic acids. The approach of designing these compounds was a semi-synthetic approach since most of the backbone or core structures are constituted of phenolic acids and, hydroxybenzoic acid which is widely distributed in the plant kingdom as secondary metabolite as previously explained in the literature review in Chapter 2. There are numerous plant species on Earth (400,000–500,000 species). Each plant is a mini-factory producing chemicals such as secondary metabolites, small molecules such as phenolics and polyphenols, terpenoids, essential oils, alkaloids, and others<sup>348</sup>. Many of these compounds exhibit antimicrobial activity, but they are not given the attention they deserve by medical practitioners because almost all antibiotics and antivirals available on the market are either produced by bacteria and fungi or synthesized chemically. These drugs are derived from natural sources such as microorganisms, plants, or marine organisms, or they are chemically synthesized in laboratories<sup>348</sup>. Approximately 75% of antibacterial compounds introduced into clinical practice in the last 40 years are derived from natural products since natural products are scaffolds with potential in drug discovery<sup>349</sup>. Hence, our attention on the design of the amide and ester compounds was by modifying a naturally occurring product to also help limit the adverse effect that might occur when the compound is hundred percent synthetic without any natural traces.

The antibacterial activity of amide compounds was dominated by the overall activity (broad bacterial spectrum) of compounds KAM 1 and KAM 4 with a mean MIC of  $0.40 \pm 0.22$  and  $0.42 \pm 0.57$  mg/mL respectively, whilst for ester compounds C1 and C9 showed the overall best activity with the mean MIC of  $0.28 \pm 0.26$  and  $0.38 \pm 0.30$  mg/mL respectively. Compound KAM 1 showed high activity against *S. pyogenes* with a mean MIC of 0.16 mg/mL and MBC of 1.25 mg/mL and good activity against *S. aureus* with a mean MIC of 0.21 mg/mL and MBC of 0.31 mg/mL. Compound KAM 11 on the other hand, showed the highest activity against *E. coli* with a mean MIC of 0.04 mg/mL and MBC of 0.63 mg/mL and the highest activity against *P. aeruginosa* with a mean MIC of 0.08 mg/mL and MBC of 0.63 mg/mL. The amide compound KAM 11 was the only compound which showed considerable high value of MIC against both Gram-negative bacterial species (*E. coli* and *P. aeruginosa*). The ester compounds C1 and C9 were more active against *S. aureus* with MIC value of 0.16 mg/mL in comparison to amide compounds. Interestingly, both ester compounds that showed high activity were very active against Gram-positive bacterial strains *S. aureus* and *S. pyogenes*, with compound C1 showing the highest activity against the latter pathogen with a MIC of 0.02 mg/mL and MBC of 0.04 mg/mL. Gram-negative bacteria showed resistance in comparison to the Gram-positive strains against most of the compounds.

Gram-negative bacteria have a thin peptidoglycan cell wall that is surrounded by an outer lipopolysaccharide-containing membrane<sup>350</sup>. Gram-positive bacteria lack an outer membrane but are surrounded by peptidoglycan layers that are many times thicker than those found in Gram-negative bacteria<sup>350</sup>. However, due to the lack of an outer membrane, Gram-positive bacteria are more susceptible to most antibiotics and synthesized compounds. This outer membrane makes Gram-negative bacteria resistant to various antibiotics and certain drugs<sup>350,351</sup>. Hence, Gram-positive bacteria were more susceptible to both the amide and ester compounds.

Backbone structures contribute significantly to the activity of these four compounds. Compound KAM1 (N,N'-(1,3-phenylene)bis(3-hydroxybenzamide)) was the conjugation of 3-hydroxybenzoic acid and 1,3-diaminobenzene while compound C1 (3-acetylphenyl 3-hydroxybenzoate) was formed from the conjugation of 3-hydroxybenzoic acid and 3-hydroxyacetophenone. Therefore, 3-hydroxybenzoic acid proved to be very effective as a backbone structural motif for Gram-positive bacteria either as an amide or an ester derivative. Satupte and co-workers studied a series of ester derivatives of 3-hydroxybenzoic acid and their findings showed activity against both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*)



bacteria<sup>352</sup>. A similar, effect from this study was observed by Plech and co-researchers<sup>353</sup> where they did a series of thiosemicarbazides derived from hydroxybenzoic acid. Their results, showed that these compounds were more active towards Gram-positive than Gram-negative strains and the 3-hydroxybenzoic acid derivative also showed more activity against Gram-positive bacteria.

Antimicrobial properties of phenolic acid alkyl esters and 3-hydroxybenzoic acid alkyl ester were found to have good antibacterial activity against *S.aureus* in a study done by Feldeková and fellow researchers<sup>354</sup>. Ohlan and co-workers<sup>101</sup> also synthesized and evaluated the antimicrobial activity of a series of urea inclusion complexes. Their results showed that the 3-hydroxybenzoic acid complex showed superior activity against *S. aureus* in comparison to other complexes<sup>101</sup>. Furthermore, in this study, compound C9 had a backbone structure of 3,5-dihydroxybenzoic acid and also showed activity against Gram-positive bacteria.

Compound KAM 11 (*N,N'*-(4-hydroxy-1,3-phenylene)bis(3,4,5-trihydroxy benzamide)) which showed the highest activity against Gram-negative bacteria is the result of conjugation of gallic acid and 2,4-diaminophenol. The observed antimicrobial activity could be attributed to more hydroxyl moieties found in both the gallic acid and 2,4-diaminophenol. Friedman et al. (2003) reported antibacterial activity of phenolic benzaldehydes and benzoic Acids against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. In their study, the number of hydroxyl moiety on the periphery of the structure was taken to consideration and the findings showed that trihydroxybenzoic acids, more specifically gallic acid derivatives had more activity compared to mono- and di- substituted compounds against a broad bacterial spectrum<sup>355</sup>. Contrary to their, obtained results relating to the activity of gallic acid derivative against *P. aeruginosa*, Boaventura and co-workers in their study observed a weak antibacterial activity against *P. aeruginosa* and better activity against *E. coli*<sup>356</sup>.

The findings of compound KAM 11 are particularly intriguing because Gram-negative bacteria are becoming increasingly resistant to known antibiotics, necessitating the development of new drugs<sup>356</sup>. *E. coli* in particular, is a concern not only because it causes potentially fatal diseases such as pneumonia and meningitis, but also because it causes opportunistic bloodstream infections associated with wounds and surgical infections<sup>356</sup>.

The aforementioned compounds gave satisfactory antibacterial results. However, fungal species also pose serious threat as opportunistic infections to immunocompromised patients in hospital settings. Therefore, finding compounds that could have broad antimicrobial spectrum

could go a long way in addressing the challenges encountered in the co-administration of drugs involving the resistance of microbial species to the administered drugs.

#### 7.4.2. Antifungal activity of Amides and Ester derivatives

*Candida* yeasts cause a variety of infectious diseases, including *candidiasis*<sup>357</sup> and *candidemia*<sup>358</sup>. Because of their adaptability to different host niches, these yeasts are commensal in healthy humans but may cause systemic infection in immunocompromised individuals<sup>357</sup>. The compounds in this study mostly showed different levels of activity against the studied *Candida* species.

The number of hydroxyl group on the backbone structure and the mono-, di-, and tripodal (e.g. phloroglucinol) substitutions and the type of bonding (ester or amide) had a huge effect on the activity of these synthesized compounds. Ester compounds showed moderately good activity in comparison to most of the amide compounds with compounds C1 and C4 showing good activity in a broad fungal species. Compound C4 showed the highest antifungal activity with the mean MIC value of  $0.39 \pm 0.16$  mg/mL. While compound C1 showed good activity with the mean MIC value  $0.43 \pm 0.24$  mg/mL. Compound C4 is a tripodal substituted compound which is a conjugation of 3-hydroxybenzoic acid with phloroglucinol. On the other hand, compound C1 is a mono-substituted compound of 3-hydroxybenzoic acid conjugated to 3-hydroxyacetophenone. The commonality of these two ester compounds is the 3-hydroxybenzoic acid backbone, further showing good activity across the broad microbial spectrum.

Compound C1 showed the highest activity against *C. parapsilosis* with an MIC value of 0.16 mg/mL and MBC of 0.31 mg/mL. C1 also gave good activity against *C. tropicalis* with an MIC value of 0.31 mg/mL and MBC value of 0.63 mg/mL. Moderate activity of C1 was observed against the other two *Candida* species. While compound C4 showed good activity against *C. albicans*, *C. parapsilosis* and *C. tropicalis* with an MIC value of 0.31 mg/mL and MBC of 0.63 mg/mL for all the three strains. Ester compounds showed moderate activity against *C. albicans* except for compound C4 which showed good activity. *C. glabrata* on the other hand, showed moderate sensitivity to all the ester compounds. Krátký and coworkers reported antimicrobial activity of sulfonamide derivatives from the 5-chloro-2-hydroxybenzoic acid scaffold. The compounds showed high activities against many antimicrobial strains but showed moderate activity against *C. glabrata*. *C. glabrata*, resistance is most commonly mediated by overexpression of the drug-efflux transporter genes CgCDR1, CgCDR2, and CgSNQ2 via a

gain-of-function (GOF) mutation in the transcription factor pleiotropic drug-resistance (PDR)<sup>359–362</sup>. Perhaps similar resistance mechanism is occurring with amide and esters studied in work.

The amide compounds on the other hand, showed moderate activity in most microbes with KAM 5 being the exception since it outperformed other compounds with a mean MIC value of  $0.47 \pm 0.18$  mg/mL across the fungal species studied. KAM 5 gave good results against *C. albicans* and *C. tropicalis* with an MIC value of 0.31 mg/mL and an MBC value of 0.63 mg/mL for both strains. Compound KAM 5 is a product of gallic acid and *m*-aminophenol. Compounds KAM1-KAM 8 gave good results against *C. tropicalis* with an MIC value of 0.31 mg/mL. Like esters, all the amide compounds showed moderate activity against *C. glabrata*.

Previous studies have also shown the antimicrobial activity of the compounds synthesized from gallic acid, and this further corroborated the results obtained in this work. Compound KAM 5 was synthesized from gallic acid derived compound showed activity in most of the bacterial and fungal strains studied. Lima and co-workers<sup>363</sup> also showed antifungal activity of un-modifications gallic acid. According to Rivero-Buceta et al.<sup>364</sup> gallic acid substitution either as a mono-, di-, or tri-amide substitution had major effect on the anti-hepatitis C virus (HCV) activity, further showing a versatile activity of gallic acid.

The results above have shown the potential activity of the designed and synthesized compounds against both the Gram-negative and Gram-positive bacteria as well as the selected fungal strains. However, the need arose for compounds that showed good overall activity to be screened for their synergistic effect on all the studied microorganisms. Combination therapy has since been known to increase potency of antibiotics, hence, in this study one ester and one amide compound that showed overall good activity in the microbial assays studied were mixed in a ratio of 1:1 to check the potential effect that might arise from combining two compounds. The need for combination therapy was prompted by the moderate activity observed for compound C1 and KAM 1 in other strains while they showed good activities in others.

#### **7.4.3. Combination of amide and ester compounds**

Combination therapy involves the combination of two or more drugs in order to restore or increase the efficacy of both drugs against a bacterial pathogen that is resistant to standard antibiotics. With the development of new antibacterial drugs slowing for a variety of reasons, scientists are looking into combination therapy for use against bacteria or fungi.

The results from the combination of KAM 1 and C1 in a 1:1 ratio has shown minimal to no effect on the overall antimicrobial activity, suggesting antagonistic overall effect on the studied microorganisms. However, synergistic effect was observed on *S.auresus*. However, a minimal effect was observed on *C. glabrata* which showed resistance to separate individual compounds.

Therefore, the combination of compounds overall was not effective; the exception was only observed on a few strains, with slightly better effect. This then called for a new approach to improve the antimicrobial activity of the compounds which showed potential. Compound C1 and KAM 1 were loaded onto the polymeric nanoparticles, which have gained interest as drug vehicles.

#### **7.4.4. Antimicrobial activity of nanoparticle-loaded Amides and Ester derivatives**

Nanotechnology has revolutionized the medical and pharmaceutical field since its inception. The drugs that were deemed inactive due to degradation and instability in the blood circulation have been revived and repurposed through this technological advancement. Nanotechnology has the potential to significantly improve the treatment and prevention of several diseases including microbial infections <sup>365</sup>. In this study, polymer nanoparticles were used as drug delivery system for the synthesized compounds that showed great potential in the broad antimicrobial spectrum. Compound C1 from esters and KAM 1 from amides were selected and loaded onto the novel polymeric nanoparticles.

The derivate loaded nanoparticles performed way better than the independent un-loaded compounds. However, the blank nanoparticles also showed good activity that is comparable to the loaded nanoparticles thus signifying that the designed novel nanoparticles were active due to the trans-cinnamic acid moiety used in the design of these nanoparticles. Trans-cinnamic acid is a phenolic acid, like gallic acid that is found mainly in fruits and vegetables as a secondary metabolite. Ruwizhi and Aderibigbe, showed both antimicrobial and anticancer activity of trans-cinnamic acid derivatives <sup>366</sup>. In the study reported by Sun and co-workers functionalizing Ag nanoparticles with cinnamic acid yielded good antimicrobial activity <sup>367</sup>.

The antibacterial results of the studied blank and loaded nanoparticles showed similar results in all the strains except for *P. aeruginosa* when treated by blank nanoparticles. The results showed better activity when *P. aeruginosa* was treated with only the nanoparticles without compounds. These results are particularly interesting since *P. aeruginosa* as previously stated is known to be resistant to most antibacterial agents. Furthermore, *S. aureus* was shown to

have minimal resistance against the loaded and un-loaded nanoparticles showing MIC value 0.25 mg/ml for all the studied nanoparticles. The most sensitive strain amongst the bacterial strains was *S. pyeogenes* which showed an MIC value of 0.0625 mg/mL for all the studied nanoparticles. The nanoparticles both loaded and un-loaded showed good antibacterial activity against Gram-negative and Gram-positive. The overall or mean MIC value was found to be  $0.14 \pm 0.079$ ,  $0.15 \pm 0.079$ , and  $0.15 \pm 0.079$  mg/mL for blank nanoparticles, C1-loaded, and KAM 1-loaded respectively, these values were lower compared to un-loaded compounds signifying enhanced activity compared to un-loaded compounds. The un-loaded compounds showed overall MIC of  $0.40 \pm 0.22$  and  $0.28 \pm 0.26$  mg/mL for KAM 1 and C1 respectively. The same observation was reported by Sobhani and co-workers, when they loaded ciprofloxacin to chitosan and observed a 50% increase in activity against both Gram-positive and Gram-negative bacteria <sup>368</sup>. Therefore, the results obtained in the aforementioned study further corroborate the findings obtained in this study, indicating the importance of loading compounds onto nanoparticles to enhance the overall activity.

The results obtained for antibacterial activity were also further observed for the antifungal activity. The loading of compounds also enhanced the antifungal activity of compound C1 and KAM 1. All the studied nanoparticles loaded, and blank showed similar good activity in all the fungal strains with an MIC value of 0.125 mg/mL for *C. albicans*, *C. glabrata*, and *C. parapsilosis*. These strains more especially *C. albicans* and *C. glabrata* showed resistance to un-loaded compounds further reiterating the importance of the nanomaterial used in this study. *C. tropicalis* was the most sensitive strain towards the studied nanomaterial with an MIC value of 0.0625 mg/mL.

Numerous studies have been conducted to improve or enhance the efficacy of antibacterial agents loaded onto polymeric nanoparticles. These findings are related to a variety of factors, including improved drug penetration into bacterial cells, improved drug delivery to the site of action, and increased stability of the encapsulated drug in nanoparticles <sup>369–371</sup>. The antibacterial activity and antifungal activity of the loaded nanoparticles was in the excess of 5-fold high in comparison to the un-loaded compounds further justifying the use of nanoparticles in combating microbial infection even for the most resistant Gram-negative bacteria associated infections.

## 7.5 Conclusions

Structure-activity relationship studies of phenolic acids show that some parameters, such as the basic chemical structure, the position and number of hydroxyl groups on the phenolic ring, as well as their substituents, and the esterification or amidation of the carboxyl group, can affect antimicrobial activity. Ester compounds in this study, showed better antibacterial activity against Gram-positive bacteria compared to Gram-negative bacteria. Compound C1 and C9 were the most effective compounds for Gram-positive bacteria on esters. Moreover, compound KAM 1 on amides showed good activity against Gram-positive bacteria. While compound KAM 11 showed very good activity against both Gram-negative bacterial strains. The combination therapy approach overall proved to be ineffective against the studied strains. However, the polymeric nanoparticles on the other hand enhanced the activity of the selected compounds by several fold in comparison to un-loaded compounds.

The antimicrobial activity and potential of these designed and synthesized compounds necessitated further investigation of the cytotoxicity and anticancer capabilities of these compounds.

## Chapter 8: Cell Viability and Proliferation

---

### 8.1 Introduction

The reason for the selection of the pathogens discussed in Chapter 7 emanates from the fact that cancer patients are prone to be immunocompromised, and therefore become susceptible to secondary or opportunistic infections. Despite notable developments in medical science, cancer continues to be an incurable disease and a leading cause of death worldwide <sup>372</sup>. In this study, pancreatic cancer (PC) was selected as the cell line of choice because of its poor prognosis, high mortality rate, and 5% survival rate once diagnosed <sup>373</sup>. PC has been reported to be among the most aggressive and lethal malignancies <sup>374</sup>. According to the most recent estimates, it is Europe's fourth leading cause of cancer-related fatalities and ranks seventh worldwide <sup>375,376</sup>. One of the most aggressive malignancies of the digestive system is pancreatic ductal adenocarcinoma (PDAC), which accounts for 90% of PCs <sup>373</sup>. The pancreatic ductal tree gives rise to an infiltrating neoplasm known as pancreatic ductal adenocarcinoma (PDAC), which has glandular differentiation <sup>373</sup>. Despite significant research efforts and therapeutic advances, the median survival rate for patients with PC is approximately six months after diagnosis, and the 5-year survival rate for patients with PC is still 2-9% <sup>376</sup>.

The best chemotherapy drugs are those that specifically restrict the proliferation of only aberrant cells while having minimal or no impact on normal cells <sup>372</sup>. However, the available anticancer drugs frequently affect cells with rapid metabolism and proliferation and are unable to distinguish between cancer and normal cells. Therefore, one of the most active areas in medicinal chemistry and drug design is the search for novel, potent, selective, and less toxic anticancer agents <sup>372</sup>.

Cell viability assays are typically used to evaluate the impact of a particular drug on a specific cell population or cell line. There are several dyes that have been previously and currently used to test the viability of the cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye is one of the most used dyes, and it relies on the enzyme succinic dehydrogenase in viable cells to convert tetrazolium salt into purple formazan crystals. Because the formazan product is impermeable to the cell membrane, it accumulates in healthy cells, and the number of viable cells can be determined by absorbance readings with an ELISA reader

following the solubilization of the formed formazan crystals. In this study, the compounds were serially diluted, and the best compound concentration was the one that caused the least damage to normal cells.

## **8.2. Methods and materials**

### **8.2.1 Cell Culture:**

Human embryonic kidney (HEK 293) and Mia-PaCa-2 cells were obtained from Mintek. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (Pen/Strep) in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>.

### **8.2.2 Test Compounds:**

The test compounds used in this study included hydroxybenzoic acid derivatives and positive controls (methotrexate, camptothecin and auranofin). These compounds were dissolved in 10% DMSO and 90% growth media to prepare stock solutions of 2.5 mg/mL and stored at 4 °C.

### **8.2.3 Cytotoxicity Assay**

HEK-293 (Human Embryonic Kidney) and MiaPaCa-2 cells were automatically counted in a Bio-rad TC20<sup>TM</sup> cell counter before being plated in 96-well plates containing the synthesized compounds at a density of 10,000 cells/well in a final volume of 100 µL. The cells were seeded in 96-well tissue culture plates and placed in an incubator for 24 h to attach, and over 80% confluence was achieved. Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (Pen/Strep) was used as the growth medium. The amide and ester compounds were dissolved in 10% DMSO and 90% growth medium to obtain a 2.5 mg/mL stock solution. The selected loaded nanoparticles were also dissolved similarly to the above-mentioned compounds to make a 0.5 mg/mL stock solution. The cells were exposed to serially diluted compounds/loaded and blank nanoparticles at concentrations of 0.00977 – 1.25 mg/mL (9.77 µM – 1250 µM) for unloaded compounds and 0.002 – 0.25 mg/mL (2 µM – 250 µM) for nanoparticles and loaded nanoparticles, which were then incubated for 72 h in 5% CO<sub>2</sub>, 95% humidity at 37 °C. After incubation, the cells were stained with 20 µL MTT dye and incubated in 5% CO<sub>2</sub> for 4 h at 37°C. After the 4-hour incubation, the medium was removed and replaced with DMSO to dissolve the formazan



crystals. A microtitre plate reader at 550 nm was used to measure the absorbance using DMEM and untreated cells as the background control.

Cell viability and inhibition can then be calculated by the following formula:

$$\%Cell\ viability = \frac{Aver.\ abs.\ treated\ cells}{Aver.\ abs.\ untreated} \times 100\%$$

where Aver. is the average and abs. is the absorbance of the synthesized compound.

The selectivity index (SI) is the most important variable when determining an overall active and non-toxic drug. The selectivity index is a ratio that quantifies the relationship between cytotoxicity and anticancer activity. The formula for calculating SI is given below:

$$SI = \frac{CC_{50}}{IC_{50}}$$

where CC<sub>50</sub> is the half-maximal cytotoxic concentration and IC<sub>50</sub> is the half-maximal inhibitory concentration of the compound.

## 8.3 Results

### 8.3.1 Cytotoxicity and anticancer activity of the amide compounds

#### 8.3.1.1 Cytotoxicity of amides against HEK-293 cells

The designed and synthesized amide compounds were screened for their *in vitro* cytotoxicity in normal human cell lines. In this case, the normal cell line used was HEK-293 (Human Embryonic Kidney) cells. The results in Fig. 8.1 show that the compounds had dose-dependent responses because higher concentrations had a toxic effect on normal cells. Fig. 8.1(a) shows that in the lowest concentration (0.00977 mg/ml or 9.77 µM) the compounds had a percentage cell viability range of 81% – 96%, while auranofin had a 76% viability at this concentration. The percentage viability of the compound's highest concentrations was in the range of 24% - 37%, with auranofin having a percentage viability of 8% at the highest concentration. Figure 8.1(b) shows the CC<sub>50</sub> (The effective concentration of a cytotoxic compound that causes 50% of the compound's maximum possible cell death) of the amide compounds. From the graph, one could deduce that compound KAM 1 (CC<sub>50</sub> of 0.870 mg/mL or 870 µM) was the less toxic compound, followed by compound KAM 3 (CC<sub>50</sub> of 0.471 mg/mL or 471 µM), and KAM 6 (CC<sub>50</sub> of 0.447 mg/mL or 447 µM) compared to other compounds and auranofin.

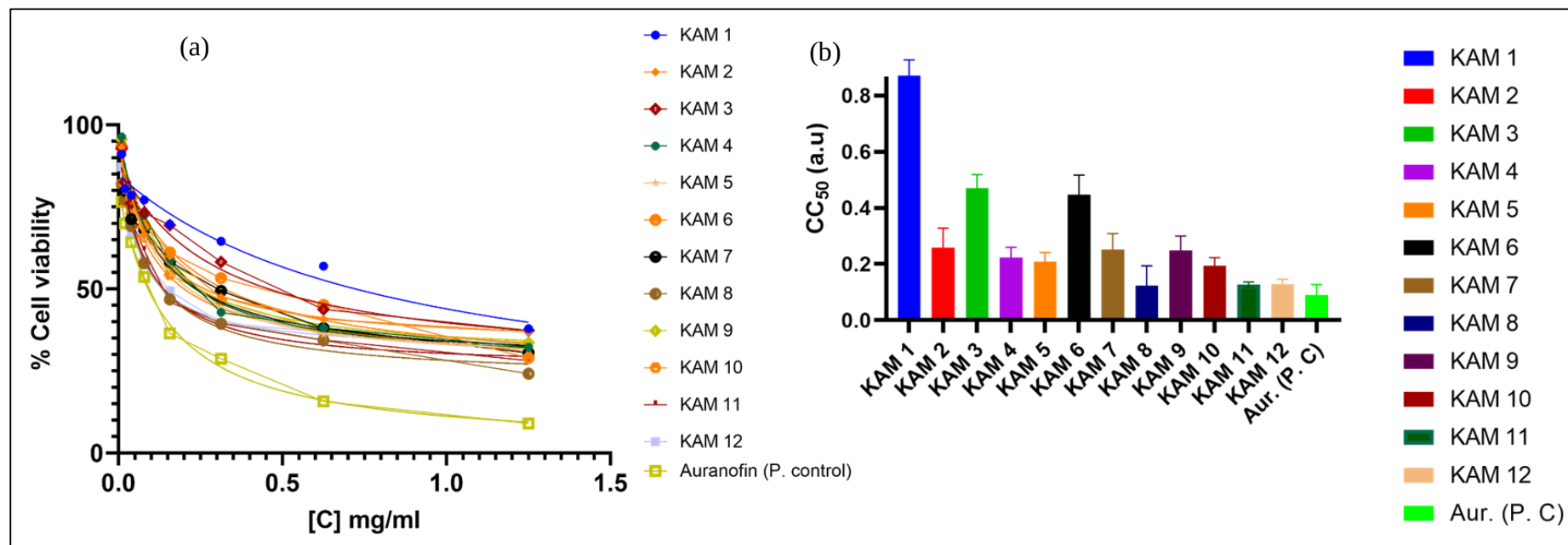


**Figure 8. 1:** Effect of amide compounds (KAM1 to KAM 12) on HEK 293 cells (a) % cell viability vs. concentration curve and (b) cell cytotoxicity at half-maximum (CC<sub>50</sub>) of amide compounds against HEK-293 cells.

#### **8.3.1.2 Anticancer activity of amides against MiaPaCa-2 cells**

The anticancer activity of the synthesized amides was also determined. The pancreatic cancer cell line (MiaPaCa-2) was exposed to the synthesized compounds. Fig. 8.2(a) shows that the activity of these compounds was concentration dependent because lower concentrations showed lower activity and higher concentrations showed higher inhibition. The percentage inhibition for the compounds ranged from 24% - 39% for the lowest concentration (9.77  $\mu$ M), while the highest concentration (1250  $\mu$ M) ranged from 72% - 85%. Methotrexate had a 32% inhibition at lower concentrations and gave 86% inhibition at the highest concentration. Fig. 8.2(b) shows that compounds KAM 6 and KAM 8 performed better than the other compounds, as seen from the bar graph. Methotrexate was the most active compound and showed a lower  $IC_{50}$  value.

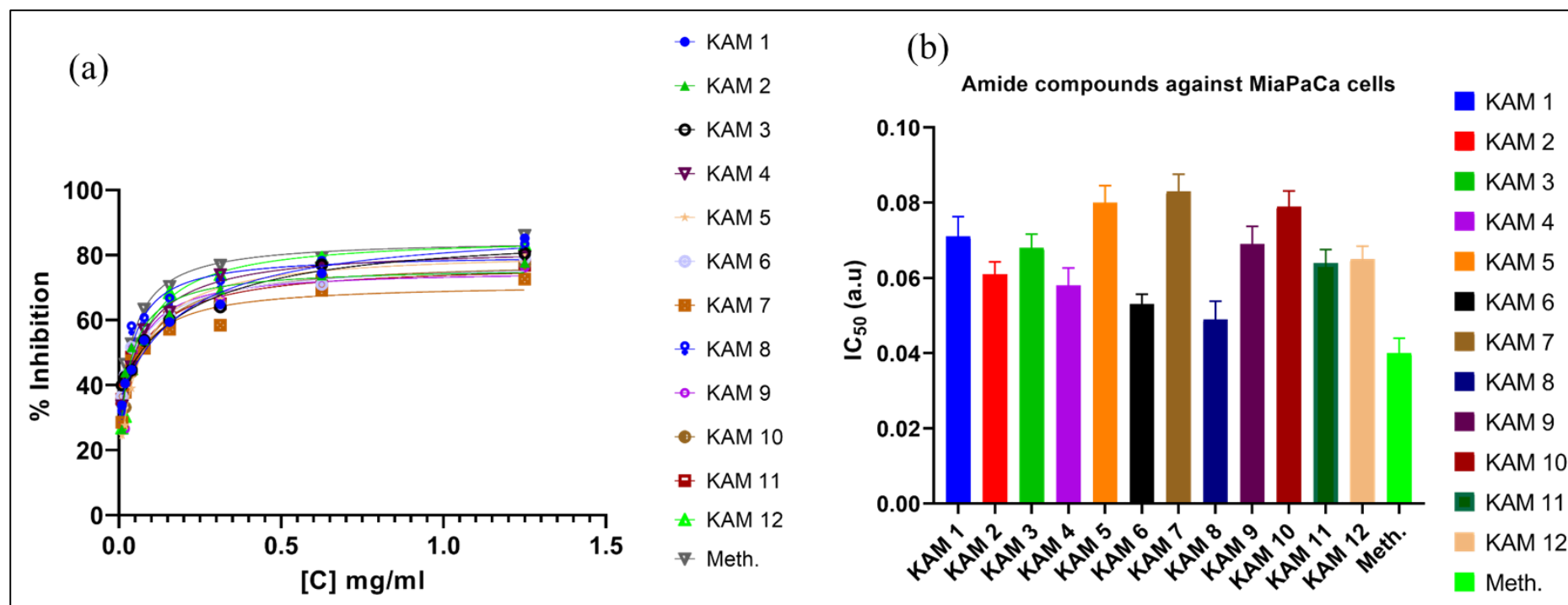


**Figure 8. 2:** Effect of amide compounds (KAM1 to KAM 12) on Mia-PaCa- 2 cells (a) % inhibition vs. concentration curve and (b) inhibition concentration at half maximum ( $IC_{50}$ ) of amide compounds against MiaPaCa-2 cells.

### 8.3.1.3 Summary of the cytotoxicity and anticancer activity of the amide compounds

Table 8.1 below summarizes the overall activity of the synthesized amide compounds. In the obtained results of the CC<sub>50</sub> compound, KAM 1, KAM 3, and KAM 6 showed high values of  $0.870 \pm 0.0051$ ,  $0.471 \pm 0.0042$ , and  $0.447 \pm 0.0131$  mM, respectively. Compounds KAM 2, KAM 4, KAM 6, and KAM 8 showed high IC<sub>50</sub> values of  $0.061 \pm 0.0068$ ,  $0.058 \pm 0.0022$ ,  $0.053 \pm 0.0048$  and  $0.049 \pm 0.0053$  mM, respectively. However, only four compounds gave an SI value that is  $\geq 4$ , namely, compounds KAM 1, KAM 2, KAM 3, and KAM 6, with SI values of 12.3, 4.23, 6.93, and 8.43, respectively, indicating high selectivity for cancer cells. Compounds KAM 11 and KAM 12 of 1.98 and 1.97, respectively indicating toxic traits and low selectivity. The CC<sub>50</sub> for auranofin was  $0.090 \pm 0.0052$  mM, which was many fold higher than that of the above-mentioned compounds. The IC<sub>50</sub> value for methotrexate was found to be  $0.040 \pm 0.0010$  mM, which was the highest in comparison with the synthesized amide compounds.

**Table 8. 1:** Summary of the cytotoxicity, anticancer, and selectivity index (SI) of the amide compounds.

| Compounds | Cytotoxicity<br>(CC <sub>50</sub> , mM) $\pm$ SD<br>HEK-293 | Anticancer activity<br>(IC <sub>50</sub> , mM) $\pm$ SD<br>MiaPaCa-2 | Selectivity<br>Index (SI)<br>CC <sub>50</sub> /IC <sub>50</sub> |
|-----------|-------------------------------------------------------------|----------------------------------------------------------------------|-----------------------------------------------------------------|
| KAM 1     | <b>0.870 <math>\pm</math> 0.0051</b>                        | 0.071 $\pm$ 0.0041                                                   | <b>12.3</b>                                                     |
| KAM 2     | 0.258 $\pm$ 0.0024                                          | <b>0.061 <math>\pm</math> 0.0068</b>                                 | <b>4.23</b>                                                     |
| KAM 3     | <b>0.471 <math>\pm</math> 0.0042</b>                        | 0.068 $\pm$ 0.0056                                                   | <b>6.93</b>                                                     |
| KAM 4     | 0.223 $\pm$ 0.0038                                          | <b>0.058 <math>\pm</math> 0.0022</b>                                 | 3.84                                                            |
| KAM 5     | 0.209 $\pm$ 0.0044                                          | 0.080 $\pm$ 0.0048                                                   | 2.61                                                            |
| KAM 6     | <b>0.447 <math>\pm</math> 0.0131</b>                        | <b>0.053 <math>\pm</math> 0.0048</b>                                 | <b>8.43</b>                                                     |
| KAM 7     | 0.252 $\pm$ 0.0020                                          | 0.083 $\pm$ 0.0025                                                   | 3.04                                                            |
| KAM 8     | 0.123 $\pm$ 0.0043                                          | <b>0.049 <math>\pm</math> 0.0053</b>                                 | 2.51                                                            |

|           |                |                       |      |
|-----------|----------------|-----------------------|------|
| KAM 9     | 0.249 ± 0.0047 | 0.069 ± 0.0056        | 3.61 |
| KAM 10    | 0.193 ± 0.0021 | 0.079 ± 0.0045        | 2.44 |
| KAM 11    | 0.127 ± 0.0056 | 0.064 ± 0.0035        | 1.98 |
| KAM 12    | 0.128 ± 0.0039 | 0.065 ± 0.0026        | 1.97 |
| Aur/Meth. | 0.090 ± 0.0052 | <b>0.040 ± 0.0010</b> | -    |

---

### 8.3.2 Cytotoxicity and anticancer activity of ester compounds

#### 8.3.2.1 Cytotoxicity of esters against HEK-293 cells

The ester compounds were also synthesized and screened for their toxicity against HEK-293 cells *in vitro*. A similar trend to amide compounds was observed for the ester compounds because they also followed a dose-dependent response, as seen in Fig. 8.3 (a), with higher concentrations showing lower percentage cell viability, while lower concentrations showed high percentage cell viability. The percentage cell viability at the highest concentration (1250 µM or 1.25 mM) ranged from 22% to 46%, whereas auranofin had a 29% cell viability at the highest concentration. Compound C1 showed the highest percentage cell viability of 46% at the highest concentration and compound C7 showed the lowest percentage cell viability of 22% at the highest concentration. The lowest concentration, on the other hand, showed a percentage cell viability that ranged from 74% to 97%, while auranofin showed a percentage cell viability of 62%. Compound C15 showed the highest percentage cell viability of 97% at the lowest concentration, and compound C3 showed low percentage cell viability of 74% compared with other synthesized compounds. Fig. 8.3 (b) shows the CC<sub>50</sub> values, and this bar graph clearly shows that compounds C1, C5, C11, and C15 had the highest CC<sub>50</sub> against HEK-293 cells, which is indicative of the less toxic traits possessed by these compounds. Compound C7 and auranofin showed the lowest CC<sub>50</sub> values as indicated on the bar graph which were indicating toxic traits from the aforementioned compounds

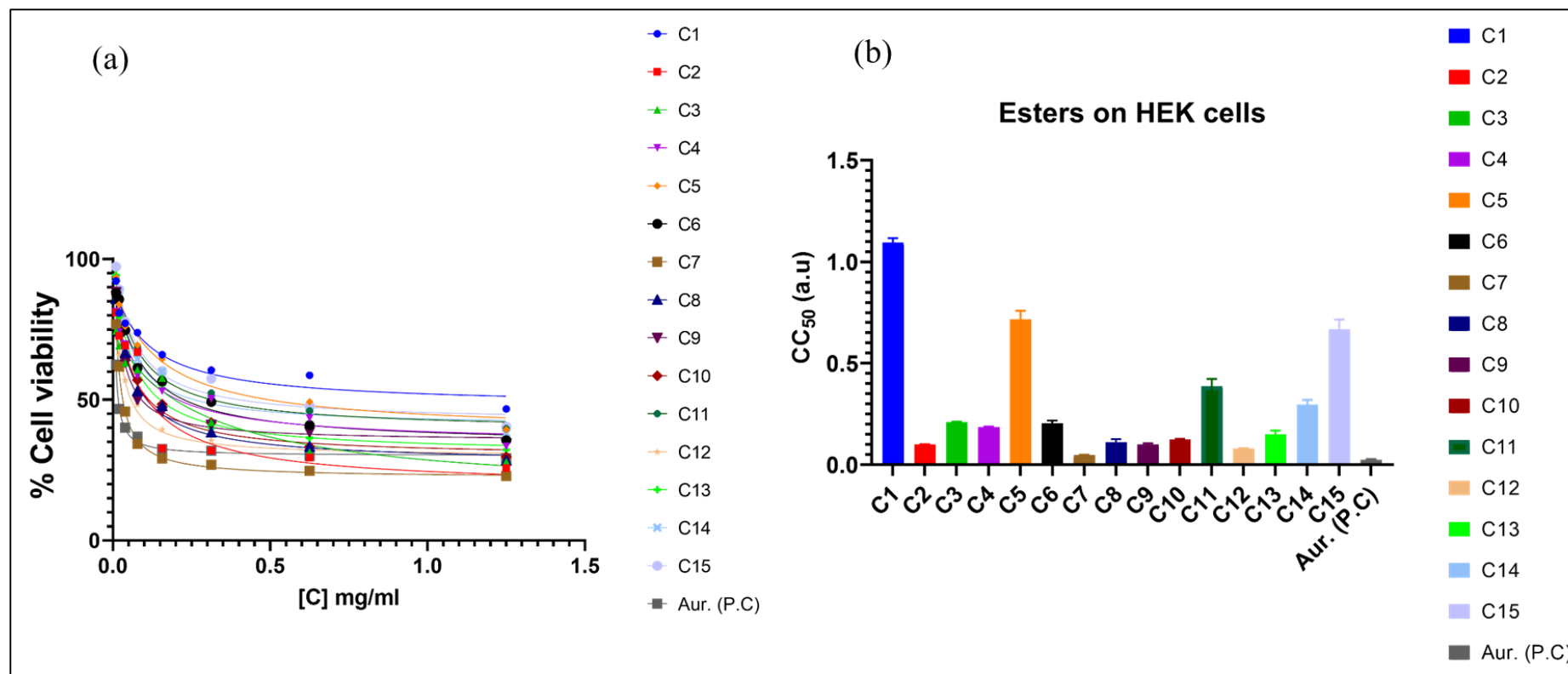


**Figure 8. 3:** Effect of ester compounds (C1 to C15) on HEK-293 cells (a) % cell viability vs. concentration curve and (b)  $CC_{50}$  bar graph of ester compounds against HEK-293 cells.

#### **8.3.2.2 Anticancer activity of esters against MiaPaCa-2 cells**

The ester compounds were also screened for their anticancer capabilities against MiaPaCa-2 cells. Fig. 8.4 (a) shows a similar trend to that of the HEK-293 cell line; it was dose-dependent, and the higher concentration showed higher percentage inhibition and the lower concentrations showed lower percentage inhibition. The percentage inhibition ranged from 24% to 37% for the lowest concentration of the synthesized esters and was 55% for methotrexate. The percentage inhibition for the highest concentration ranged from 72% to 97% for ester compounds, and the positive control (methotrexate) showed a percentage inhibition of 95%. Compound C14 showed a high percentage inhibition of 37% at the lowest concentration, whereas compound C15 showed the highest percentage inhibition of 97% at the highest concentration. Fig. 8.4 (b) shows that compounds C4, C7, C13, C14, and C15 had good  $IC_{50}$  values compared with other ester compounds. Compound C4 showed the greatest  $IC_{50}$  value compared to the other ester compounds and the positive control.



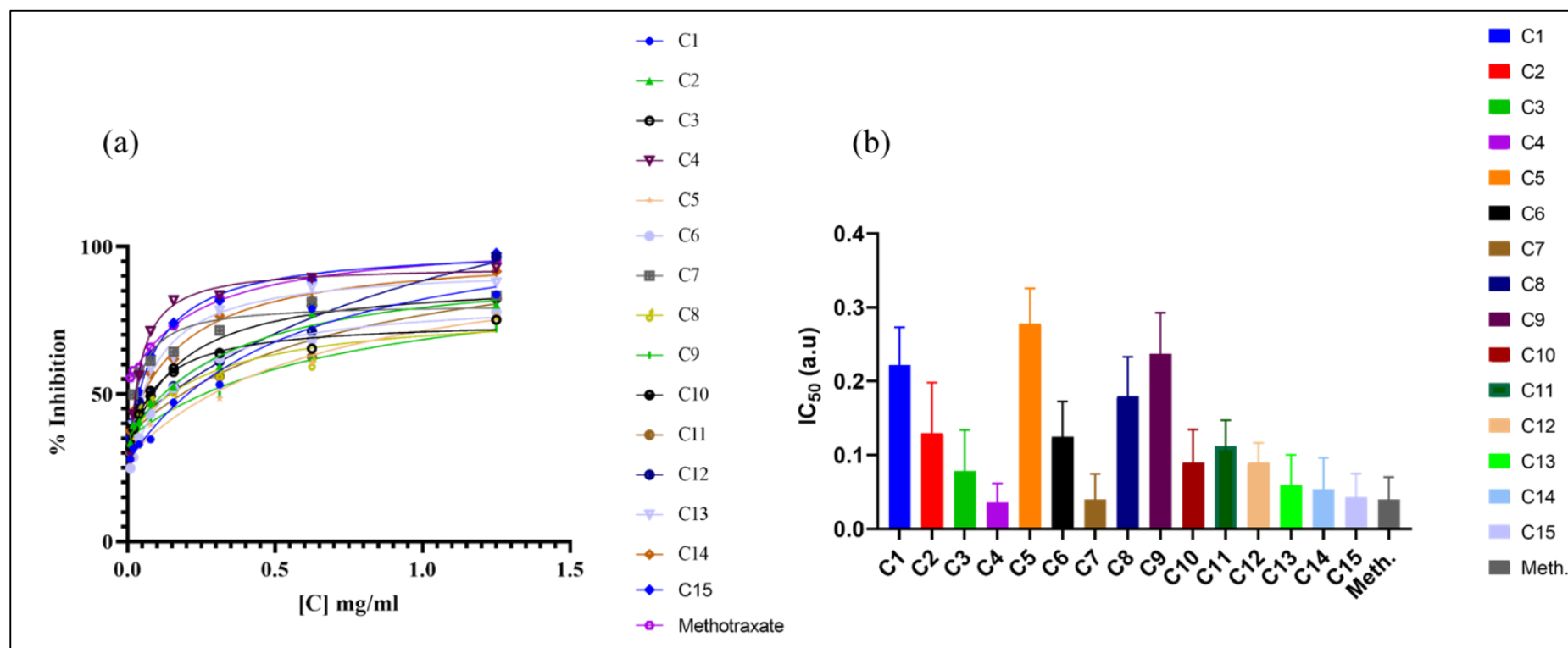


**Figure 8. 4:** Effect of ester compounds (C1 to C15) on Mia-PaCa-2 cells (a) % inhibition vs. concentration curve and (b) IC<sub>50</sub> bar graph of ester compounds against MiaPaCa-2 cells.

### 8.3.2.3 Summary of the cytotoxicity and anticancer activity of ester compounds

In the summarized Table 8.2 below, it can be seen that compound C1 had the greatest CC<sub>50</sub> value of  $1.095 \pm 0.0211$  mM, followed by compound C5 with  $0.715 \pm 0.0440$  mM and C15 with  $0.668 \pm 0.0473$  mM. However, compounds C1 and C5 did not reciprocate the same effect on cancer cells because they showed moderate activity on the Mia-PaCa-2 cells. However, regardless of the moderate activity of compound C1, the SI index was greater than 4. Compound C3 showed high activity ( $0.078 \pm 0.016$  mM) against the MiaPaCa-2 cells, however, the SI was low which indicates low selectivity for cancer cells. Compounds C4 ( $0.036 \pm 0.012$  mM), C7 ( $0.040 \pm 0.025$  mM), C13 ( $0.059 \pm 0.015$  mM), C14 ( $0.052 \pm 0.022$  mM), and C15 ( $0.043 \pm 0.012$  mM) also showed high anticancer activity. However, only compounds C4, C14, and C15 showed a great SI value  $\geq 4$ , indicating high selectivity<sup>377</sup>. Compounds C7, C8, C9, C10, and C12 gave very low SI values of 1.20, 1.04, 0.42, 1.38, and 0.89, respectively, indicating high toxicity traits and very low selectivity. Auranofin showed high toxicity on HEK-293 cells with a CC<sub>50</sub> of  $0.024 \pm 0.0037$  mM, whereas methotrexate showed high activity against Mia-PaCa-2 cells with an IC<sub>50</sub> of  $0.040 \pm 0.012$  mM.

**Table 8. 2:** Summary of the cytotoxicity, anticancer, and selectivity index (SI) of the ester compounds.

| Compounds | Cytotoxicity<br>(CC <sub>50</sub> , mM) $\pm$ SD<br>HEK-293 | Anticancer activity<br>(IC <sub>50</sub> , mM) $\pm$ SD<br>MiaPaCa-2 | Selectivity<br>Index (SI)<br>CC <sub>50</sub> /IC <sub>50</sub> |
|-----------|-------------------------------------------------------------|----------------------------------------------------------------------|-----------------------------------------------------------------|
| C1        | <b>1.095 <math>\pm</math> 0.0211</b>                        | 0.222 $\pm$ 0.051                                                    | <b>4.93</b>                                                     |
| C2        | 0.100 $\pm$ 0.0013                                          | 0.130 $\pm$ 0.038                                                    | 0.77                                                            |
| C3        | 0.209 $\pm$ 0.0033                                          | 0.078 $\pm$ 0.016                                                    | 2.77                                                            |
| C4        | 0.186 $\pm$ 0.0026                                          | <b>0.036 <math>\pm</math> 0.012</b>                                  | <b>5.17</b>                                                     |
| C5        | <b>0.715 <math>\pm</math> 0.0440</b>                        | 0.278 $\pm$ 0.048                                                    | 2.58                                                            |
| C6        | 0.205 $\pm$ 0.0133                                          | 0.125 $\pm$ 0.048                                                    | 1.64                                                            |
| C7        | 0.048 $\pm$ 0.0020                                          | <b>0.040 <math>\pm</math> 0.025</b>                                  | 1.20                                                            |
| C8        | 0.112 $\pm$ 0.0141                                          | 0.108 $\pm$ 0.053                                                    | 1.04                                                            |
| C9        | 0.100 $\pm$ 0.0056                                          | 0.237 $\pm$ 0.056                                                    | 0.42                                                            |
| C10       | 0.124 $\pm$ 0.0034                                          | 0.090 $\pm$ 0.045                                                    | 1.38                                                            |

|           |                       |                      |             |
|-----------|-----------------------|----------------------|-------------|
| C11       | 0.387 ± 0.0356        | 0.112 ± 0.035        | 3.46        |
| C12       | 0.080 ± 0.0025        | 0.090 ± 0.026        | 0.89        |
| C13       | 0.150 ± 0.0185        | <b>0.059 ± 0.015</b> | 2.54        |
| C14       | 0.296 ± 0.0237        | <b>0.052 ± 0.022</b> | <b>5.69</b> |
| C15       | <b>0.668 ± 0.0473</b> | <b>0.043 ± 0.012</b> | <b>15.5</b> |
| Aur/Meth. | 0.024 ± 0.0037        | <b>0.040 ± 0.012</b> | -           |

---

### 8.3.3 Cytotoxicity and anticancer activity of blank and loaded nanoparticles

#### 8.3.3.1 Cytotoxicity of blank and loaded nanoparticles against HEK-293 cells

The compounds that showed good activity in antimicrobial and less cytotoxicity were selected for loading onto nanoparticles and further anticancer screening was done. This was done for continuation and to determine if the results observed in microbial studies of enhanced activity after loading will still be seen or not. From the obtained results, there was clear evidence that the blank and loaded nanoparticles were less toxic compared to auranofin, and this was also confirmed statistically, since the comparison of nanoparticles with auranofin had a p-value of less than 0.05, which is considered statistically significant. Furthermore, the toxicity of the nanoparticles was dose-dependent since lower concentrations showed a percentage cell viability in the range of 95 to 99% and the highest concentrations with percentage cell viability range of 58 to 62% for nanoparticles compared to 31% cell viability of auranofin, as seen in Fig. 8.5 (a). There was no statistically significant difference between the nanoparticles, as shown in the bar graph in Fig. 8.5 (b). Composite C1-L showed the highest mean CC<sub>50</sub> value of 0.05733 ± 0.00677 mg/mL, followed by KAM1-L, which gave a CC<sub>50</sub> value of 0.05364 ± 0.00586 mg/mL. The blank nanoparticles showed a low CC<sub>50</sub> value of 0.04397 ± 0.00736 mg/mL. Auranofin gave the lowest CC<sub>50</sub> value of 0.01531 ± 0.00376mg/mL.

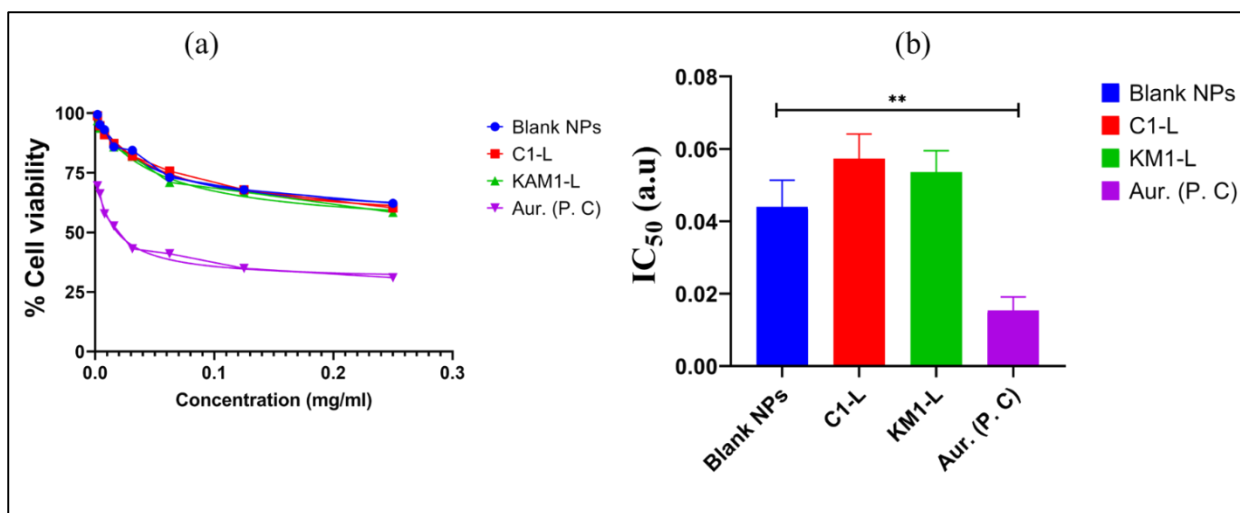


**Figure 8. 5:** Graphs representing (a) % cell viability vs. concentration curve and (b) IC<sub>50</sub> bar graph of blank and loaded nanoparticles against HEK 293 cells.

### 8.3.3.2 Anticancer activity of nanoparticles and loaded nanoparticles against MiaPaCa-2 cells

The effect of blank and loaded nanoparticles was further examined in the Mia-PaCa-2 cancer cell line. The results showed that the nanoparticles and camptothecin were active and their activity was dose-dependent, as illustrated in Fig. 8.6 (a). Fig 8.6 (b) shows the bar graph of the IC<sub>50</sub> values and the statistical difference as shown by the asterisks on top of the bar graphs. The results in Fig. 8.6 (a) show that at higher concentrations, there was a percentage inhibition range of 93 to 99% for nanoparticles and camptothecin, and the lowest concentration had a percentage inhibition that ranged from 46% to 60% inhibition among the studied nanocomposites and a positive control. The 46% inhibition was for the blank nanoparticles at ~2  $\mu$ M (0.00195 mg/ml), while C1-L and KAM1-L had 57% and 52% inhibition, respectively, at similar lower concentrations. This indicates an increase in activity upon loading compounds onto the nanoparticles. Camptothecin, however, performed better than all the nanoparticles at ~2  $\mu$ M (0.00195 mg/mL), since there was over 60% inhibition of the cancer cells at the lowest concentration. However, the IC<sub>50</sub> value of camptothecin was similar to that of C1-L of 0.01180 mg/mL, as shown in Fig. 8.6 (b). Composite KAM1-L gave the best IC<sub>50</sub> value of  $0.003841 \pm 0.001$  mg/mL followed by blank nanoparticles with an IC<sub>50</sub> value of  $0.005976 \pm 0.0012$  mg/mL. Statistical difference between C1-L and blank nanoparticles was observed, because the p-value was less than 0.05, between these two nanomaterials. The p-value was also significant when comparing KAM1-L and C1-L. However, no statistically significant difference was observed when comparing blank NPs and KAM1-L. The results showed that loading C1 to nanoparticles

decreased the overall inhibition activity, whereas loading KAM1 improved the overall inhibition activity.

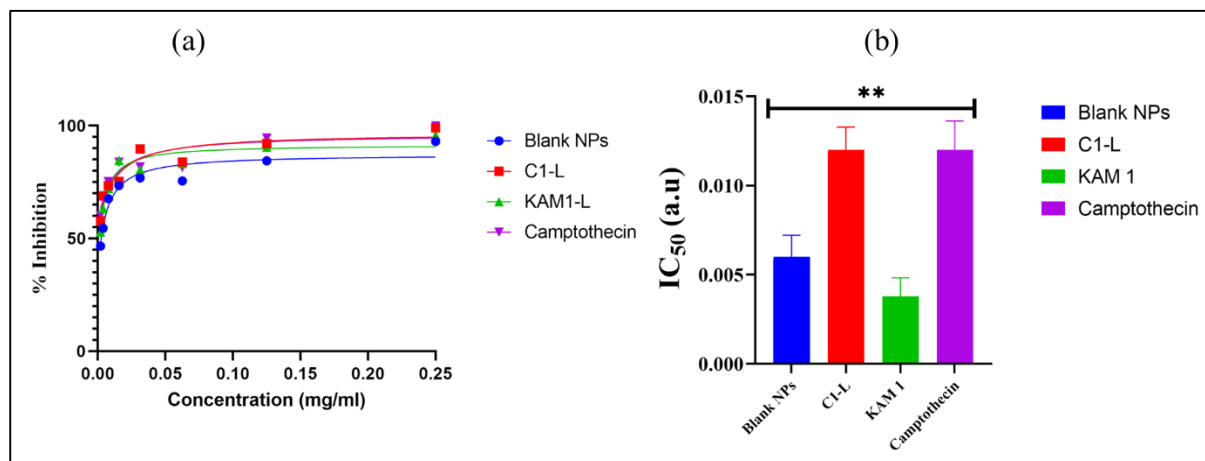


**Figure 8. 6:** The graphs representing (a) % inhibition vs. concentration curve and (b) the IC<sub>50</sub> bar graph of blank and loaded nanoparticles against MiaPaCa-2 cells.

### 8.3.3.3 Summary of the cytotoxicity and anticancer activity of nanoparticles

The summarized results shown in table 8.3 show that the nanoparticles were more potent on cancer cells than on normal cells. The selectivity index also showed that nanoparticles were several -fold selective to cancer cells. From the tabled results, it is clear that nanocomposite KAM1-L was the nanomaterial that gave the highest IC<sub>50</sub> and SI values. The SI value was 13.96 for KAM1-L and 7.36 for blank nanoparticles. The SI value was 4.86 for nanocomposite C1-L. The results prove that compound KAM1 was more compatible when loaded to nanoparticles, whereas compound C1 was less compatible. However, KAM1 and C1 were significantly enhanced in terms of their inhibition ability after loading on nanoparticles.

**Table 8. 3:** Summary of the cytotoxicity, anticancer, and selectivity index (SI) of the loaded and blank nanoparticles

| Compounds | Cytotoxicity<br>(CC <sub>50</sub> , mM) ± SD<br>HEK-293 | Anticancer activity<br>(IC <sub>50</sub> , mM) ± SD<br>MiaPaCa-2 | Selectivity<br>Index (SI)<br>CC <sub>50</sub> /IC <sub>50</sub> |
|-----------|---------------------------------------------------------|------------------------------------------------------------------|-----------------------------------------------------------------|
| Blank NPs | 0.0440 ± 0.00736                                        | <b>0.00598 ± 0.0012</b>                                          | <b>7.36</b>                                                     |
| C1-Load   | <b>0.0573 ± 0.00677</b>                                 | <b>0.0118 ± 0.0010</b>                                           | <b>4.86</b>                                                     |
| KAM1-Load | <b>0.0536 ± 0.00586</b>                                 | <b>0.00384 ± 0.0010</b>                                          | <b>13.96</b>                                                    |
| Aur/CPT.  | <b>0.0153 ± 0.00376</b>                                 | <b>0.0118 ± 0.0020</b>                                           | -                                                               |

## 8.4 Discussion

### 8.4.1 Cytotoxicity and anticancer activity of the amide compounds

To determine the cytotoxic activity of synthesized compounds in pancreatic cancer (MiaPaCa-2) and human embryonic kidney (HEK-293) cells, the cells were treated for 48 h with different serially diluted concentrations (0.00977 – 1.25 mM) of the synthetic compounds. The cytotoxicity of synthesized amides and methotrexate against cells was measured using the MTT assay. The CC<sub>50</sub> and IC<sub>50</sub> values obtained in this study were all dose-dependent, where higher concentrations showed toxic traits for both cancer and normal cells.

Compound KAM 1 (carrying 3-hydroxybenzoic acid moiety as a backbone) showed the highest CC<sub>50</sub> and SI values among the amide compounds. However, KAM 1 showed moderate anticancer activity against Mia-PaCa-2 cells. Compound KAM 3 (carrying 3,5-dihydroxybenzoic acid and 2,4-diaminophenol moiety) was the second highest compound to show low toxicity traits. Meanwhile, compound KAM 8 (carrying gallic acid moiety) showed the lowest IC<sub>50</sub> value. These results indicates that compound KAM 8 was the most potent compound among the synthesized amide compounds and, it was only eclipsed by methotrexate (a positive control). However, compound KAM 8 showed a very low SI value, indicative of non-selectivity and toxicity traits. The compound that showed the second lowest IC<sub>50</sub> value was compound KAM 6 (a conjugation of gallic acid and 1,3-diaminobenzene), which demonstrated an IC<sub>50</sub> value of 0.053 ± 0.0048 mM and an SI value of 8.43, indicating potency

and selectivity only towards cancer cells. This less toxic trait is seen from the third highest CC<sub>50</sub> value.

Compounds KAM 10, KAM 11, and KAM 12 showed toxicity traits with very low SI values. The obtained CC<sub>50</sub> values for these compounds were close to the CC<sub>50</sub> value obtained for auranofin (positive control), which is widely known to be very toxic.

The results show that compounds that bear the gallic acid moiety (KAM 6 and KAM 8) in their backbone can be used as scaffolds for designing more potent amide compounds. Gallic acid potency has been previously reported by Liu and co-workers, who focused on the apoptotic pathways responsible for inhibiting MiaPaCa-2 cells<sup>105</sup>. Another report by Cedò et al.<sup>378</sup> also showed that gallic acid, as an active component in grape seed procyanidins, affected apoptotic biomarkers such as Bax and Bcl-2<sup>378</sup>. Sales and co-workers reported the effect of octyl gallate and gallic acid on cell cycle progression by altering the expression of the cell cycle regulators (Cyclin D1, D3, CDK-4, CDK-6, p18 INK4, p21Waf-1, and p27 KIP)<sup>379</sup>.

These previously mentioned studies show that most of the activity of gallic acid emanates from affecting apoptotic pathways as a mechanism of action, with another study alluding that other pathways might also be altered by gallic acid and its derivatives. However, future molecular studies are necessary to validate the mechanism of action of the gallic acid compounds synthesized in this study.

#### **8.4.2 Cytotoxicity and anticancer activity of the ester compounds**

Cytotoxicity studies on hydroxybenzoic acid esters have been widely explored, however, most of these studies focus on alkyl derivatives, and few to no reports exist on aryl ester derivatives of hydroxybenzoic acids. Previous studies have shown the activity of alkyl esters, opening doors for the exploration of aryl derivatives.

The cytotoxicity of ester compounds against the HEK 293 cells showed that most of the compounds had high toxic traits, except for compound C1, C5, and C15. Compound C1 (bearing a 3-hydroxybenzoic acid backbone) showed the highest CC<sub>50</sub>, indicating that it is less toxic compound and has good selectivity towards cancer cells. However, compound C15 (conjugate of gallic acid and N-3-hydroxyphenylacetamide) had the highest selectivity compared with C1 and C15.

The anticancer activity of these compounds was led by compound C4 (conjugate of 3-hydroxybenzoic acid and phloroglucinol), which had the highest activity with an IC<sub>50</sub> value of

and SI value. C4 had a higher activity compared with methotrexate (positive control). Compound C7 (bearing 3,5-dihydroxybenzoic acid and 3-hydroxyacetophenone) showed the second highest activity with an IC<sub>50</sub> value and low SI value. Compound C7 showed an IC<sub>50</sub> value similar to methotrexate with good activity, however, the SI value was very low, indicative of a very toxic compound, and this was evident from the extremely low CC<sub>50</sub> value that was obtained. Compound C15 (bearing gallic acid moiety) also demonstrated good potency against Mia-PaCa-2 cells and a very high SI value of 15.5, which was the highest among all the compounds, including amide compounds, and this indicates high selectivity of this compound. Compound C13 (conjugation of gallic acid and phloroglucinol) and compound C14 (conjugation of gallic acid and 3,5-dihydroxyacetophenone) also showed good activity. The SI values for compounds C13 and C14 were 2.54 and 5.69, respectively, further indicating that compound C13 shows toxicity traits, whereas compound C14 shows good potency and less toxic traits.

The results indicate that 3-hydroxybenzoic acid conjugated with phloroglucinol to give a tripodal hydroxybenzoate compound was the best among both the amide and ester compounds against Mia-PaCa-2 cells. Compound C13 also a tripodal conjugate of gallic acid and phloroglucinol, showed good anticancer activity. However, C13 (Mw: 588 Da) also showed toxicity against HEK 293 cells, and this could be attributed to the bulky structure and high molecular weight that exceed 500 Da, which is one of the physicochemical properties that contribute significantly to the toxicity traits of a molecule. In addition, the phloroglucinol compound that also showed toxicity was compound C9 (bearing 3,5-dihydroxybenzoic acid, Mw: 534 Da), which had the lowest SI value of 0.42. Therefore, C4 (Mw: 486 Da) was better in terms of molecular weight; hence, it showed less toxicity traits compared with C13 and C9 because they were all phloroglucinol conjugates. Monohydroxybenzoic acid in this case performed much better than the dihydroxy- and trihydroxybenzoic acid ester tripodal counterparts in terms of the highest anticancer activity. The trend was 3-hydroxybenzoic acid > gallic acid > 3,5-dihydroxybenzoic acid for the tripodal compounds.

The superior efficacy of 3-hydroxybenzoic acid compared with the renowned gallic acid was also observed by Alim et al.<sup>102</sup> in their study on the *in vitro* inhibition of rat kidney aldose reductase enzyme for diabetes mellitus application<sup>380</sup>. In contrast, gallic acid-based compounds have been previously shown to possess activity on a broad spectrum of cancer cell lines. In addition, phloroglucinol has been shown to possess strong anticancer activity over a broad spectrum of cancer cell lines. Chauthe et al.<sup>381</sup> synthesized a series of dimeric



phloroglucinol compounds and tested them against various cancer cell lines. Their results showed that phloroglucinol compounds exhibited a strong broad anticancer activity <sup>381</sup>. Chapman and co-workers also showed the effectiveness of phloroglucinol sulfonate esters to have strong anticancer/antimalaria activity <sup>382</sup>. A recent study by Xu et al. further emphasized the importance of having a phloroglucinol motif as a nucleus in the synthesis of novel Bis-Artemisinin-Phloroglucinol hybrid molecules with dual anticancer and immunomodulatory activities <sup>383</sup>.

#### **8.4.3 Cytotoxicity and anticancer activity of the nanoparticles**

The development of a new drug is a difficult and costly process; additionally, the successful administration of recent drugs is not applicable in clinical trials because of their inefficient performance <sup>384</sup>. Typically, drugs are destroyed in normal tissues, resulting in severe pathologic side effects. This is because the drugs used in conventional medicine are unstable and toxic, with only short-term therapeutic effects <sup>384</sup>. Furthermore, some drugs have solubility limitations. To address the aforementioned issues, various new drug-carrier systems in the micro- and nanometer size ranges were developed <sup>385</sup>. These nanometer-sized delivery systems provide distinct advantages for drug delivery. Polymer nanoparticles fall in the bracket of these delivery systems, and more research is required to fully understand their toxicity profile and how well they will sustain the drug for drug delivery purposes. Moreover, their toxicity is mainly controlled by the monomers used in their synthesis, therefore, the use of biodegradable monomer aids in limiting the toxicity of these nanoparticles.

In this study, polymer nanoparticles were used as drug delivery systems for compounds C1 and KAM1. The nanoparticles used were a conjugation of trans-cinnamic acid and N-isopropyl acrylamide (NIPAM). The choice of these monomers was mainly based on their potential for responsive stimuli or inherent bioactivity. In this case, NIPAM was used because it has inherent thermoresponsive ability and trans-cinnamic acid has potential as an anticancer active compound. The hybrid system of these two molecules would form a novel system that could potentially enhance the anticancer activity of the loaded compound.

In the current report, the pancreatic cancer cell line (Mia-PaCa-2) and HEK 293 cells were treated with blank and loaded nanoparticles to evaluate the effect of nanoparticles. The results showed that both loaded and blank nanoparticles exerted outstanding antiproliferative properties compared with the unloaded compounds. However, the loading also had an effect on the normal cells because the CC<sub>50</sub> value was very low compared with the unloaded

compounds. The SI values corresponded well even though the CC<sub>50</sub> values were low, indicating good selectivity for cancer cells compared with normal cells.

The blank nanoparticles were exceptionally effective against cancer cells, with an extremely low IC<sub>50</sub> value of ~6  $\mu$ M ( $0.005976 \pm 0.0012$  mg/ml) when compared with both C1-Loaded and camptothecin, which both had an IC<sub>50</sub> value that was ~12  $\mu$ M. Camptothecin was used as a positive control because of its well-known anticancer activity. KAM1-Loaded outperformed all of the tested compounds including camptothecin.

The loading of nanoparticles enhanced the anticancer activity of compounds C1 and KAM1 both of which had moderate to poor anticancer activity before loading to the nanoparticles, as reported in the above sections. Enhancement of drugs by nanoparticles has previously been reported. Ashour et al.<sup>386</sup> used polymer nanoparticles to load 5-flourouracil (5-FU), and their results showed that 5-FU-loaded NPs significantly increased 5-FU cytotoxicity on cancer cells compared with pure 5-FU<sup>386</sup>. Udompornmongkol and Chiang also showed that polymeric nanoparticles enhanced curcumin anti-colorectal cancer activity<sup>387</sup>. The size and shape of nanoparticles are the main driving force in their enhancement abilities. When optimal sizes and/or shapes are achieved, there is a significant increase in cellular internalization which contributes to high activity.

However, in this study not only size and shape of nanoparticles played a role in the increased anticancer activity of the synthesized compounds but also the material used had a significant activity enhancement. The anticancer ability of the blank nanoparticles is attributed to the inherent anticancer action of trans-cinnamic acid. Previous studies have investigated the potential anticancer mechanism of action of cinnamic acid and its derivatives, which involves cell death caused by irreversible DNA damage after the induction of apoptosis<sup>388</sup>. Pontiki and co-workers have shown that bonding cinnamic acid on the conjugated double bond increases anticancer activity more than bonding on the carboxylic acid because all their compounds that had an intact conjugated double bond were inactive or moderately active<sup>389</sup>. Nanoparticles in this study were also conjugated on the conjugated double bond of trans-cinnamic acid.

## 8.5 Conclusion

The above results showed that most of the synthesized compounds were nontoxic with C1 and KAM1 being the most notable compounds against HEK-293 cells. The two compounds were 3-hydroxybenzoic acid conjugates, indicating that compounds derived from 3-hydroxybenzoic

acid are potentially safe precursors for the synthesis of amide and ester compounds. However, these two compounds showed moderate activity against a cancer cell line (Mia-PaCa-2). However, the high selectivity index of these compounds showed the potential of the compounds to be selectively active only against Mia-PaCa-2 cell line upon modification or loading onto nanoparticles. This was also evident for compound C15, which has gallic acid as a precursor. Compound C15 was the highest in terms of activity against Mia-PaCa-2 and showed low toxicity on HEK-293 cells. The selectivity index of this compound was the highest in comparison with the synthesized amide and ester compounds. Compound C4, a tripodal compound of 3-hydroxybenzoic acid and phloroglucinol, showed the highest  $IC_{50}$  value against Mia-PaCa-2 cells with a good SI value.

The loading of compounds C1 and KAM1 onto nanoparticles showed a significant increase in the activity against cancer cells. The blank nanoparticles also showed high activity against the cancer cell line, which is attributed to the trans-cinnamic acid in the polymer backbone. A synergistic effect on the loaded materials was observed for the KAM1-loaded nanocomposite. The KAM1-loaded nanocomposite showed the highest  $IC_{50}$  value compared to C1-loaded, blank nanoparticles and camptothecin. The SI values of the nanomaterials was high indicating high selectivity to cancer cells. The enhanced activity of the nanoparticle-based materials was attributed to the small size that permits the entrance of these materials to cell membrane. The future work will be to load compound C4, C14 and C15 onto nanoparticles for further enhancement since they performed extremely well as un-loaded compounds.

## Chapter 9: General Discussion

---

Cancer is a complex and multifaceted disease characterized by uncontrolled cell growth and proliferation <sup>390</sup>. It's one of the leading causes of death worldwide, with various factors contributing to its development, including genetic predisposition, environmental exposures, lifestyle choices, and aging <sup>391</sup>. Given its complexity, tackling cancer requires a multi-pronged approach involving prevention, early detection, diagnosis, treatment, and ongoing research.

In recent years, there has been significant progress in understanding the molecular mechanisms underlying cancer development and progression <sup>392</sup>. This understanding has led to the development of targeted therapies that aim to specifically interfere with pathways and molecules involved in cancer growth and spread. Additionally, immunotherapy has emerged as a promising approach, harnessing the body's immune system to recognize and eliminate cancer cells<sup>393</sup>. However, cancer alone is no longer the only threat in hospital patients. Due to weak immunity in cancer patients, secondary opportunistic infections severely threatens human health. There finding a drug that could have dual effect on cancer and microbial infections would go a long way in addressing this lingering issue.

One avenue of research in combating cancer and possible microbial infections involves *in-silico* methods, which utilize computational tools and techniques to analyze biological data, model cellular processes, and predict drug responses. These approaches have the potential to accelerate drug discovery and development by identifying novel drug targets, simulating drug interactions with biological molecules, and optimizing treatment regimens.

Before conducting any bioassay studies, it was crucial to design novel compounds and predict the bioactivity and potential toxicity of these compounds through *in-silico* studies. The computer toxicity assessments showed no signs of toxicity from the designed compounds. Additionally, computational studies revealed an intriguing trend in ester compounds regarding bioactivity, suggesting potential activity based on the number of substituted hydroxyl groups in each designed compound. Specifically, tri-substituted compounds demonstrated superior potential activity, followed by di-substituted compounds, and mono-substituted compounds. Among the tri-substituted compounds, compound **C13**, derived from phloroglucinol and gallic acid, exhibited the most potency against the studied proteins, namely 7NEZ (an ABCG2 protein

responsible for multidrug resistance<sup>394</sup>) and 1K4T (a DNA topoisomerase I protein crucial in cellular processes such as DNA transcription, replication, recombination, and DNA repair<sup>395</sup>).

The activity demonstrated by the designed tri-substituted, di-substituted, and mono-substituted compounds for both amide and ester compounds prompted further exploration through *in-vitro* antimicrobial and anticancer studies. These compounds were successfully synthesized and characterized using spectroscopic techniques, confirming successful conjugation. However, the potentially potent tri-substituted compounds were found to interact with the stationary phase due to their polarity, resulting in impurity peaks in the proton NMR studies. Nevertheless, other peaks belonging to the studied compounds were observed, confirming successful conjugation, and these results were further corroborated by FTIR and mass spectroscopy results.

The *in-vitro* results for both antimicrobial and anticancer assays contradicted the *in-silico* studies, as esters derived from 3-hydroxybenzoic acid (specifically C1 in most cases) emerged as the most potent, displaying remarkable antimicrobial and anticancer properties. The 3-hydroxybenzoic acid-derived amides (particularly KAM 1) also exhibited high potency among the designed and synthesized amides. Several potential reasons may explain why the 3-hydroxybenzoic acid-derived compounds displayed increased activity compared to 3,5-dihydroxybenzoic acid and gallic acid-derived compounds. These reasons include steric hindrance in the latter molecules with additional hydroxyl groups, potentially leading to a decrease in hydrogen bonding (contrary to *in-silico* studies) of the compounds with biomolecules. Another possible reason could be the structural flexibility of the 3-hydroxybenzoic acid-derived compounds, allowing for conformational changes that optimize interactions with target biomolecules and enhance binding affinity and potency compared to more rigid structures.

The disparity between the *in-vitro* and *in-silico* studies underlines the complexity of predicting bioactivity solely based on computational models and emphasizes the importance of experimental validation. Nonetheless, the compounds demonstrate potential as precursors or scaffolds in the design of more potent derivatives for antimicrobial and anticancer applications despite the disparity in computational and experimental findings.

Then to further enhance the activity of the designed compounds and successfully deliver the selected potent amide and ester compounds, the novel polymer nanoparticles were utilized. The

polymer nanoparticles were successfully synthesized and characterized by FTIR, TEM, and DLS. To determine the interaction of monomers the FTIR showed interesting results that the *N*-isopropyl acrylamide interacted via the amide moiety instead of the anticipated alkene part, a strange but interesting phenomenon that normally occurs on supramolecules. This interaction results with gel-like particles that could be of interest in other field such wound-healing since it forms a nanogel-like nanoparticles.

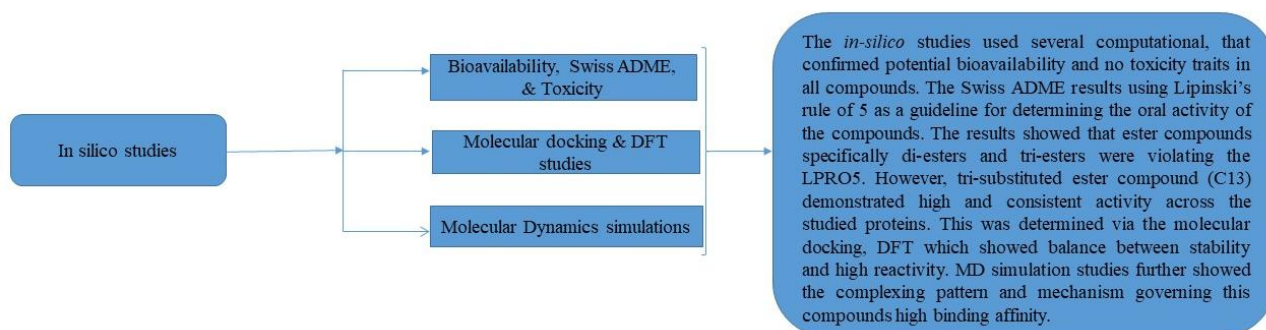
Furthermore, the nanoparticle sizes were confirmed by both DLS and TEM techniques that showed an optimum molar ratio of 50:50 (NIPAM: TCA) to be the best amongst other molar ratios, because of the good dispersity ( $PDI < 0.5$ )<sup>377</sup> and small-sized particles.

These nanoparticles were then used as a drug-delivery system for the selected active compounds KAM 1 and C1. The loaded compounds showed enhanced activity compared to the unloaded compounds for both *in-vitro* antimicrobial and anticancer studies. These further confirm the importance of using nanomaterials as drug vehicles and probe further exploration of different types of nanomaterials for other types of cancer cells and microbial strains.

Figure 9.1 below shows summarized work and scope of the work that is already discussed in the upper section of this chapter.

### Part A: *In silico* drug design of ester and amide derivative compounds

The *in silico* drug design approach was undertaken to determine compounds potential bioactivity when screened against biological targets such as DNA topoisomerase I enzyme, an enzyme that is responsible for the transcription and replication of cells, and ABCG2 transporter protein that is responsible for most of the drug resistance of cancer cells. The compounds were screened for physicochemical properties using various online computational tools to determine their bioavailability and drug-likeness amongst other screening procedures. These tools confirmed the potential bioavailability and non-toxicity of the designed compounds probing further *in silico* screening. The molecular docking and molecular dynamic simulations further corroborated the results obtained from the online tools of bioavailability testing. Molecular docking showed that ester compounds were more active compared to amide compounds. Then by virtue of the high activity of ester compounds, one ester compound (C13) was selected based on the merit of the undertaken screening procedures. C13 (CE12) was a tripodal gallic acid compound that showed good overall activity and this compound's molecular dynamic simulation results showed the stability of the compound with studied proteins that resulted in high binding affinities.

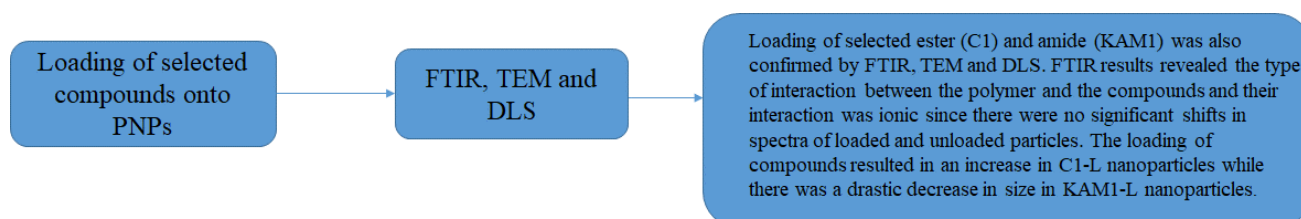
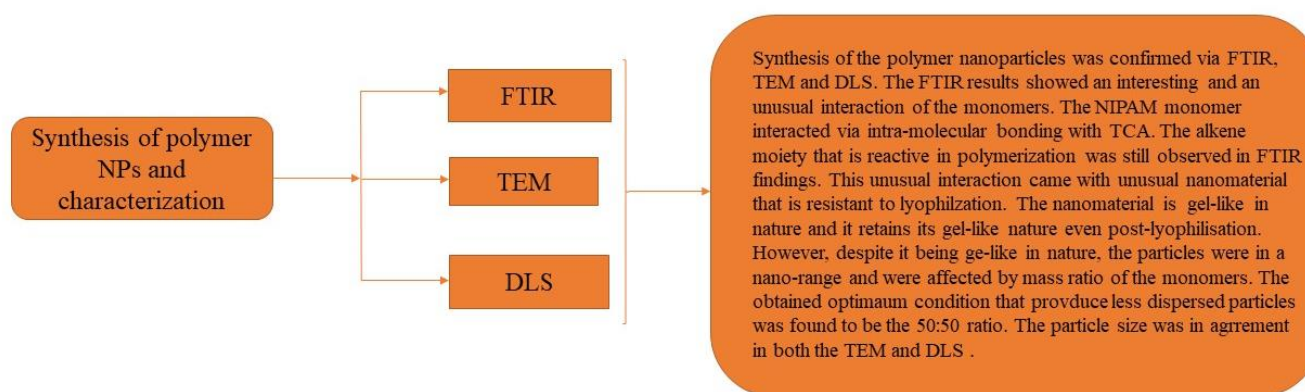
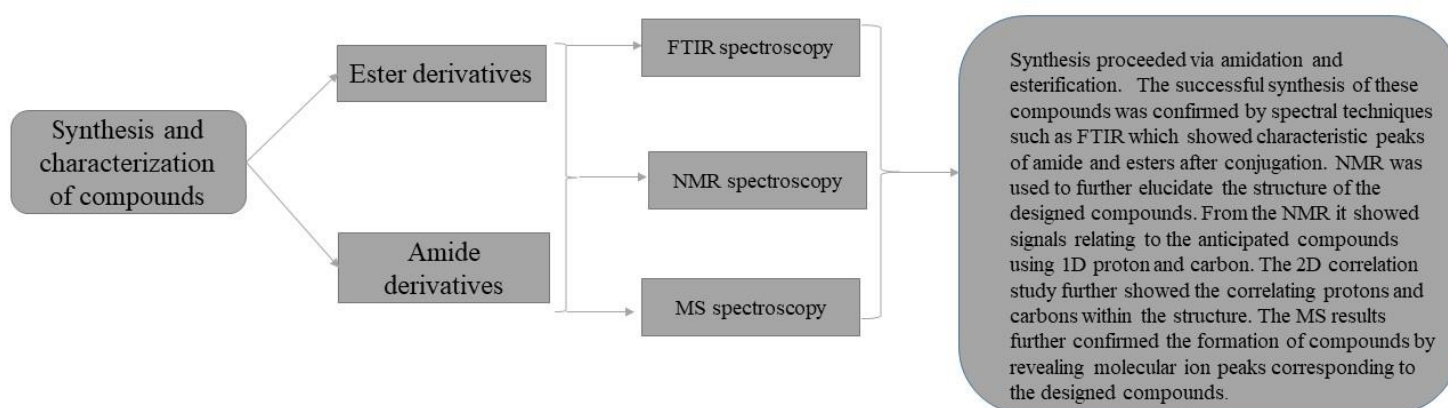


### Part B: Synthesis and characterization of design compounds and their polymer nanocomposites

The designed compounds were then synthesized via esterification and amidation one-pot synthesis method. Their synthesis was confirm by spectroscopic techniques such as FTIR, NMR and MS. All the amide and ester compounds showed similar patterns that confirmed similar library of compounds and the characteristic peaks or signals were observed on FTIR and NMR results. The MS results on the other hand showed the molecular ion peak of the desired compounds further confirming successful synthesis of the compounds.

The synthesis of polymer nanoparticles was also achieved from nanoprecipitation method. Successful conjugation of monomers was confirmed by FTIR spectroscopy that showed peaks that confirmed successful polymerization. The size and morphology of the nanoparticles were obtained on TEM images that showed the effect of monomer ratios on the size and shape of particles. The 50:50 monomer ratio was selected as the optimum ratio because of the monodispersed particle size obtained in this ratio. The DLS results also confirmed the particle sizes obtained from TEM. The polydispersity index results confirmed the monodispersed nanoparticles for the selected monomer ratio (50:50). The surface charge was also determined on DLS.

The compounds that showed the best overall bioactivity were loaded onto the polymer nanoparticles. The interaction between the polymer and the loaded compounds were determined using FTIR spectroscopy. The sizes of the compound-loaded nanoparticles were obtained from DLS and TEM results. Loading compounds onto the NPs slightly increased the particle size.

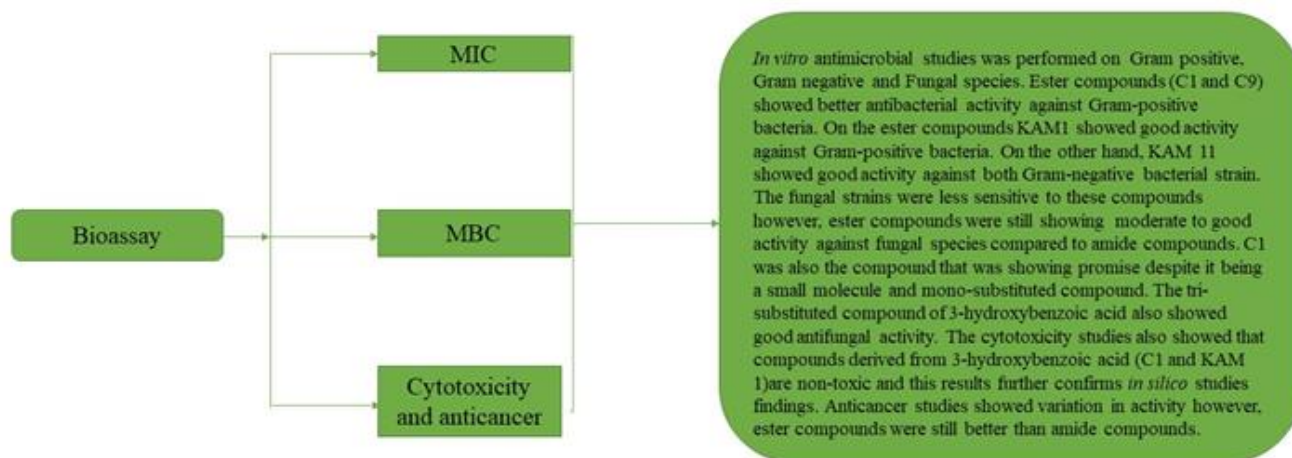




### Part C: Bioactivity of designed compounds and their nanocomposites

Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) were determined by methods reported in Chapter 7. The MIC, MBC, and MFC showed that compound C1 on the ester compound and KAM 1 on the amide compounds were the overall best antimicrobial compounds. Loading these compounds resulted in slightly improved activity against the tested strains.

The anticancer activity of these two compounds (C1 and KAM 1) was determined from the MTT assay. The results obtained showed good potential for most compounds tested against a pancreatic cancer cell line (MiaPaCa-2). These compounds were also tested for their toxicity against human embryonic kidney cell line (HEK 293). The cytotoxicity results showed compounds C1 and KAM 1 to have less toxic effects in comparison to other studied compounds. The loaded on the other hand showed slightly toxic effects in comparison to the un-loaded compounds. However, the loaded compounds showed significantly higher activity compared to un-loaded compounds.



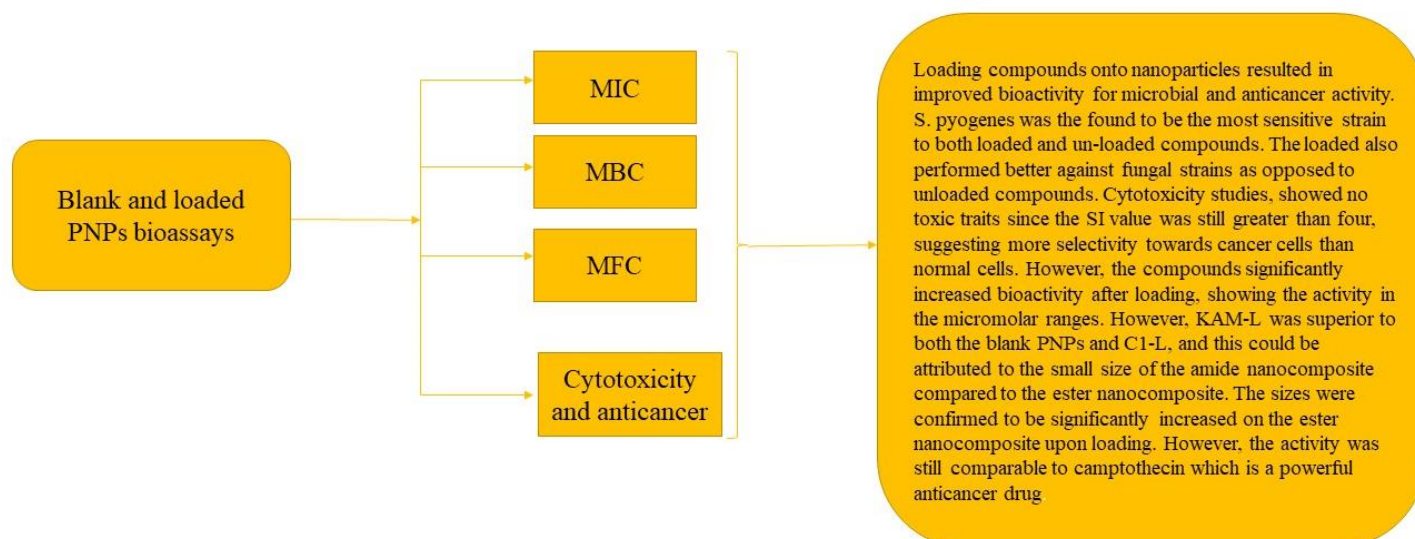


**Figure 9. 1:** Summarized flowchart representation of the study

## Chapter 10: Overall conclusion and recommendations

---

### 10.1 Conclusion

A comprehensive investigation into the design, synthesis, and bioactivity evaluation of hydroxybenzoic acid amides and esters, as well as their polymer nanocomposites, has provided valuable insights into their potential as antimicrobial and anticancer agents. This study aimed to address the challenge of finding non-toxic compounds with dual activity against microbial infections and cancer, particularly given the increased susceptibility of immunocompromised cancer patients to secondary infections in hospital settings.

The compounds were identified through *in-silico* screening, followed by synthesis and characterization using spectroscopic techniques. The broth microdilution assay was employed to assess the antimicrobial activity of the synthesized compounds and their polymer nanocomposites. Additionally, cytotoxicity screening using the MTT assay was conducted to evaluate the toxicity and anticancer potential of these compounds in doing so we were able to address the aims and objectives of the study.

Our findings indicate that while both amides and esters demonstrate promising bioactivity profiles, the esters consistently exhibit superior activity across all bioassays. The successful synthesis of the designed compounds, facilitated by the *in-silico* approach, led to the discovery of novel compounds with potential as scaffolds or precursors for highly active compounds, thus highlighting their clinical relevance.

Furthermore, the study achieved the successful synthesis of small-sized nanoparticles, which showed activity even as blanks nanomaterial and were compatible with the selected compounds. These nanoparticles enhanced both the anticancer and antimicrobial activities of the compounds.

For future research, it would be valuable to explore the synthesized novel nanomaterials in applications such as wound healing. Investigating their surface properties and understanding their mechanisms of action in biological systems could aid in identifying similar materials and expanding the knowledge base of drug delivery systems.

## 10.2 Limitations of the study

- There was a prolonged clean-up time of the synthesized compounds because of their interaction with silica
- Inability to do drug entrapment efficiency and stimuli-responsive drug-release studies because of the nature of our novel polymer. It would have been easier to explain the activity if there were drug-release kinetics studies.
- Scanning electron microscopy would have shown us the actual morphology and surface properties like pores for drug entrapment purposes
- For purification and obtaining pure compounds, the variable temperature NMR (VT-NMR) would have assisted in varying the temperature effect of the sticky materials

## 10.3 Recommendations of the study

- Further exploration of the structure-activity relationship (SAR) of hydroxybenzoic acid derivatives to gain deeper insights into the molecular mechanisms underlying their bioactivity.
- Investigation of the pharmacokinetic properties and toxicity profiles of these compounds *in vivo* to assess their potential development as therapeutic agents.
- In-depth studies on the mode of action of the most active compounds to identify novel targets for antimicrobial and anticancer interventions.
- Exploration of potential synergistic effects of hydroxybenzoic acid derivatives with existing antimicrobial and anticancer drugs to enhance therapeutic efficacy.
- Continued research into different types of nanomaterials for drug delivery systems to optimize their efficacy in targeting specific cancer cell types and microbial strains.
- Drug-delivery and drug entrapment efficiency studies with a new set of lyophilizable polymers.
- Further structural exploration of the synthesized novel polymer since it possesses interesting structural features for potential use as wound-healing nanomaterial due to its gel-like nature.
- Characterize compounds using X-ray crystallography for crystal structure determination

- Use HPLC to determine compound purity for all the synthesized compounds

## References

1. Baker, R. E. *et al.* Infectious disease in an era of global change. *Nat. Rev. Microbiol.* **20**, 193–205 (2022).
2. Li, Y. *et al.* Healthy lifestyle and life expectancy free of cancer, cardiovascular disease, and type 2 diabetes: prospective cohort study. *BMJ* **368**, l6669 (2020).
3. Kocarnik, J. M. *et al.* Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life Years for 29 Cancer Groups From 2010 to 2019 A Systematic Analysis for the Global Burden of Disease Study 2019. *JAMA Oncol.* **8**, 420–444 (2022).
4. Apostol, T. V. *et al.* Synthesis, in silico and in vitro evaluation of antimicrobial and toxicity features of new 4-[(4-chlorophenyl)sulfonyl] benzoic acid derivatives. *Molecules* **26**, 1–23 (2021).
5. Nussbaumer, S., Bonnabry, P., Veuthey, J. L. & Fleury-Souverain, S. Analysis of anticancer drugs: A review. *Talanta* **85**, 2265–2289 (2011).
6. Holohan, C., Van Schaeybroeck, S., Longley, D. B. & Johnston, P. G. Cancer drug resistance: an evolving paradigm. *Nat. Rev. Cancer* **13**, 714–726 (2013).
7. Nitulescu, G. M. *et al.* Akt inhibitors in cancer treatment: The long journey from drug discovery to clinical use (Review). *Int. J. Oncol.* **48**, 869–885 (2016).
8. Champoux, J. J. DNA Topoisomerases: Structure, Function, and Mechanism. *Annu. Rev. Biochem.* **70**, 369–413 (2001).
9. Corbett, K. D. & Berger, J. M. Structure, molecular mechanisms, and evolutionary relationships in DNA topoisomerases. *Annu. Rev. Biophys. Biomol. Struct.* **33**, 95–118 (2004).
10. Chen, S. H., Chan, N. L. & Hsieh, T. S. New mechanistic and functional insights into DNA topoisomerases. *Annu. Rev. Biochem.* **82**, 139–170 (2013).
11. Vos, S. M., Tretter, E. M., Schmidt, B. H. & Berger, J. M. All tangled up: How cells direct, manage and exploit topoisomerase function. *Nat. Rev. Mol. Cell Biol.* **12**, 827–841 (2011).
12. Wang, J. C. DNA topoisomerases. *Annu. Rev. Biochem.* **65**, 635–692 (1996).
13. Wani, A. K. *et al.* Targeting Apoptotic Pathway of Cancer Cells with Phytochemicals and Plant-Based Nanomaterials. *Biomolecules* **13**, 1–34 (2023).
14. Wang, H. *et al.* Preloading of hydrophobic anti-cancer drug into multifunctional nanocarrier for multi-modal imaging, NIR-responsive drug release and synergistic therapy. *Small* **12**, 6388–6397 (2016).
15. Díaz, M. R. & Vivas-Mejia, P. E. Nanoparticles as drug delivery systems in cancer medicine: Emphasis on RNAi-containing nanoliposomes. *Pharmaceuticals* **6**, 1361–1380 (2013).
16. Chatelut, E., Delord, J. P. & Canal, P. Toxicity patterns of cytotoxic drugs. *Invest. New Drugs* **21**, 141–148 (2003).

17. Abd El Razik, H. A. & Abdel Wahab, A. E. Full Paper Synthesis and Biological Evaluation of Some Novel Fused Pyrazolopyrimidines as Potential Anticancer and Antimicrobial Agents \*. *Arch Pharm Chem. Life Sci.* **11**, 184–196 (2011).
18. Zhao, X. *et al.* Functionalized graphene oxide nanoparticles for cancer cell- specific delivery of antitumor drug. *Bioconjug. Chem.* **26**, 128–136 (2015).
19. Zhao, X. *et al.* Biocompatible Graphene Oxide Nanoparticle-Based Drug Delivery Platform for Tumor Microenvironment-Responsive Triggered Release of Doxorubicin. *Langmuir* **30**, 10419–10429 (2014).
20. Gao, W., Liu, W., Christensen, T., Zalutsky, M. R. & Chilkoti, A. In situ growth of a PEG-like polymer from the C terminus of an intein fusion protein improves pharmacokinetics and tumor accumulation. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 16432–16437 (2010).
21. Park, J.-H. *et al.* Biodegradable luminescent porous silicon nanoparticles for in vivo applications. *Nat. Mater.* **8**, 331–336 (2009).
22. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646–674 (2011).
23. Cooper, G. M. & Hausman, R. E. *The cell: a molecular approach. Choice Reviews Online* vol. 35 (Sinauer Associates, 2000).
24. Bray, F., Laversanne, M., Weiderpass, E. & Soerjomataram, I. The ever-increasing importance of cancer as a leading cause of premature death worldwide. *Cancer* **127**, 3029–3030 (2021).
25. Sung, H. *et al.* Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA. Cancer J. Clin.* **71**, 209–249 (2021).
26. Siegel, R. L., Miller, K. D., Wagle, N. S. & Jemal, A. Cancer statistics, 2023. *CA. Cancer J. Clin.* **73**, 17–48 (2023).
27. American Cancer Society. Cancer Facts and Figures 2021. 1–67 (2021).
28. Abbas, Z. & Rehman, S. An Overview of Cancer Treatment Modalities. in *IntechOpen* (ed. Shahzad, H.) 1–21 (IntechOpen, 2016).
29. Wu, H.-C., Chang, D. & Huang, C. Targeted-therapy for cancer Related papers. *J. Cancer Mol.* **2**, 57–66 (2006).
30. Airley, R. *Cancer Chemotherapy*. (Wiley-Blackwell, 2009).
31. Fischer, D. S. *et al.* *Lippincott's Cancer Chemotherapy Handbook. Cancer Chemotherapy Handbook* (Lippincott Williams & Wilkins publishers, 2001).
32. Anand, U. *et al.* Cancer chemotherapy and beyond: Current status, drug candidates, associated risks and progress in targeted therapeutics. *Genes Dis.* **10**, 1367–1401 (2023).
33. Wong, R. S. Y. Apoptosis in cancer: From pathogenesis to treatment. *J. Exp. Clin. Cancer Res.* **30**, 1–14 (2011).
34. Basu, A. K. DNA damage, mutagenesis and cancer. *Int. J. Mol. Sci.* **19**, 1–13 (2018).

35. Sun, Y., Liu, Y., Ma, X. & Hu, H. The influence of cell cycle regulation on chemotherapy. *Int. J. Mol. Sci.* **22**, 1–25 (2021).
36. Parkinson, E. I. & Hergenrother, P. J. Deoxynyboquinones as NQO1-Activated Cancer Therapeutics. *Acc. Chem. Res.* **48**, 2715–2723 (2015).
37. Gudiol, C., Albasanz-Puig, A., Cuervo, G. & Carratalà, J. Understanding and Managing Sepsis in Patients With Cancer in the Era of Antimicrobial Resistance. *Front. Med.* **8**, (2021).
38. Raj, G. M. & Raveendran, R. *Introduction to basics of pharmacology and toxicology: Volume 1: General and molecular pharmacology: Principles of drug action*. Springer vol. 1 (Springer Nature Singapore, 2019).
39. Sakaue-Sawano, A., Kobayashi, T., Ohtawa, K. & Miyawaki, A. Drug-induced cell cycle modulation leading to cell-cycle arrest, nuclear mis-segregation, or endoreplication. *BMC Cell Biol.* **12**, 1–12 (2011).
40. Konstantinov, S. M. & Berger, M. R. Antimetabolites. in *Encyclopedia of Molecular Pharmacology* (eds. Offermanns, S. & Rosenthal, W.) 147–152 (Springer Berlin Heidelberg, 2008). doi:10.1007/978-3-540-38918-7\_220.
41. Weber, G. F. DNA Damaging Drugs. *Molecular Therapies of Cancer* 9–112 (2014) doi:10.1007/978-3-319-13278-5\_2.
42. Scholar, E. Alkylating Agents. in (eds. Enna, S. J. & Bylund, D. B. B. T. T. C. P. R.) 1–4 (Elsevier, 2007). doi:https://doi.org/10.1016/B978-008055232-3.61034-7.
43. Balduzzi, A. & Castiglione-Gertsch, M. Leukemia Risk after Adjuvant Treatment of Early Breast Cancer. *Women's Heal.* **1**, 73–85 (2005).
44. Sreerama, L. Alkylating agents. *Encycl. Cancer* **15**, 1–6 (2014).
45. Lu, J. J., Bao, J. L., Chen, X. P., Huang, M. & Wang, Y. T. Alkaloids isolated from natural herbs as the anticancer agents. *Evidence-based Complement. Altern. Med.* **2012**, 1–12 (2012).
46. Moudi, M., Go, R., Yien, C. Y. S. & Nazre, M. Vinca alkaloids. *Int. J. Prev. Med.* **4**, 1231–5 (2013).
47. Buzun, K., Bielawska, A., Bielawski, K. & Gornowicz, A. DNA topoisomerases as molecular targets for anticancer drugs. *J. Enzyme Inhib. Med. Chem.* **35**, 1781–1799 (2020).
48. Forterre, P., Gribaldo, S., Gadelle, D. & Serre, M. C. Origin and evolution of DNA topoisomerases. *Biochimie* **89**, 427–446 (2007).
49. Spakman, D. *et al.* Unravelling the mechanisms of Type 1A topoisomerases using single-molecule approaches. *Nucleic Acids Res.* **49**, 5470–5492 (2021).
50. Capitini, C. M., Otto, M., DeSantes, K. B. & Sondel, P. M. Immunotherapy in pediatric malignancies: current status and future perspectives. *Futur. Oncol.* **10**, 1659–1678 (2014).
51. Zitvogel, L., Apetoh, L., Ghiringhelli, F. & Kroemer, G. Immunological aspects of cancer chemotherapy. *Nat. Rev. Immunol.* **8**, 59–73 (2008).



52. Reang, P., Gupta, M. & Kohli, K. Biological response modifiers in cancer. *MedGenMed* **8**, 33 (2006).
53. Baskar, R., Lee, K. A., Yeo, R. & Yeoh, K. W. Cancer and radiation therapy: Current advances and future directions. *Int. J. Med. Sci.* **9**, 193–199 (2012).
54. Baskar, R., Dai, J., Wenlong, N., Yeo, R. & Yeoh, K. W. Biological response of cancer cells to radiation treatment. *Front. Mol. Biosci.* **1**, 1–9 (2014).
55. da Silva, M. S. DNA Double-Strand Breaks: A Double-Edged Sword for Trypanosomatids. *Front. Cell Dev. Biol.* **9**, 1–9 (2021).
56. Hall, N. & Rouge, B. The physics of proton therapy. *Phys. Med. Biol.* **60**, R155–R209 (2015).
57. Tuieng, R. J., Cartmell, S. H., Kirwan, C. C. & Sherratt, M. J. The effects of ionising and non-ionising electromagnetic radiation on extracellular matrix proteins. *Cells* **10**, 1–25 (2021).
58. Yu, X. *et al.* Optimization of Transmission X-ray Target for Intense Pulsed Electron Beam Accelerators. *Appl. Sci.* **12**, 1–15 (2022).
59. Gebauer, J., Higham, C., Langer, T., Denzer, C. & Brabant, G. Long-Term Endocrine and Metabolic Consequences of Cancer Treatment: A Systematic Review. *Endocr. Rev.* **40**, 711–767 (2018).
60. De Campos Vieira Abib, S. *et al.* International Society of Paediatric Surgical Oncology (IPSO) Surgical Practice Guidelines. *Ecancermedicalscience* **16**, 1–274 (2022).
61. Lordick, F. & Gockel, I. Chances, risks and limitations of neoadjuvant therapy in surgical oncology. *Innov. Surg. Sci.* **1**, 3–11 (2020).
62. Hiller-Sturmhöfel, S. & Bartke, A. The endocrine system. *Alcohol Heal. Res. World* **22**, 153–164 (1998).
63. Del Río, J. P. *et al.* Steroid Hormones and Their Action in Women's Brains: The Importance of Hormonal Balance. *Front. Public Heal.* **6**, 1–15 (2018).
64. Nair, B. C. & Vadlamudi, R. K. Regulation of hormonal therapy resistance by cell cycle machinery. *Gene Ther. Mol. Biol.* **12**, 395–404 (2008).
65. Ulm, M. *et al.* Therapeutic advances in hormone-dependent cancers: Focus on prostate, breast and ovarian cancers. *Endocr. Connect.* **8**, R10–R26 (2019).
66. Abraham, J. & Staffurth, J. Hormonal therapy for cancer. *Medicine (Baltimore)*. **44**, 30–33 (2016).
67. Santos, R. B., Lemos, C. & Saraiva, M. Gender-Affirming Hormone Therapy: Physical and Sociopsychological Effects, Impact and Satisfaction. *Cureus* **15**, 1–11 (2023).
68. Senapati, S., Mahanta, A. K., Kumar, S. & Maiti, P. Controlled drug delivery vehicles for cancer treatment and their performance. *Signal Transduct. Target. Ther.* **3**, 1–19 (2018).
69. Cheng, Z., Li, M., Dey, R. & Chen, Y. Nanomaterials for cancer therapy: current progress and perspectives. *J. Hematol. Oncol.* **14**, 1–27 (2021).

70. Debela, D. T. *et al.* New approaches and procedures for cancer treatment: Current perspectives. *SAGE Open Med.* **9**, 1–10 (2021).
71. Akhoun, N. Precision Medicine: A New Paradigm in Therapeutics. *Int. J. Prev. Med.* **12**, 1–7 (2021).
72. Pfeffer, C. M. & Singh, A. T. K. Apoptosis: A target for anticancer therapy. *Int. J. Mol. Sci.* **19**, 1–10 (2018).
73. Wang, J. C. Interaction between DNA and an Escherichia coli protein  $\omega$ . *J. Mol. Biol.* **55**, 523–533 (1971).
74. Bansal, S., Bajaj, P., Pandey, S. & Tandon, V. Topoisomerases: Resistance versus Sensitivity, How Far We Can Go? *Med. Chem. Res.* **00**, 1–34 (2016).
75. Rasheed, Z. A. & Rubin, E. H. Mechanisms of resistance to topoisomerase I-targeting drugs. *Oncogene* **22**, 7296–7304 (2003).
76. Pommier, Y., Leo, E., Zhang, H. & Marchand, C. DNA Topoisomerases and Their Poisoning by Anticancer and Antibacterial Drugs. *Chem. Biol.* **17**, 421–433 (2010).
77. Kathiravan, M. K., Khilare, M. M., Nikoomanesh, K., Chothe, A. S. & Jain, K. S. Topoisomerase as target for antibacterial and anticancer drug discovery. *J. Enzyme Inhib. Med. Chem.* **28**, 419–435 (2013).
78. Burden, D. A. & Osheroff, N. Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. *Biochim. Biophys. Acta - Gene Struct. Expr.* **1400**, 139–154 (1998).
79. Pommier, Y. Drugging Topoisomerases: Lessons and Challenges. (2013).
80. Bender, R. P. & Osheroff, N. DNA Topoisomerases as Targets for the Chemotherapeutic Treatment of Cancer. in *Checkpoint Responses in Cancer Therapy* (ed. Dai, W.) 57–91 (Humana Press, 2008). doi:10.1007/978-1-59745-274-8\_3.
81. Nitiss, J. L. Targeting DNA topoisomerase II in cancer chemotherapy. *Nat. Rev. Cancer* **9**, 338–350 (2009).
82. Nagaraja, V., Godbole, A. A., Henderson, S. R. & Maxwell, A. DNA topoisomerase I and DNA gyrase as targets for TB therapy. *Drug Discov. Today* **22**, 510–518 (2017).
83. Manzo, S. G. *et al.* DNA Topoisomerase I differentially modulates R-loops across the human genome. *DNA Topoisomerase I Differ. Modul. R-loops across Hum. genome* 257303 (2018) doi:10.1101/257303.
84. Baker, N. M., Rajan, R. & Mondragón, A. Structural studies of type I topoisomerases. *Nucleic Acids Res.* **37**, 693–701 (2009).
85. Viard, T. & de la Tour, C. B. Type IA topoisomerases: A simple puzzle? *Biochimie* **89**, 456–467 (2007).
86. Pommier, Y. Topoisomerase I inhibitors: Camptothecins and beyond. *Nat. Rev. Cancer* **6**, 789–802 (2006).
87. Nitiss, J. L. Targeting DNA topoisomerase II in cancer chemotherapy. *Nat. Rev. Cancer* **9**, 338–350 (2009).
88. Vann, K. R., Oviatt, A. A. & Osheroff, N. Topoisomerase II Poisons: Converting

- Essential Enzymes into Molecular Scissors. *Biochemistry* **60**, 1630–1641 (2021).
89. Wall, M. E., Wani, M. C., Cook, C. E. & Palmer, K. H. The Isolation and Structure of Camptothecin, a Novel Alkaloidal Leukemia and Tumor Inhibitor from *Camptotheca acuminata*. *J. Am. Chem. Soc.* **88**, 3888–3890 (1966).
  90. Wall, M. E. & Wani, M. C. Camptothecin and Taxol: discovery to clinic—Thirteenth Bruce F. Cain Memorial Award lecture. *Cancer Res.* **55**, 753–760 (1995).
  91. Li, F., Jiang, T., Li, Q. & Ling, X. Camptothecin analogues and their molecular targets. *Am. J. Cancer Res.* **7**, 2350–2394 (2017).
  92. Musiol, R. An overview of quinoline as a privileged scaffold in cancer drug discovery. *Expert Opin. Drug Discov.* **12**, 583–597 (2017).
  93. Wahid, M. & Bano, Q. Camptothecin and Its Analogs Antitumor Activity By Poisoning Topoisomerase I, Their Structureactivityrelationshipandclinicaldevelopment Perspectiveofanalogs. *Rev. Artic. CAMPTOTHECIN ITS Analog. ANTITUMOR Act. BY POISONING TOPOISOMERASE I* **386**, 286–295 (2014).
  94. Kingsbury, W. D. *et al.* Synthesis of water-soluble (aminoalkyl)camptothecin analogues: inhibition of topoisomerase I and antitumor activity. *J. Med. Chem.* **34**, 98–107 (1991).
  95. Ardizzoni, A. Camptothecin analogues in the treatment of non-small cell lung cancer. *Lung Cancer* **12 Suppl 1**, S177-85 (1995).
  96. Gounder, M. K. *et al.* Effects of drug efflux proteins and topoisomerase I mutations on the camptothecin analogue gimatecan. *Invest. New Drugs* **26**, 205–213 (2008).
  97. Murkovic, M. PHENOLIC COMPOUNDS. in *Encyclopedia of Food Sciences and Nutrition* 4507–4514 (Academic Press, 2003).
  98. Martinez, K. B., Mackert, J. D. & McIntosh, M. K. *Polyphenols and Intestinal Health. Nutrition and Functional Foods for Healthy Aging* (Elsevier Inc., 2017). doi:10.1016/B978-0-12-805376-8.00018-6.
  99. Khadem, S. & Marles, R. J. Monocyclic phenolic acids; hydroxy- and polyhydroxybenzoic acids: Occurrence and recent bioactivity studies. *Molecules* **15**, 7985–8005 (2010).
  100. Kumar, P. *et al.* Phytochemical constituents, antimicrobial and antioxidant activities of Kumaun Himalayan Hoop Pine bark extract. *Nat. Prod. Res.* **0**, 1–5 (2020).
  101. Ohlan, R., Narasimhan, B., Ohlan, S., Narang, R. & Judge, V. Synthesis and antimicrobial evaluation of urea inclusion complexes. *Org. Commun.* **1**, 24–32 (2008).
  102. Koksai, Z., Alim, Z., Beydemir, S. & Ozdemir, H. Potent Inhibitory Effects of Some Phenolic Acids on Lactoperoxidase. *J. Biochem. Mol. Toxicol.* **30**, 533–538 (2016).
  103. Aslan, H. E. & Beydemir. Phenolic compounds: The inhibition effect on polyol pathway enzymes. *Chem. Biol. Interact.* **266**, 47–55 (2017).
  104. Kalinowska, M. *et al.* Plant-Derived and Dietary Hydroxybenzoic Acids—A Comprehensive Study of Structural, Anti-/Pro-Oxidant, Lipophilic, Antimicrobial, and Cytotoxic Activity in MDA-MB-231 and MCF-7 Cell Lines. *Nutrients* **13**, 1–33 (2021).

105. Liu, C. *et al.* 3,5-Dihydroxybenzoic acid, a specific agonist for hydroxycarboxylic acid 1, inhibits lipolysis in adipocytes. *J. Pharmacol. Exp. Ther.* **341**, 794–801 (2012).
106. Sultana, N., Akhter, M. & Khatoon, Z. Nematicidal natural products from the aerial parts of *Rubus niveus*. *Nat. Prod. Res.* **24**, 407–415 (2010).
107. Kahkeshani, N. *et al.* Pharmacological effects of gallic acid in health and disease: A mechanistic review. *Iran. J. Basic Med. Sci.* **22**, 225–237 (2019).
108. Subramanian, A. P. *et al.* Gallic acid: Prospects and molecular mechanisms of its anticancer activity. *RSC Adv.* **5**, 35608–35621 (2015).
109. Borges, A., Ferreira, C., Saavedra, M. J. & Simões, M. Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. *Microb. Drug Resist.* **19**, 256–265 (2013).
110. Sorrentino, E. *et al.* Antimicrobial activity of gallic acid against food-related *Pseudomonas* strains and its use as biocontrol tool to improve the shelf life of fresh black truffles. *Int. J. Food Microbiol.* **266**, 183–189 (2018).
111. Sarjit, A., Wang, Y. & Dykes, G. A. Antimicrobial activity of gallic acid against thermophilic *Campylobacter* is strain specific and associated with a loss of calcium ions. *Food Microbiol.* **46**, 227–233 (2015).
112. Li, K., Guan, G., Zhu, J., Wu, H. & Sun, Q. Antibacterial activity and mechanism of a laccase-catalyzed chitosan–gallic acid derivative against *Escherichia coli* and *Staphylococcus aureus*. *Food Control* **96**, 234–243 (2019).
113. Kang, M. S., Oh, J. S., Kang, I. C., Hong, S. J. & Choi, C. H. Inhibitory effect of methyl gallate and gallic acid on oral bacteria. *J. Microbiol.* **46**, 744–750 (2008).
114. Shao, D. *et al.* Inhibition of Gallic Acid on the Growth and Biofilm Formation of *Escherichia coli* and *Streptococcus mutans*. *J. Food Sci.* **80**, M1299–M1305 (2015).
115. Borges, A., Saavedra, M. J. & Simões, M. The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. *Biofouling* **28**, 755–767 (2012).
116. Ohno, Y. *et al.* Induction of apoptosis by gallic acid in lung cancer cells. *Anticancer. Drugs* **10**, 845–851 (1999).
117. Devi, R. & gill, sandeep. A squared bossed diaphragm piezoresistive pressure sensor based on CNTs for low pressure range with enhanced sensitivity. *Microsyst. Technol.* **27**, 1–9 (2021).
118. Devi, Y. P., Uma, A., Narasu, M. L. & Kalyani, C. Anticancer activity of gallic acid on cancer cell lines, HCT15 AND MDA MB 231. *Int. J. Res. Applied, Nat. Soc. Sci.* **2**, 269–272 (2014).
119. Chandramohan Reddy, T. *et al.* Anti-leukemic effects of gallic acid on human leukemia K562 cells: Downregulation of COX-2, inhibition of BCR/ABL kinase and NF- $\kappa$ B inactivation. *Toxicol. Vitro.* **26**, 396–405 (2012).
120. Liu, Z., Li, D., Yu, L. & Niu, F. Gallic acid as a cancer-selective agent induces apoptosis in pancreatic cancer cells. *Chemotherapy* **58**, 185–194 (2012).
121. Variya, B. C., Bakrania, A. K. & Patel, S. S. Antidiabetic potential of gallic acid from *Emblica officinalis*: Improved glucose transporters and insulin sensitivity through

- PPAR- $\gamma$  and Akt signaling. *Phytomedicine* **73**, 1–11 (2020).
122. Li, Y. *et al.* The anti-inflammatory effect of feiyangchangweiyuan capsule and its main components on pelvic inflammatory disease in rats via the regulation of the NF- $\kappa$ B and BAX/BCL-2 Pathway. *Evidence-based Complement. Altern. Med.* **2019**, (2019).
  123. De Figueiredo, R. M., Suppo, J. S. & Campagne, J. M. Nonclassical Routes for Amide Bond Formation. *Chem. Rev.* **116**, 12029–12122 (2016).
  124. Jiang, Y. Y., Zhu, L., Liang, Y., Man, X. & Bi, S. Mechanism of Amide Bond Formation from Carboxylic Acids and Amines Promoted by 9-Silafluorenyl Dichloride Derivatives. *J. Org. Chem.* **82**, 9087–9096 (2017).
  125. Carey, J. S., Laffan, D., Thomson, C. & Williams, M. T. Analysis of the reactions used for the preparation of drug candidate molecules. *Org. Biomol. Chem.* **4**, 2337–2347 (2006).
  126. Chen, L. *et al.* Effect of alkylglycerone phosphate synthase on the expression levels of lncRNAs in glioma cells and its functional prediction. *Oncol. Lett.* **20**, 1–15 (2020).
  127. Chen, L. *et al.* Amide derivatives of Gallic acid: Design, synthesis and evaluation of inhibitory activities against in vitro  $\alpha$ -synuclein aggregation. *Bioorganic Med. Chem.* **28**, (2020).
  128. Mahdavi, B., Hosseyni-Tabar, S. M., Rezaei-Seresht, E., Rezaei-Seresht, H. & Falanji, F. Synthesis and biological evaluation of novel hybrid compounds derived from gallic acid and the 2-aminothiophene derivatives. *J. Iran. Chem. Soc.* **17**, 809–815 (2020).
  129. Manuja, R., Sachdeva, S., Jain, A. & Chaudhary, J. A comprehensive review on biological activities of P-hydroxy benzoic acid and its derivatives. *Int. J. Pharm. Sci. Rev. Res.* **22**, 109–115 (2013).
  130. Qin, W. Use of hydroxybenzoic acid ester compounds for the manufacture of medicament for the prevention and treatment of HPV infection. vol. 24 1–12 (2008).
  131. Figueroa-Espinoza, M.-C. & Villeneuve, P. Phenolic Acids Enzymatic Lipophilization. *J. Agric. Food Chem.* **53**, 2779–2787 (2005).
  132. Zieniuk, B., Wołoszynowska, M., Białecka-Florjańczyk, E. & Fabiszewska, A. Synthesis of industrially useful phenolic compounds esters by means of biocatalysts obtained along with waste fish oil utilization. *Sustainability* **12**, 1–18 (2020).
  133. Białecka-Florjańczyk, E., Fabiszewska, A. & Zieniuk, B. Phenolic Acids Derivatives - Biotechnological Methods of Synthesis and Bioactivity. *Current Pharmaceutical Biotechnology* vol. 19 1098–1113 (2018).
  134. Roby, M. H. H. Synthesis and characterization of phenolic lipids. in *Phenolic compounds - Natural Sources, Importance and Applications* vol. 32 90–116 (2017).
  135. Dofe, V. S., Sarkate, A. P., Kathwate, S. H. & Gill, C. H. Synthesis, antimicrobial activity and anti-biofilm activity of novel tetrazole derivatives. *Heterocycl. Commun.* **23**, 325–330 (2017).
  136. Balloux, F. & van Dorp, L. Q&A: What are pathogens, and what have they done to and for us? *BMC Biol.* **15**, 4–9 (2017).
  137. Revelas, A. Healthcare - associated infections: A public health problem. *Niger. Med. J.*

- 53, 59–64 (2012).
138. Bhatt, K. *et al.* High mortality co-infections of COVID-19 patients: mucormycosis and other fungal infections. *Discoveries* **9**, 1–12 (2021).
  139. Mevik, B.-H. & Wehrens, R. Invasive Candidiasis. *N. Engl. J. Med.* **373**, 1445–1456 (2015).
  140. Pappas, P. G., Lionakis, M. S., Arendrup, M. C., Ostrosky-Zeichner, L. & Kullberg, B. J. Invasive candidiasis. *Nat. Rev. Dis. Prim.* **4**, 1–20 (2018).
  141. Pappas, P. G. *et al.* A prospective observational study of candidemia: Epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. *Clin. Infect. Dis.* **37**, 634–643 (2003).
  142. Magill, S. S. *et al.* Multistate Point-Prevalence Survey of Health Care–Associated Infections for the Emerging Infections Program Healthcare-Associated Infections and Antimicrobial Use Prevalence Survey Team \* Centers for Disease Control and Prevention (. St. Paul (R.L.); Connect. Dep. Public Heal. Oakl. (J.N. Decatur (S.M.R **370**, 1198–1208 (2014).
  143. McCarty, T. P. & Pappas, P. G. Invasive Candidiasis. *Infect. Dis. Clin. North Am.* **30**, 103–124 (2016).
  144. Lowy, F. D. Staphylococcus aureus Infections. *N. Engl. J. Med.* **339**, 520–532 (1998).
  145. Tong, S. Y. C., Davis, J. S., Eichenberger, E., Holland, T. L. & Fowler, V. G. Staphylococcus aureus infections: Epidemiology, pathophysiology, clinical manifestations, and management. *Clin. Microbiol. Rev.* **28**, 603–661 (2015).
  146. DeLeo, F. R., Diep, B. A. & Otto, M. Host Defense and Pathogenesis in Staphylococcus aureus Infections. *Infect. Dis. Clin. North Am.* **23**, 17–34 (2009).
  147. Kaper, J. B., Nataro, J. P. & Mobley, H. L. T. Pathogenic Escherichia coli. *Nat. Rev. Microbiol.* **2**, 123–140 (2004).
  148. Braz, V. S., Melchior, K. & Moreira, C. G. Escherichia coli as a Multifaceted Pathogenic and Versatile Bacterium. *Front. Cell. Infect. Microbiol.* **10**, 1–9 (2020).
  149. Islam, M. A., Kabir, S. M. L. & Rahman, M. T. Molecular Detection and Characterization of Staphylococcus Aureus Isolated From Raw Milk Sold in Different Markets of Bangladesh. *Bangladesh J. Vet. Med.* **14**, 277–282 (2017).
  150. Diggle, S. P. & Whiteley, M. Microbe profile: Pseudomonas aeruginosa: Opportunistic pathogen and lab rat. *Microbiol. (United Kingdom)* **166**, 30–33 (2020).
  151. Tuon, F. F., Dantas, L. R., Suss, P. H. & Tasca Ribeiro, V. S. Pathogenesis of the Pseudomonas aeruginosa Biofilm: A Review. *Pathogens* **11**, (2022).
  152. Stover, C. K. *et al.* Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature* **406**, 959–964 (2000).
  153. Reynolds, D. & Kollef, M. The Epidemiology and Pathogenesis and Treatment of Pseudomonas aeruginosa Infections: An Update. *Drugs* **81**, 2117–2131 (2021).
  154. Wu, D. C., Chan, W. W., Metelitsa, A. J., Fiorillo, L. & Lin, A. N. Pseudomonas skin infection: Clinical Features, Epidemiology, and Management. *Am. J. Clin. Dermatol.*

- 12, 157–169 (2011).
155. Castro, S. A. & Dorfmueller, H. C. A brief review on Group A Streptococcus pathogenesis and vaccine development. *R. Soc. Open Sci.* **8**, 1–17 (2021).
  156. Avire, N. J., Whiley, H. & Ross, K. A review of streptococcus pyogenes: Public health risk factors, prevention and control. *Pathogens* **10**, 1–18 (2021).
  157. Ramla, S., Sharma, V. & Patel, M. Influence of cancer treatment on the Candida albicans isolated from the oral cavities of cancer patients. *Support. Care Cancer* **24**, 2429–2436 (2016).
  158. Bensadoun, R. J., Patton, L. L., Lalla, R. V. & Epstein, J. B. Oropharyngeal candidiasis in head and neck cancer patients treated with radiation: Update 2011. *Support. Care Cancer* **19**, 737–744 (2011).
  159. Mikulska, M. *et al.* Aetiology and resistance in bacteraemias among adult and paediatric haematology and cancer patients. *J. Infect.* **68**, 321–331 (2014).
  160. Holland, T., Fowler, V. G. & Shelburne, S. A. Invasive gram-positive bacterial infection in cancer patients. *Clin. Infect. Dis.* **59**, 331–334 (2014).
  161. Bhat, S., Muthunatarajan, S., Mulki, S. S., Archana Bhat, K. & Kotian, K. H. Bacterial Infection among Cancer Patients: Analysis of Isolates and Antibiotic Sensitivity Pattern. *Int. J. Microbiol.* **2021**, 1–7 (2021).
  162. Nogami, Y. *et al.* Case of streptococcal toxic shock syndrome caused by rapidly progressive group A hemolytic streptococcal infection during postoperative chemotherapy for cervical cancer. *J. Obstet. Gynaecol. Res.* **40**, 250–254 (2014).
  163. Saghir, S., Faiz, M., Saleem, M., Younus, A. & Aziz, H. Characterization and antimicrobial susceptibility of gram-negative bacteria isolated from cancer patients on chemotherapy in Pakistan. *Indian J. Med. Microbiol.* **27**, 341–347 (2009).
  164. Morita, F. *et al.* The first case of pseudomonas aeruginosa bacteremic pneumonia in a cancer patient receiving pegfilgrastim. *Intern. Med.* **56**, 2039–2042 (2017).
  165. Bai, C. *et al.* Clinical Analysis of Bloodstream Infection of Escherichia coli in Patients with Pancreatic Cancer from 2011 to 2019. *Can. J. Infect. Dis. Med. Microbiol.* **2022**, 1–8 (2022).
  166. Pappas, P. G. *et al.* Clinical Practice Guidelines for the Management of Candidiasis: 2009 Update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **48**, 503–535 (2009).
  167. Hollier, L. M. & Cox, S. M. Fluconazole (Diflucan®). *Infect. Dis. Obstet. Gynecol.* **3**, 222–225 (1995).
  168. Forastiero, A. *et al.* Candida tropicalis antifungal cross-resistance is related to different azole target (Erg11p) modifications. *Antimicrob. Agents Chemother.* **57**, 4769–4781 (2013).
  169. Baginski, M. & Czub, J. Amphotericin B and Its New Derivatives – Mode of Action. *Curr. Drug Metab.* **10**, 459–469 (2009).
  170. Qin, J., Yang, H., Shan, Z., Jiang, L. & Zhang, Q. Clinical efficacy and safety of antifungal drugs for the treatment of Candida parapsilosis infections: A systematic

- review and network meta-analysis. *J. Med. Microbiol.* **70**, 1–8 (2021).
171. Lotfali, E. *et al.* Antifungal susceptibility analysis of clinical isolates of candida parapsilosis in Iran. *Iran. J. Public Health* **45**, 322–328 (2016).
  172. Baran, A., Kwiatkowska, A. & Potocki, L. Antibiotics and Bacterial Resistance—A Short Story of an Endless Arms Race. *Int. J. Mol. Sci.* **24**, 1–34 (2023).
  173. Gao, Y. *et al.* Antibiotics for cancer treatment: A double-edged sword. *J. Cancer* **11**, 5135–5149 (2020).
  174. Zaman, S. Bin *et al.* A Review on Antibiotic Resistance: Alarm Bells are Ringing. *Cureus* **9**, 1–9 (2017).
  175. Khatkar, A., Nanda, A., Kumar, P. & Narasimhan, B. Synthesis, antimicrobial evaluation and QSAR studies of gallic acid derivatives. *Arab. J. Chem.* **10**, S2870–S2880 (2017).
  176. Sherin, L., Shujaat, S., Sohail, A. & Arif, F. Synthesis and biological evaluation of novel gallic acid analogues as potential antimicrobial and antioxidant agents. *Croat. Chem. Acta* **91**, 551–565 (2018).
  177. Voet, A. R. D., Kumar, A., Berenger, F. & Zhang, K. Y. J. Combining in silico and in cerebro approaches for virtual screening and pose prediction in SAMPL4. *J. Comput. Aided. Mol. Des.* **28**, 363–373 (2014).
  178. Heikamp, K. & Bajorath, J. The Future of Virtual Compound Screening. *Chem. Biol. Drug Des.* **81**, 33–40 (2013).
  179. Ou-Yang, S. S. *et al.* Computational drug discovery. *Acta Pharmacol. Sin.* **33**, 1131–1140 (2012).
  180. Donoso, L. A. Financial anatomy of biomedical research: Commentary. *JAMA* **294**, 1333–1342 (2005).
  181. Lahana, R. How many leads from HTS? *Drug Discov. Today* **4**, 447–448 (1999).
  182. Lobanov, V. Using artificial neural networks to drive virtual screening of combinatorial libraries. *Drug Discov. Today BIOSILICO* **2**, 149–156 (2004).
  183. Shekhar, C. In Silico Pharmacology: Computer-Aided Methods Could Transform Drug Development. *Chem. Biol.* **15**, 413–414 (2008).
  184. Song, C. M., Lim, S. J. & Tong, J. C. Recent advances in computer-aided drug design. *Brief. Bioinform.* **10**, 579–591 (2009).
  185. Zhang, S. Computer-aided drug discovery and development. *Methods Mol. Biol.* **716**, 23–38 (2011).
  186. Xiang, M., Cao, Y., Fan, W., Chen, L. & Mo, Y. Computer-Aided Drug Design: Lead Discovery and Optimization. *Comb. Chem. High Throughput Screen.* **15**, 328–337 (2012).
  187. Jorgensen, W. L. The Many Roles of Computation in Drug Discovery. *Science (80-. )*. **303**, 1813–1818 (2004).
  188. Tang, Y., Zhu, W., Chen, K. & Jiang, H. New technologies in computer-aided drug design: Toward target identification and new chemical entity discovery. *Drug Discov.*



- Today Technol.* **3**, 307–313 (2006).
189. Chen, L. *et al.* From Laptop to Benchtop to Bedside: Structure-based Drug Design on Protein Targets. *Curr. Drug Metab.* **18**, 1217–1239 (2012).
  190. Acharya, C., Coop, A., E. Polli, J. & D. MacKerell, A. Recent Advances in Ligand-Based Drug Design: Relevance and Utility of the Conformationally Sampled Pharmacophore Approach. *Curr. Comput. Aided-Drug Des.* **7**, 10–22 (2010).
  191. Wang, F. *et al.* Computational screening for active compounds targeting protein sequences: Methodology and experimental validation. *J. Chem. Inf. Model.* **51**, 2821–2828 (2011).
  192. Lu, W., Zhang, R., Jiang, H., Zhang, H. & Luo, C. Computer-aided drug design in epigenetics. *Front. Chem.* **6**, 1–23 (2018).
  193. Rognan, D. Chemogenomic approaches to rational drug design. *Br. J. Pharmacol.* **152**, 38–52 (2007).
  194. Chen, X. W. & Jeong, J. C. Sequence-based prediction of protein interaction sites with an integrative method. *Bioinformatics* **25**, 585–591 (2009).
  195. Gallet, X., Charleatoux, B., Thomas, A. & Brasseur, R. A fast method to predict protein interaction sites from sequences. *J. Mol. Biol.* **302**, 917–926 (2000).
  196. Bhalani, D. V., Nutan, B., Kumar, A. & Singh Chandel, A. K. Bioavailability Enhancement Techniques for Poorly Aqueous Soluble Drugs and Therapeutics. *Biomedicines* **10**, 1–33 (2022).
  197. Allen, T. M. & Cullis, P. R. Drug Delivery Systems: Entering the Mainstream. *Science (80-. )*. **303**, 1818–1822 (2004).
  198. Langer, R. Drug delivery and targeting. *Nature* **392**, 5–10 (1998).
  199. Chien, Y. *Novel drug delivery systems*. CRC Press vol. 1 103 (1991).
  200. Vargason, A. M., Anselmo, A. C. & Mitragotri, S. The evolution of commercial drug delivery technologies. *Nat. Biomed. Eng.* **5**, 951–967 (2021).
  201. Gidal, B. E. *et al.* Gabapentin bioavailability: Effect of dose and frequency of administration in adult patients with epilepsy. *Epilepsy Res.* **31**, 91–99 (1998).
  202. Serajuddin, A. T. M. Solid dispersion of poorly water-soluble drugs: Early promises, subsequent problems, and recent breakthroughs. *J. Pharm. Sci.* **88**, 1058–1066 (1999).
  203. Patra, J. K. *et al.* Nano based drug delivery systems: Recent developments and future prospects. *J. Nanobiotechnology* **16**, 1–33 (2018).
  204. Jahangirian, H., Lemraski, E. G., Webster, T. J., Rafiee-Moghaddam, R. & Abdollahi, Y. A review of drug delivery systems based on nanotechnology and green chemistry: Green nanomedicine. *Int. J. Nanomedicine* **12**, 2957–2978 (2017).
  205. Watkins, R., Wu, L., Zhang, C., Davis, R. M. & Xu, B. Natural product-based nanomedicine: Recent advances and issues. *Int. J. Nanomedicine* **10**, 6055–6074 (2015).
  206. Liu, Z., Tabakman, S., Welsher, K. & Dai, H. Carbon nanotubes in biology and medicine: In vitro and in vivo detection, imaging and drug delivery. *Nano Res.* **2**, 85–

- 120 (2009).
207. Orive, G., Gascón, A. R., Hernández, R. M., Domínguez-Gil, A. & Pedraz, J. L. Techniques: New approaches to the delivery of biopharmaceuticals. *Trends Pharmacol. Sci.* **25**, 382–387 (2004).
  208. De Jong, W. H. & Borm, P. J. a. Drug delivery and nanoparticles: applications and hazards. *Int. J. Nanomedicine* **3**, 133–149 (2008).
  209. Wang, Y. *et al.* The influence of nanoparticle properties on oral bioavailability of drugs. *Int. J. Nanomedicine* **15**, 6295–6310 (2020).
  210. Emeje, M. O., Obidike, I. C., Akpabio, E. I. & Ofoefule, S. I. Nanotechnology in Drug Delivery. in *Recent Advances in Novel Drug Carriers Systems* 69–106 (IntechOpen, 2012).
  211. Bayda, S., Adeel, M., Tuccinardi, T., Cordani, M. & Rizzolio, F. The history of nanoscience and nanotechnology: From chemical-physical applications to nanomedicine. *Molecules* **25**, 1–15 (2020).
  212. Khan, I., Saeed, K. & Khan, I. Nanoparticles: Properties, applications and toxicities. *Arab. J. Chem.* **12**, 908–931 (2019).
  213. Shi, J., Votruba, A. R., Farokhzad, O. C. & Langer, R. Nanotechnology in Drug Delivery and Tissue Engineering: From Discovery to Applications Jinjun. *Nano Lett.* **10**, 3223–3230 (2010).
  214. Soppimath, K. S. K., Aminabhavi, T. M. T. M., Kulkarni, A. R. A. R. & Rudzinski, W. E. Biodegradable polymeric nanoparticles as drug delivery devices. *J. Control. Release* **70**, 1–20 (2001).
  215. Agustin, C. G., Carlos, G. D. & Alberto, E. *Carbon-based nanomaterials in analytical chemistry*. (The Royal Society of Chemistry, 2019). doi:10.1039/9781788012751-fp001.
  216. Yan, H. *et al.* Toxicity of carbon nanotubes as anti-tumor drug carriers. *Int. J. Nanomedicine* **14**, 10179–10194 (2019).
  217. Gholamine, B., Karimi, I., Salimi, A., Mazdarani, P. & Becker, L. A. Neurobehavioral toxicity of carbon nanotubes in mice: Focus on brain-derived neurotrophic factor mRNA and protein. *Toxicol. Ind. Health* **33**, 340–350 (2017).
  218. Singh, B., Lohan, S., Sandhu, P. S., Jain, A. & Mehta, S. K. Functionalized carbon nanotubes and their promising applications in therapeutics and diagnostics. *Nanobiomaterials Med. Imaging* **4**, 455–478 (2016).
  219. Wong, B. S. *et al.* Carbon nanotubes for delivery of small molecule drugs. *Adv. Drug Deliv. Rev.* **65**, 1964–2015 (2013).
  220. Sharma, S., Parveen, R. & Chatterji, B. P. Toxicology of Nanoparticles in Drug Delivery. *Curr. Pathobiol. Rep.* **9**, 133–144 (2021).
  221. Park, E. J. *et al.* Subchronic immunotoxicity and screening of reproductive toxicity and developmental immunotoxicity following single instillation of HIPCO-single-walled carbon nanotubes: purity-based comparison. *Nanotoxicology* **10**, 1188–1202 (2016).
  222. Lee, S., Khang, D. & Kim, S. H. High dispersity of carbon nanotubes diminishes

- immunotoxicity in spleen. *Int. J. Nanomedicine* **10**, 2697–2710 (2015).
223. Bottini, M. *et al.* Multi-walled carbon nanotubes induce T lymphocyte apoptosis. *Toxicol. Lett.* **160**, 121–126 (2006).
  224. Larner, S. F. *et al.* In vitro neurotoxicity resulting from exposure of cultured neural cells to several types of nanoparticles. *J. Cell Death* **10**, 8–14 (2017).
  225. Aragon, M. J. *et al.* Serum-borne bioactivity caused by pulmonary multiwalled carbon nanotubes induces neuroinflammation via blood-brain barrier impairment. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E1968–E1976 (2017).
  226. Chen, H. *et al.* Single-walled carbon nanotubes modulate pulmonary immune responses and increase pandemic influenza A virus titers in mice. *Viol. J.* **14**, 1–15 (2017).
  227. Pourmand, A. & Abdollahi, M. Current opinion on nanotoxicology. *DARU, J. Pharm. Sci.* **20**, 1–3 (2012).
  228. Bahadar, H., Maqbool, F., Niaz, K. & Abdollahi, M. Toxicity of nanoparticles and an overview of current experimental models. *Iran. Biomed. J.* **20**, 1–11 (2016).
  229. Navya, P. N. & Daima, H. K. Rational engineering of physicochemical properties of nanomaterials for biomedical applications with nanotoxicological perspectives. *Nano Converg.* **3**, 1–14 (2016).
  230. Begines, B. *et al.* Polymeric nanoparticles for drug delivery: Recent developments and future prospects. *Nanomaterials* **10**, 1–41 (2020).
  231. Thang, N. H., Chien, T. B. & Cuong, D. X. Polymer-Based Hydrogels Applied in Drug Delivery: An Overview. *Gels* **9**, 1–38 (2023).
  232. Bamrungsap, S. *et al.* Nanotechnology in therapeutics: a focus on nanoparticles as a drug delivery system. *Nanomedicine* **7**, 1253–1271 (2012).
  233. Elsabahy, M. & Wooley, K. L. Design of polymeric nanoparticles for biomedical delivery applications. *Chem. Soc. Rev.* **41**, 2545–2561 (2012).
  234. Sridhar, R. & Ramakrishna, S. Electrosprayed nanoparticles for drug delivery and pharmaceutical applications. *Biomatter* **3**, e24281-12 (2013).
  235. Kulkarni, S. A. & Feng, S.-S. Effects of Particle Size and Surface Modification on Cellular Uptake and Biodistribution of Polymeric Nanoparticles for Drug Delivery. *Pharm. Res.* **30**, 2512–2522 (2013).
  236. He, C., Hu, Y., Yin, L., Tang, C. & Yin, C. Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. *Biomaterials* **31**, 3657–3666 (2010).
  237. Banik, B. L., Fattahi, P. & Brown, J. L. Polymeric nanoparticles: The future of nanomedicine. *Wiley Interdiscip. Rev. Nanomedicine Nanobiotechnology* **8**, 271–299 (2016).
  238. Srividya, B., Cardoza, R. M. & Amin, P. D. Sustained ophthalmic delivery of ofloxacin from a pH triggered in situ gelling system. *J. Control. Release* **73**, 205–211 (2001).

239. Yang, Y., Wang, Z., Peng, Y., Ding, J. & Zhou, W. A smart pH-sensitive delivery system for enhanced anticancer efficacy via paclitaxel endosomal escape. *Front. Pharmacol.* **9**, 1–11 (2019).
240. Jin, X. *et al.* Dual temperature- and pH-responsive ibuprofen delivery from poly(N-isopropylacrylamide-co-acrylic acid) nanoparticles and their fractal features. *Polym. Bull.* **74**, 3619–3638 (2017).
241. Shinn, J., Kwon, N., Lee, S. A. & Lee, Y. Smart pH-responsive nanomedicines for disease therapy. *J. Pharm. Investig.* **52**, 427–441 (2022).
242. Chen, S., Jiang, X. & Sun, L. Reaction mechanisms of n-isopropylacrylamide soap-free emulsion polymerization based on two different initiators. *J. Macromol. Sci. Part A Pure Appl. Chem.* **51**, 447–455 (2014).
243. Do Nascimento Marques, N., Da Silva Maia, A. M. & De Carvalho Balaban, R. Development of dual-sensitive smart polymers by grafting chitosan with poly (N-isopropylacrylamide): an overview. *Polimeros* **25**, 237–246 (2015).
244. Johnson, R. P. *et al.* Dual Stimuli-Responsive Poly ( N - isopropylacrylamide ) - b - poly ( L - histidine ) Chimeric Materials for the Controlled Delivery of Doxorubicin into Liver Carcinoma. *Biomacromolecules* **14**, 1434–1443 (2013).
245. Rwei, S. P., Tuan, H. N. A., Chiang, W. Y. & Way, T. F. Synthesis and characterization of pH and thermo dual-responsive hydrogels with a semi-IPN structure based on N-isopropylacrylamide and itaconamic acid. *Materials (Basel)*. **11**, 1–21 (2018).
246. Gao, X. *et al.* pH- and thermo-responsive poly(N-isopropylacrylamide- co-acrylic acid derivative) copolymers and hydrogels with LCST dependent on pH and alkyl side groups. *J. Mater. Chem. B* **1**, 5578–5587 (2013).
247. Lanzalaco, S. & Armelin, E. Poly(N-isopropylacrylamide) and copolymers: A review on recent progresses in biomedical applications. *Gels* **3**, 1–31 (2017).
248. Aseyev, V., Tenhu, H. & Winnik, F. M. Non-ionic thermoresponsive polymers in water. *Adv. Polym. Sci.* **242**, 29–89 (2011).
249. Yan, H. & Tsujii, K. Potential application of poly(N-isopropylacrylamide) gel containing polymeric micelles to drug delivery systems. *Colloids Surfaces B Biointerfaces* **46**, 142–146 (2005).
250. Chung, J. E. *et al.* Thermo-responsive drug delivery from polymeric micelles constructed using block copolymers of poly(N-isopropylacrylamide) and poly(butylmethacrylate). *J. Control. Release* **62**, 115–127 (1999).
251. Brogi, S., Ramalho, T. C., Kuca, K., Medina-Franco, J. L. & Valko, M. Editorial: In silico Methods for Drug Design and Discovery. *Front. Chem.* **8**, 1–5 (2020).
252. Shaker, B., Ahmad, S., Lee, J., Jung, C. & Na, D. In silico methods and tools for drug discovery. *Comput. Biol. Med.* **137**, 1–15 (2021).
253. Paul, S. M. *et al.* How to improve RD productivity: The pharmaceutical industry's grand challenge. *Nat. Rev. Drug Discov.* **9**, 203–214 (2010).
254. Huh, D., Hamilton, G. A. & Ingber, D. E. From 3D cell culture to organs-on-chips.

- Trends Cell Biol.* **21**, 745–754 (2011).
255. Ismail, M. A. H., Tratat, C. & Haroun, M. G. Molecular modelling design , synthesis and potential topoisomerase-I inhibitory activity cytotoxic evaluation of certain substituted 2- ( galloylamino ) benzo [ d ] thiazole derivatives having potential topoisomerase-I inhibitory activity. *J. Enzyme Inhib. Med. Chem.* **28**, 1331–1345 (2013).
  256. Champoux, J. J. DNA topoisomerases: structure, function, and mechanism. *Annu. Rev. Biochem.* **70**, 369–413 (2001).
  257. Jain, C. K., Majumder, H. K. & Roychoudhury, S. Natural Compounds as Anticancer Agents Targeting DNA Topoisomerases. *Curr. Genomics* **18**, 75–92 (2017).
  258. Wang, J. C. Cellular roles of DNA topoisomerases: A molecular perspective. *Nat. Rev. Mol. Cell Biol.* **3**, 430–440 (2002).
  259. Ghilarov, D. A. & Shkundina, I. S. DNA topoisomerases and their functions in a cell. *Mol. Biol.* **46**, 47–57 (2012).
  260. Baikar, S. & Malpathak, N. Secondary metabolites as DNA topoisomerase inhibitors: A new era towards designing of anticancer drugs. *Pharmacogn. Rev.* **4**, 12–26 (2010).
  261. Tse-Dinh, Y. C. Targeting bacterial topoisomerase I to meet the challenge of finding new antibiotics. *Futur. Med Chem* **7**, 459–471 (2015).
  262. Hevener, K. E., Verstak, T. A., Lutat, K. E., Riggsbee, D. L. & Mooney, J. W. Recent developments in topoisomerase-targeted cancer chemotherapy. **8**, 844–861 (2018).
  263. Chen, A. Y., Chen, P. M. T. & Chen, Y. J. DNA topoisomerase I drugs and radiotherapy for lung cancer. *J. Thorac. Dis.* **4**, 390–397 (2012).
  264. Slichenmyer, W. J., Rowinsky, E. K., Donehower, R. C. & Kaufmann, S. H. The Current Status of Camptothecin Analogues as Antitumor Agents. *J. Natl. Cancer Inst.* **85**, 271–291 (1993).
  265. Polgar, O., Robey, R. W. & Bates, S. E. ABCG2: Structure, function and role in drug response. *Expert Opin. Drug Metab. Toxicol.* **4**, 1–15 (2008).
  266. Ferreira, R. J., Bonito, C. A., Cordeiro, M. N. D. S., Ferreira, M. J. U. & Dos Santos, D. J. V. A. Structure-function relationships in ABCG2: Insights from molecular dynamics simulations and molecular docking studies. *Sci. Rep.* **7**, 1–17 (2017).
  267. Nobili, S. *et al.* Role of ATP-binding cassette transporters in cancer initiation and progression. *Semin. Cancer Biol.* **60**, 72–95 (2020).
  268. Ibrahim, M. A. A. *et al.* In Silico Targeting Human Multidrug Transporter ABCG2 in Breast Cancer: Database Screening, Molecular Docking, and Molecular Dynamics Study. *Mol. Inform.* **41**, 1–15 (2022).
  269. Ibrahim, M. A. A. *et al.* Naturally occurring plant-based anticancerous candidates as prospective ABCG2 inhibitors: an in silico drug discovery study. *Mol. Divers.* 1–23 (2022) doi:10.1007/s11030-022-10389-6.
  270. Almasirad, A., Mousavi, Z., Tajik, M., Assarzadeh, M. J. & Shafiee, A. Synthesis, analgesic and anti-inflammatory activities of new methyl-imidazolyl-1,3,4-oxadiazoles and 1,2,4-triazoles. *DARU J. Pharm. Sci.* **22**, 1–8 (2014).

271. Li, J., Fu, A. & Zhang, L. An Overview of Scoring Functions Used for Protein–Ligand Interactions in Molecular Docking. *Interdiscip. Sci. Comput. Life Sci.* **11**, 320–328 (2019).
272. Fu, Y., Zhao, J. & Chen, Z. Insights into the Molecular Mechanisms of Protein-Ligand Interactions by Molecular Docking and Molecular Dynamics Simulation: A Case of Oligopeptide Binding Protein. *Comput. Math. Methods Med.* **2018**, 3502514 (2018).
273. Asad, M., Siddiqui, Z. N., Asiri, A. M. & Mahmood, A. Synthesis, DFT and biological studies of novel 3,6-dimethyl-1,8-diphenyl-diazocino[3,4-c:7,8-c']bispyrazole. *J. Mol. Struct.* **1176**, 66–72 (2019).
274. Bakalbassis, E. G., Nenadis, N. & Tsimidou, M. A density functional theory study of structure-activity relationships in caffeic and dihydrocaffeic acids and related monophenols. *JAOCs, J. Am. Oil Chem. Soc.* **80**, 459–466 (2003).
275. Al Sheikh Ali, A. *et al.* Design, Synthesis, Molecular Modeling, Anticancer Studies, and Density Functional Theory Calculations of 4-(1,2,4-Triazol-3-ylsulfanylmethyl)-1,2,3-triazole Derivatives. *ACS Omega* **6**, 301–316 (2021).
276. Bharadwaj, S., Dubey, A., Yadava, U. & Mishra, S. K. Exploration of natural compounds with anti-SARS-CoV-2 activity via inhibition of SARS-CoV-2 M pro. **00**, 1–17 (2021).
277. Kumar, V. *et al.* Identification of CDK7 inhibitors from natural sources using pharmacoinformatics and molecular dynamics simulations. *Biomedicines* **9**, (2021).
278. Fatriansyah, J. F., Rizqillah, R. K., Yandi, M. Y., Fadilah & Sahlan, M. Molecular docking and dynamics studies on propolis sulabiroin-A as a potential inhibitor of SARS-CoV-2. *J. King Saud Univ. - Sci.* **34**, 1–9 (2022).
279. Pundir, H. *et al.* Using Chou's 5-steps rule to study pharmacophore-based virtual screening of SARS-CoV-2 Mpro inhibitors. *Mol. Divers.* **25**, 1731–1744 (2021).
280. Arsianti, A. *et al.* Design and screening of gallic acid derivatives as inhibitors of malarial dihydrofolate reductase by in silico docking. *Asian J. Pharm. Clin. Res.* **10**, 330–334 (2017).
281. Al-Shawabkeh, J. D., Al-Nadaf, A. H., Dahabiyeh, L. A. & Taha, M. O. Design, synthesis and structure-activity relationship of new HSL inhibitors guided by pharmacophore models. *Med. Chem. Res.* **23**, 127–145 (2014).
282. Raies, A. B. & Bajic, V. B. In silico toxicology: computational methods for the prediction of chemical toxicity. *Wiley Interdiscip. Rev. Comput. Mol. Sci.* **6**, 147–172 (2016).
283. Fewson, C. A. Microbial metabolism of mandelate: a microcosm of diversity. *FEMS Microbiol. Lett.* **54**, 85–110 (1988).
284. Daina, A., Michielin, O. & Zoete, V. SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Sci. Rep.* **7**, 1–13 (2017).
285. Hay, M., Thomas, D. W., Craighead, J. L., Economides, C. & Rosenthal, J. Clinical development success rates for investigational drugs. *Nat. Biotechnol.* **32**, 40–50 (2014).

286. Dahlin, J. L., Inglese, J. & Walters, M. A. Mitigating risk in academic preclinical drug discovery. *Nat. Rev. Drug Discov.* **14**, 279–294 (2015).
287. Lipinski, C. A., Lombardo, F., Dominy, B. W. & Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* **46**, 3–26 (2001).
288. Pany, S., Pal, A. & Sahu, P. K. In silico analysis of cyclooxygenase inhibitory activity of some natural molecules. *Int. J. Pharm. Pharm. Sci.* **5**, 410–412 (2013).
289. Benet, L. Z., Hosey, C. M., Ursu, O. & Oprea, T. I. BDDCS, the Rule of 5 and Drugability Leslie. *Adv. Drug Deliv. Rev.* **101**, 89–98 (2016).
290. Nicolaou, K. C. Organic synthesis: The art and science of replicating the molecules of living nature and creating others like them in the laboratory. *Proc. R. Soc. A Math. Phys. Eng. Sci.* **470**, 1–17 (2014).
291. Zhu, X. *et al.* Design, synthesis and biological activity of hydroxybenzoic acid ester conjugates of phenazine-1-carboxylic acid. *Chem. Cent. J.* **12**, 111 (2018).
292. Kumar, N. & Goel, N. Phenolic acids: Natural versatile molecules with promising therapeutic applications. *Biotechnol. Reports* **24**, e00370 (2019).
293. Liu, J., Du, C., Beaman, H. T. & Monroe, M. B. B. Characterization of phenolic acid antimicrobial and antioxidant structure–property relationships. *Pharmaceutics* **12**, 1–17 (2020).
294. Niphakis, M. J., Turunen, B. J. & Georg, G. I. Synthesis of 6- and 7-membered cyclic enamines: Scope and mechanism. *J. Org. Chem.* **75**, 6793–6805 (2010).
295. Howard, K. T. & Chisholm, J. D. Preparation and Applications of 4-Methoxybenzyl Esters in Organic Synthesis. *Org. Prep. Proced. Int.* **48**, 1–36 (2016).
296. Sartori, G. *et al.* Protection (and Deprotection) of Functional Groups in Organic Synthesis by Heterogeneous Catalysis. *Chem. Rev.* **104**, 199–250 (2004).
297. De Bruycker, K. *et al.* Triazolinediones as Highly Enabling Synthetic Tools. *Chem. Rev.* **116**, 3919–3974 (2016).
298. Jagtap, S. Heck reaction—State of the art. *Catalysts* **7**, (2017).
299. Dendisová, M. *et al.* The use of infrared spectroscopic techniques to characterize nanomaterials and nanostructures: A review. *Anal. Chim. Acta* **1031**, 1–14 (2018).
300. Gerald, C. F. G. C. Introduction to infrared and raman-based biomedical molecular imaging and comparison with other modalities. *Molecules* **25**, (2020).
301. Rhodes, C. J. Magnetic resonance spectroscopy. *Sci. Prog.* **100**, 241–292 (2017).
302. Dona, A. C. *et al.* A guide to the identification of metabolites in NMR-based metabolomics/metabolomics experiments. *Comput. Struct. Biotechnol. J.* **14**, 135–153 (2016).
303. Khatkar, A., Nanda, A., Kumar, P. & Narasimhan, B. Synthesis, antimicrobial evaluation and QSAR studies of gallic acid derivatives. *Arab. J. Chem.* **10**, S2870–S2880 (2017).
304. Leaym, X., Kraft, S. & Bossmann, S. H. Synthesis of water-soluble highly charged and

- methylene-bridged resorcin[4]arenes. *Synthesis (Stuttg)*. 932–942 (2008)  
doi:10.1055/s-2008-1032192.
305. Abraham, R. J. & Mobli, M. Related compounds. *Magn. Reson. Chem.* **45**, 865–877 (2007).
  306. Dai, F., Zhuang, Q., Huang, G., Deng, H. & Zhang, X. Infrared Spectrum Characteristics and Quantification of OH Groups in Coal. *ACS Omega* **8**, 17064–17076 (2023).
  307. Ibrahim, S., Ahmad, A. & Mohamed, N. S. Characterization of novel castor oil-based polyurethane polymer electrolytes. *Polymers (Basel)*. **7**, 747–759 (2015).
  308. Knop, S., Lindner, J. & Vöhringer, P. OH and NH stretching vibrational relaxation of liquid ethanolamine. *Zeitschrift für Phys. Chemie* **225**, 913–926 (2011).
  309. López-Martínez, L. M., Santacruz-Ortega, H., Navarro, R. E., Sotelo-Mundo, R. R. & González-Aguilar, G. A. A <sup>1</sup>H NMR investigation of the interaction between phenolic acids found in mango (*Mangifera indica* cv Ataulfo) and papaya (*Carica papaya* cv Maradol) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. *PLoS One* **10**, 1–11 (2015).
  310. Charisiadis, P. *et al.* <sup>1</sup>H-NMR as a structural and analytical tool of intra- and intermolecular hydrogen bonds of phenol-containing natural products and model compounds. *Molecules* **19**, 13643–13682 (2014).
  311. Kumari, S., Carmona, A. V., Tiwari, A. K. & Trippier, P. C. Amide Bond Biososteres: Strategies, Synthesis, and Successes. *J. Med. Chem.* **63**, 12290–12358 (2020).
  312. Rakipov, I. T., Petrov, A. A., Akhmediyarov, A. A., Khachatryan, A. A. & Varfolomeev, M. A. FTIR spectral study of intermolecular interactions of C=O groups of amides in solution. *J. Mol. Liq.* **354**, 118838 (2022).
  313. Myshakina, N. S., Ahmed, Z. & Asher, S. A. Dependence of Amide Vibrations on Hydrogen Bonding Nataliya. *J. Phys. Chem. B* **112**, 11873–11877 (2008).
  314. Mujika, J. I., Matxain, J. M., Eriksson, L. A. & Lopez, X. Resonance structures of the amide bond: The advantages of planarity. *Chem. - A Eur. J.* **12**, 7215–7224 (2006).
  315. Silva, A. M. S., Santos, C. M. M. & Pinto, D. C. G. A. *Advanced NMR techniques for structural characterization of heterocyclic structures. Recent Research Developments in Heterocyclic Chemistry* vol. 661 (Research signpost, 2007).
  316. Schlander, M., Hernandez-Villafuerte, K., Cheng, C. Y., Mestre-Ferrandiz, J. & Baumann, M. How Much Does It Cost to Research and Develop a New Drug? A Systematic Review and Assessment. *Pharmacoeconomics* **39**, 1243–1269 (2021).
  317. Tiwari, G. *et al.* Drug delivery systems: An updated review. *Int. J. Pharm. Investig.* **2**, 2 (2012).
  318. Mirza, A. Z. & Siddiqui, F. A. Nanomedicine and drug delivery: a mini review. *Int. Nano Lett.* **4**, 1–7 (2014).
  319. Zielinska, A. *et al.* Polymeric Nanoparticles: Production, Characterization, Toxicology and Ecotoxicology. *Molecules* **25**, 3731 (2020).
  320. Sadat, S. M. A., Jahan, S. T. & Haddadi, A. Effects of Size and Surface Charge of



- Polymeric Nanoparticles on in Vitro and in Vivo Applications. *J. Biomater. Nanobiotechnol.* **07**, 91–108 (2016).
321. Zhao, F. *et al.* Cellular uptake, intracellular trafficking, and cytotoxicity of nanomaterials. *Small* **7**, 1322–1337 (2011).
  322. Ribovski, L., Hamelmann, N. M. & Paulusse, J. M. J. Polymeric nanoparticles properties and brain delivery. *Pharmaceutics* **13**, 1–26 (2021).
  323. Bhattacharjee, S. *et al.* Surface charge-specific cytotoxicity and cellular uptake of tri-block copolymer nanoparticles. *Nanotoxicology* **7**, 71–84 (2013).
  324. Lima, L. R. M. *et al.* Thermal responsive poly-N-isopropylacrylamide/galactomannan copolymer nanoparticles as a potential amphotericin delivery carrier. *Carbohydr. Polym. Technol. Appl.* **2**, 1–10 (2021).
  325. Nowaczyk, M. *et al.* Pnipam-nanoparticle-based antiapoptotic approach for pro-regenerative capacity of skeletal myogenic cells. *Nanomaterials* **11**, 1–15 (2021).
  326. Maheswari, B., Jagadeesh Babu, P. E. & Agarwal, M. Role of N-vinyl-2-pyrrolidinone on the thermoresponsive behavior of PNIPAm hydrogel and its release kinetics using dye and vitamin-B12 as model drug. *J. Biomater. Sci. Polym. Ed.* **25**, 269–286 (2014).
  327. Çaykara, T., Kiper, S., Demirel, G., Demirci, S. & Çakanyildirim, Ç. Temperature-responsive characteristics of poly(N-isopropylacrylamide) hydrogels with macroporous structure. *Polym. Int.* **56**, 275–282 (2007).
  328. Ngadaonye, J. I., Cloonan, M. O., Geever, L. M. & Higginbotham, C. L. Synthesis and characterisation of thermo-sensitive terpolymer hydrogels for drug delivery applications. *J. Polym. Res.* **18**, 2307–2324 (2011).
  329. Chandran, K., Nithya, R., Sankaran, K., Gopalan, A. & Ganesan, V. Synthesis and characterization of sodium alkoxides. *Bull. Mater. Sci.* **29**, 173–179 (2006).
  330. Shimojo, A. A. M. *et al.* Evaluation of the influence of process parameters on the properties of resveratrol-loaded NLC using 22 full factorial design. *Antioxidants* **8**, 1–13 (2019).
  331. Hwang, B. S., Kim, J. S., Kim, J. M. & Shim, T. S. Thermogelling Behaviors of Aqueous Poly(N-Isopropylacrylamide-co-2-Hydroxyethyl Methacrylate) Microgel–Silica Nanoparticle Composite Dispersions. *Materials (Basel)*. **14**, 1–12 (2021).
  332. Futscher, M. H., Philipp, M., Müller-Buschbaum, P. & Schulte, A. The Role of Backbone Hydration of Poly(N-isopropyl acrylamide) Across the Volume Phase Transition Compared to its Monomer. *Sci. Rep.* **7**, 1–11 (2017).
  333. Nakamura, Y., Sasaki, N. & Nakata, M. Chain aggregation process of poly(methyl methacrylate) in the mixed solvent tert-butyl alcohol + water. *Macromolecules* **35**, 1365–1372 (2002).
  334. Masarudin, M. J., Cutts, S. M., Evison, B. J., Phillips, D. R. & Pigram, P. J. Factors determining the stability, size distribution, and cellular accumulation of small, monodisperse chitosan nanoparticles as candidate vectors for anticancer drug delivery: Application to the passive encapsulation of [14C]-doxorubicin. *Nanotechnol. Sci. Appl.* **8**, 67–80 (2015).

335. Omar Zaki, S. S., Ibrahim, M. N. & Katas, H. Particle size affects concentration-dependent cytotoxicity of chitosan nanoparticles towards mouse hematopoietic stem cells. *J. Nanotechnol.* **2015**, 1–5 (2015).
336. Kladko, D. V., Falchevskaya, A. S., Serov, N. S. & Prilepskii, A. Y. Nanomaterial shape influence on cell behavior. *Int. J. Mol. Sci.* **22**, 1–27 (2021).
337. Sajanalal, P. R., Sreeprasad, T. S., Samal, A. K. & Pradeep, T. Anisotropic nanomaterials: structure, growth, assembly, and functions. *Nano Rev.* **2**, 1–62 (2011).
338. Wu, Z., Yang, S. & Wu, W. Shape control of inorganic nanoparticles from solution. *Nanoscale* **8**, 1237–1259 (2016).
339. Varan, C. & Bilensoy, E. Cationic PEGylated polycaprolactone nanoparticles carrying post-operation docetaxel for glioma treatment. *Beilstein J. Nanotechnol.* **8**, 1446–1456 (2017).
340. Xu, J. *et al.* Controllable Microfluidic Production of Drug-Loaded PLGA Nanoparticles Using Partially Water-Miscible Mixed Solvent Microdroplets as a Precursor. *Sci. Rep.* **7**, 0–12 (2017).
341. Duan, X. & Li, Y. Physicochemical characteristics of nanoparticles affect circulation, biodistribution, cellular internalization, and trafficking. *Small* **9**, 1521–1532 (2013).
342. Danaei, M. *et al.* Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems. *Pharmaceutics* **10**, 1–17 (2018).
343. Inam, W. *et al.* Interactions between polymeric nanoparticles and different buffers as investigated by zeta potential measurements and molecular dynamics simulations. *View* **3**, 1–20 (2022).
344. Wilson, B. K. & Prud'homme, R. K. Nanoparticle size distribution quantification from transmission electron microscopy (TEM) of ruthenium tetroxide stained polymeric nanoparticles. *J. Colloid Interface Sci.* **604**, 208–220 (2021).
345. Asem, H. *et al.* Functional Nanocarriers for Drug Delivery by Surface Engineering of Polymeric Nanoparticle Post-Polymerization-Induced Self-Assembly. *ACS Appl. Bio Mater.* **4**, 1045–1056 (2021).
346. Dehghan Esmatabadi, M. J. *et al.* Review of new insights into antimicrobial agents. *Cell. Mol. Biol. (Noisy-le-grand)*. **63**, 40–48 (2017).
347. Eloff, J. N. The antibacterial activity of 27 southern African members of the Combretaceae. *S. Afr. J. Sci.* **95**, 148–152 (1999).
348. Alibi, S., Crespo, D. & Navas, J. Plant-derivatives small molecules with antibacterial activity. *Antibiotics* **10**, 1–19 (2021).
349. Schneider, Y. K. Bacterial natural product drug discovery for new antibiotics: Strategies for tackling the problem of antibiotic resistance by efficient bioprospecting. *Antibiotics* **10**, 1–15 (2021).
350. Jubeh, B., Breijyeh, Z. & Karaman, R. Resistance of gram-positive bacteria to current antibacterial agents and overcoming approaches. *Molecules* **25**, 1–23 (2020).
351. Exner, M. *et al.* Antibiotic resistance : What is so special about multidrug-resistant Gram-negative bacteria? *GMS Hyg. Infect. Control* **12**, 1–24 (2017).

352. Satpute, M. S., Gangan, V. D. & Shastri, I. Synthesis and antibacterial activity of novel 3-hydroxy benzoic acid hybrid derivatives [Part II]. *Rasayan J. Chem.* **12**, 1077–1084 (2019).
353. Plech, T. *et al.* Structure-activity relationship studies of microbiologically active thiosemicarbazides derived from hydroxybenzoic acid hydrazides. *Chem. Biol. Drug Des.* **85**, 315–325 (2015).
354. Feldeková, E. *et al.* Antimicrobial properties of phenolic acid alkyl esters. *Czech J. Food Sci.* **40**, 438–444 (2022).
355. Friedman, M., Henika, P. R. & Mandrell, R. E. Antibacterial Activities of Phenolic Benzaldehydes and Benzoic Acids against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J. Food Prot.* **66**, 1811–1821 (2003).
356. Boaventura, M. A. D. *et al.* Acetylcholinesterase inhibition and antimicrobial activity of hydroxyl amides synthesized from natural products derivatives. *An. Acad. Bras. Cienc.* **90**, 1945–1954 (2018).
357. Sardi, J. C. O., Scorzoni, L., Bernardi, T., Fusco-Almeida, A. M. & Mendes Giannini, M. J. S. *Candida* species: Current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J. Med. Microbiol.* **62**, 10–24 (2013).
358. Ostrosky-Zeichner, L. *et al.* Early treatment of candidemia in adults: A review. *Med. Mycol.* **49**, 113–120 (2011).
359. Korea, S. *et al.* *glabrata* Bloodstream Isolates, South Korea, 2008–2018. *Emerg. Infect. Dis.* **27**, 31–40 (2022).
360. Ferrari, S. *et al.* Gain of function mutations in CgPDR1 of *Candida glabrata* not only mediate antifungal resistance but also enhance virulence. *PLoS Pathog.* **5**, (2009).
361. Arendrup, M. C. & Patterson, T. F. Multidrug-resistant *Candida*: Epidemiology, molecular mechanisms, and treatment. *J. Infect. Dis.* **216**, S445–S451 (2017).
362. Tsai, H. F., Krol, A. A., Sarti, K. E. & Bennett, J. E. *Candida glabrata* PDR1, a transcriptional regulator of a pleiotropic drug resistance network, mediates azole resistance in clinical isolates and petite mutants. *Antimicrob. Agents Chemother.* **50**, 1384–1392 (2006).
363. Lima, V. N. *et al.* Antimicrobial and enhancement of the antibiotic activity by phenolic compounds: Gallic acid, caffeic acid and pyrogallol. *Microb. Pathog.* **99**, 56–61 (2016).
364. Rivero-Buceta, E. *et al.* Linear and branched alkyl-esters and amides of gallic acid and other (mono-, di- and tri-) hydroxy benzoyl derivatives as promising anti-HCV inhibitors. *Eur. J. Med. Chem.* **92**, 656–671 (2015).
365. Mamo, T. *et al.* Emerging nanotechnology approaches for HIV/AIDS treatment and prevention. *Nanomedicine* **5**, 269–285 (2010).
366. Ruwizhi, N. & Aderibigbe, B. A. Cinnamic acid derivatives and their biological efficacy. *Int. J. Mol. Sci.* **21**, 1–36 (2020).
367. Sun, C. *et al.* Synthesis, characterization and antimicrobial study of cinnamic acid

- functionalized Ag nanoparticles. *Nanocomposites* **8**, 95–101 (2022).
368. Sobhani, Z., Samani, S. M., Montaseri, H. & Khezri, E. Nanoparticles of chitosan loaded ciprofloxacin: Fabrication and antimicrobial activity. *Adv. Pharm. Bull.* **7**, 427–432 (2017).
  369. Jain, D. & Banerjee, R. Comparison of ciprofloxacin hydrochloride-loaded protein, lipid, and chitosan nanoparticles for drug delivery. *J. Biomed. Mater. Res. - Part B Appl. Biomater.* **86**, 105–112 (2008).
  370. Azhdarzadeh, M., Lotfipour, F., Zakeri-Milani, P., Mohammadi, G. & Valizadeh, H. Anti-bacterial performance of azithromycin nanoparticles as colloidal drug delivery system against different gram-negative and gram-positive bacteria. *Adv. Pharm. Bull.* **2**, 17–24 (2012).
  371. Yayehrad, A. T., Wondie, G. B. & Marew, T. Different Nanotechnology Approaches for Ciprofloxacin Delivery Against Multidrug-Resistant Microbes. *Infect. Drug Resist.* **15**, 413–426 (2022).
  372. Letafat, B. *et al.* Synthesis and in Vitro Cytotoxic Activity of Novel Triazole-Isoxazole Derivatives. *Iran. J. Basic Med. Sci.* **16**, 1155–1162 (2013).
  373. Luchini, C., Capelli, P. & Scarpa, A. Pancreatic Ductal Adenocarcinoma and Its Variants. *Surg. Pathol. Clin.* **9**, 547–560 (2016).
  374. Zaremba-Czogalla, M. *et al.* Evaluation of the in vitro cytotoxic activity of caffeic acid derivatives and liposomal formulation against pancreatic cancer cell lines. *Materials (Basel)*. **13**, 1–18 (2020).
  375. Rawla, P., Sunkara, T. & Gaduputi, V. Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors. *World J. Oncol.* **10**, 10–27 (2019).
  376. Ferlay, J. *et al.* Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. *Eur. J. Cancer* **103**, 356–387 (2018).
  377. Savi, L. A. *et al.* Evaluation of anti-herpetic and antioxidant activities, and cytotoxic and genotoxic effects of synthetic alkyl-esters of gallic acid. *Arzneimittel-Forschung/Drug Res.* **55**, 66–75 (2005).
  378. Cedó, L. *et al.* Gallic acid is an active component for the anticarcinogenic action of grape seed procyanidins in pancreatic cancer cells. *Nutr. Cancer* **66**, 88–96 (2014).
  379. Sales, M. S. *et al.* Octyl gallate and gallic acid isolated from *Terminalia bellarica* regulates normal cell cycle in human breast cancer cell lines. *Biomed. Pharmacother.* **103**, 1577–1584 (2018).
  380. Alim, Z., Kiliç, N., Şengül, B. & Beydemir, Ş. Inhibition behaviours of some phenolic acids on rat kidney aldose reductase enzyme: an in vitro study. *J. Enzyme Inhib. Med. Chem.* **32**, 277–284 (2017).
  381. Chauthé, S. K. *et al.* One pot synthesis and anticancer activity of dimeric phloroglucinols. *Bioorganic Med. Chem. Lett.* **22**, 2251–2256 (2012).
  382. Chapman, E. E., Langler, R. F. & Crandall, I. Phloroglucinol sulfonic acid esters as antimalarial/anticancer agents. *J. Sulfur Chem.* **29**, 607–618 (2008).
  383. Xu, W. *et al.* Novel Bis-Artemisinin-Phloroglucinol hybrid molecules with dual

- anticancer and immunomodulatory Activities: Synthesis and evaluation. *Bioorg. Chem.* **139**, 106705 (2023).
384. Salari, N. *et al.* Polymer-based drug delivery systems for anticancer drugs: A systematic review. *Cancer Treat. Res. Commun.* **32**, 1–14 (2022).
  385. Jahanshahi, M., Sanati, M. H., Hajizadeh, S. & Babaei, Z. Gelatin nanoparticle fabrication and optimization of the particle size. *Phys. Status Solidi Appl. Mater. Sci.* **205**, 2898–2902 (2008).
  386. Ashour, A. E., Badran, M. M., Kumar, A., Rishi, A. K. & Yassin, A. E. Di-Block PLCL and Tri-Block PLCLG matrix polymeric nanoparticles enhanced the anticancer activity of loaded 5-fluorouracil. *IEEE Trans. Nanobioscience* **15**, 739–747 (2016).
  387. Udompornmongkol, P. & Chiang, B. H. Curcumin-loaded polymeric nanoparticles for enhanced anti-colorectal cancer applications. *J. Biomater. Appl.* **30**, 537–546 (2015).
  388. Niero, E. L. D. O. & MacHado-Santelli, G. M. Cinnamic acid induces apoptotic cell death and cytoskeleton disruption in human melanoma cells. *J. Exp. Clin. Cancer Res.* **32**, 1–14 (2013).
  389. Pontiki, E., Hadjipavlou-Litina, D., Litinas, K. & Geromichalos, G. Novel cinnamic acid derivatives as antioxidant and anticancer agents: Design, synthesis and modeling studies. *Molecules* **19**, 9655–9674 (2014).
  390. Alzahrani, S. M., Al Doghaither, H. A. & Al-Ghafar, A. B. General insight into cancer: An overview of colorectal cancer (review). *Mol. Clin. Oncol.* **15**, 2021 (2021).
  391. Anand, P. *et al.* Cancer is a preventable disease that requires major lifestyle changes. *Pharm. Res.* **25**, 2097–2116 (2008).
  392. Liu, J., Zhang, K., Cheng, L., Zhu, H. & Xu, T. Progress in understanding the molecular mechanisms underlying the antitumour effects of ivermectin. *Drug Des. Devel. Ther.* **14**, 285–296 (2020).
  393. Zhang, Y. & Zhang, Z. The history and advances in cancer immunotherapy: understanding the characteristics of tumor-infiltrating immune cells and their therapeutic implications. *Cell. Mol. Immunol.* **17**, 807–821 (2020).
  394. Mo, W. & Zhang, J. T. Human ABCG2: Structure, function, and its role in multidrug resistance. *Int. J. Biochem. Mol. Biol.* **3**, 1–27 (2012).
  395. Mckie, S. J., Neuman, K. C. & Maxwell, A. Europe PMC Funders Group Europe PMC Funders Author Manuscripts DNA topoisomerases : Advances in understanding of cellular roles and multi-protein complexes via structure-function analysis. *Bioassays* **43**, 1–35 (2023).



# **Appendices**

---

**Design, synthesis, and bioactivity of hydroxybenzoic acid derivatives and their polymer nanocomposites**

**By**

**KHUMBLANI MNQIWU**

**(2050388)**

# Appendix A: Computational chemistry

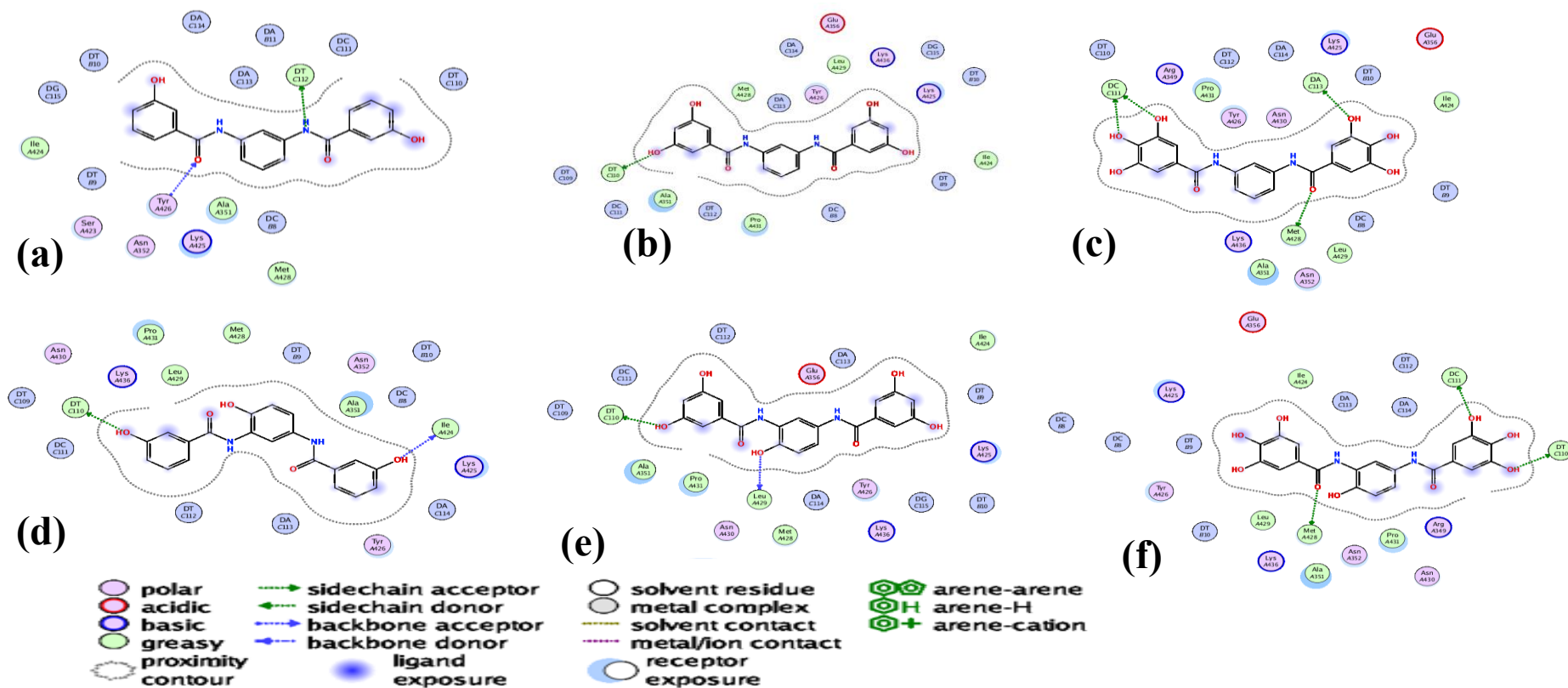


**Figure A1:** 2D protein-ligand interaction of (a) C10, (b) C11, (c) C12, (d) C13, (e) C14 and (f) C15 with 1K4T protein



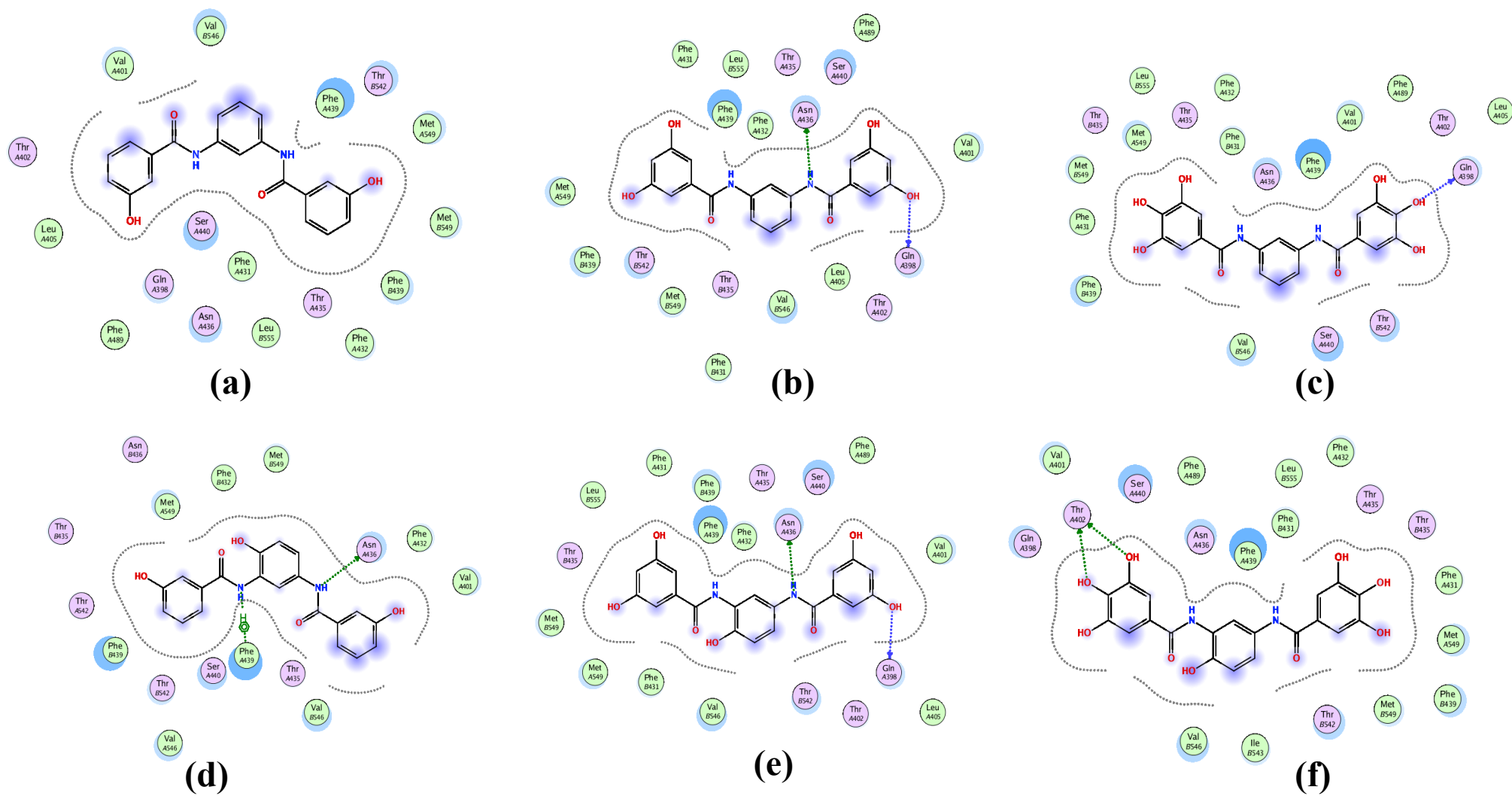


**Figure A2:** 2D protein-ligand interaction of (a) KAM 1, (b) KAM 4, (c) KAM 6, (d) KAM 2, (e) KAM 3 and (f) KAM 11

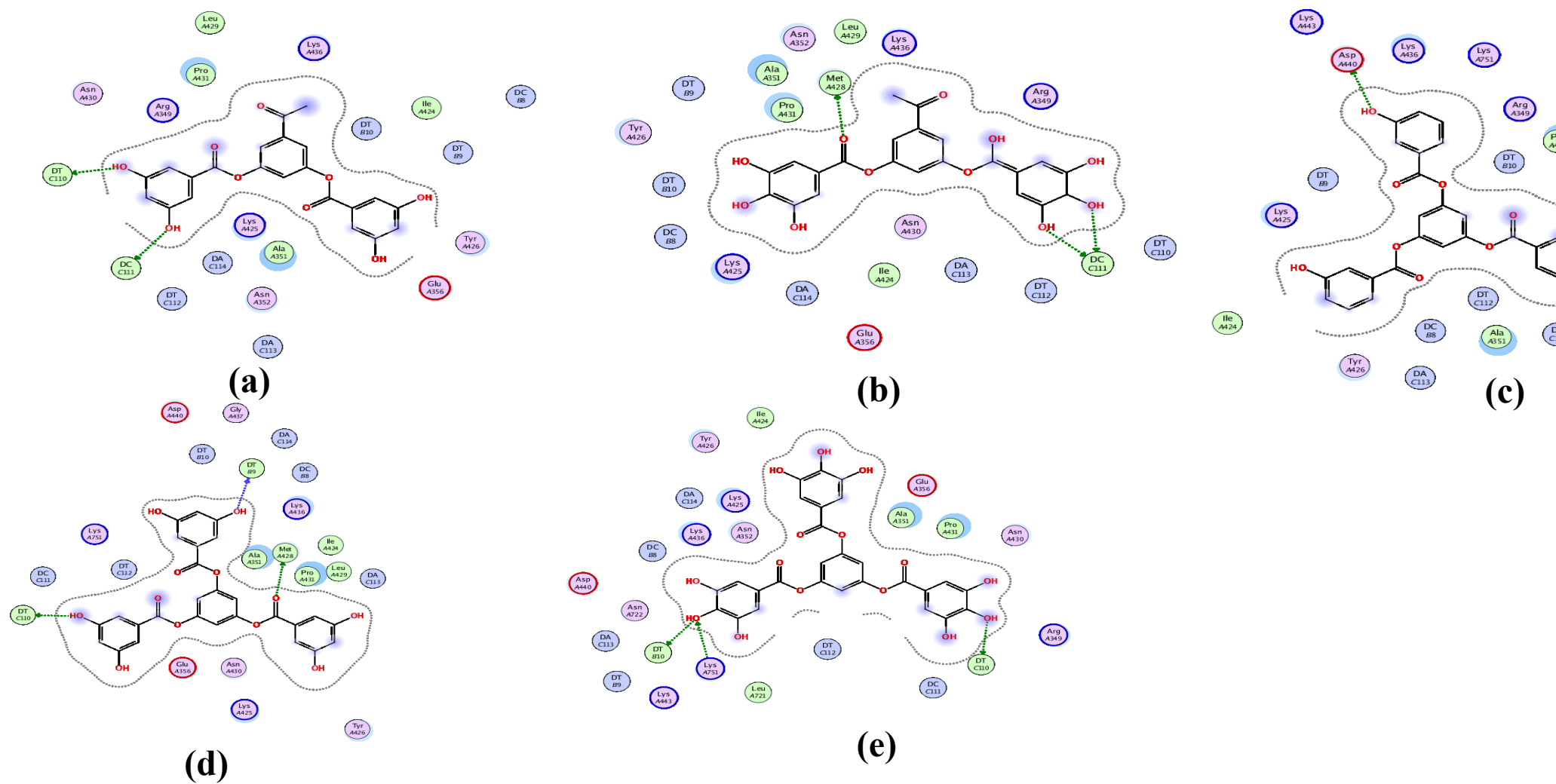


**Figure A3:** 2D protein-ligand interaction of (a) C8, (b) CE6, (c) C4, (d) C9, and (e) C13 with 1K4T protein



**Figure A4:** 2D protein-ligand interaction of (a) C8, (b) C12, (c) C4, (d) C9, and (e) C13 with 7NEZ protein

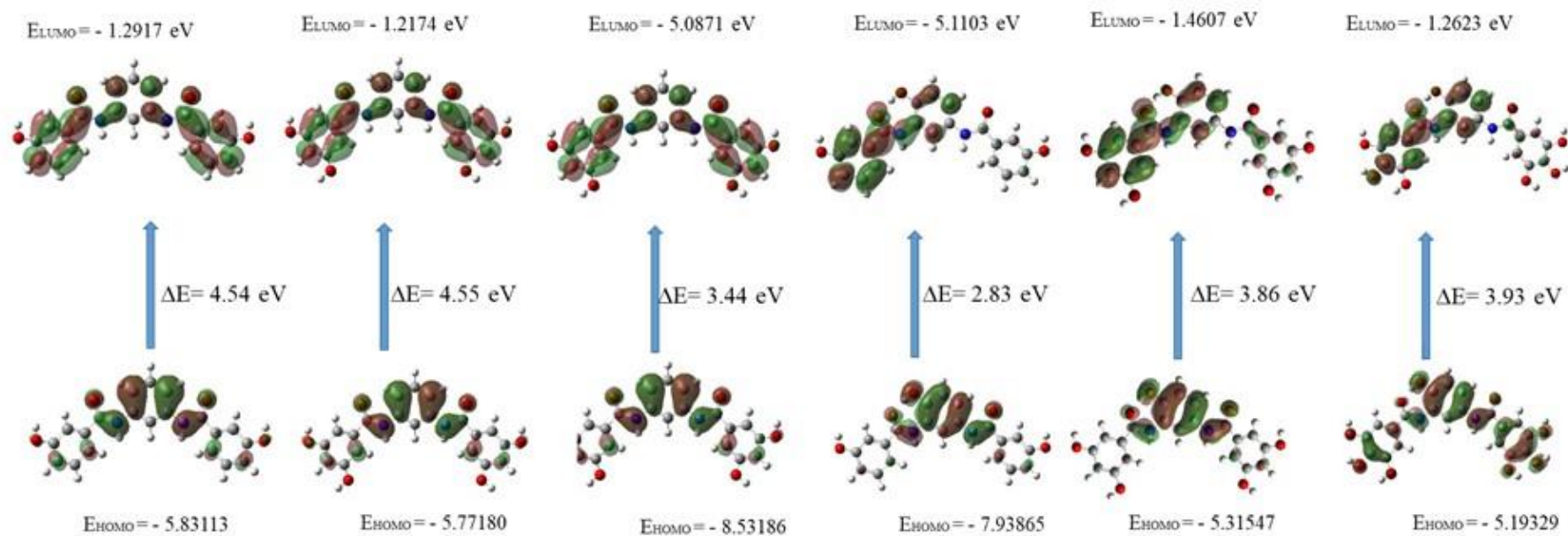


**Figure A5:** Frontier molecular descriptors of selected amide compounds

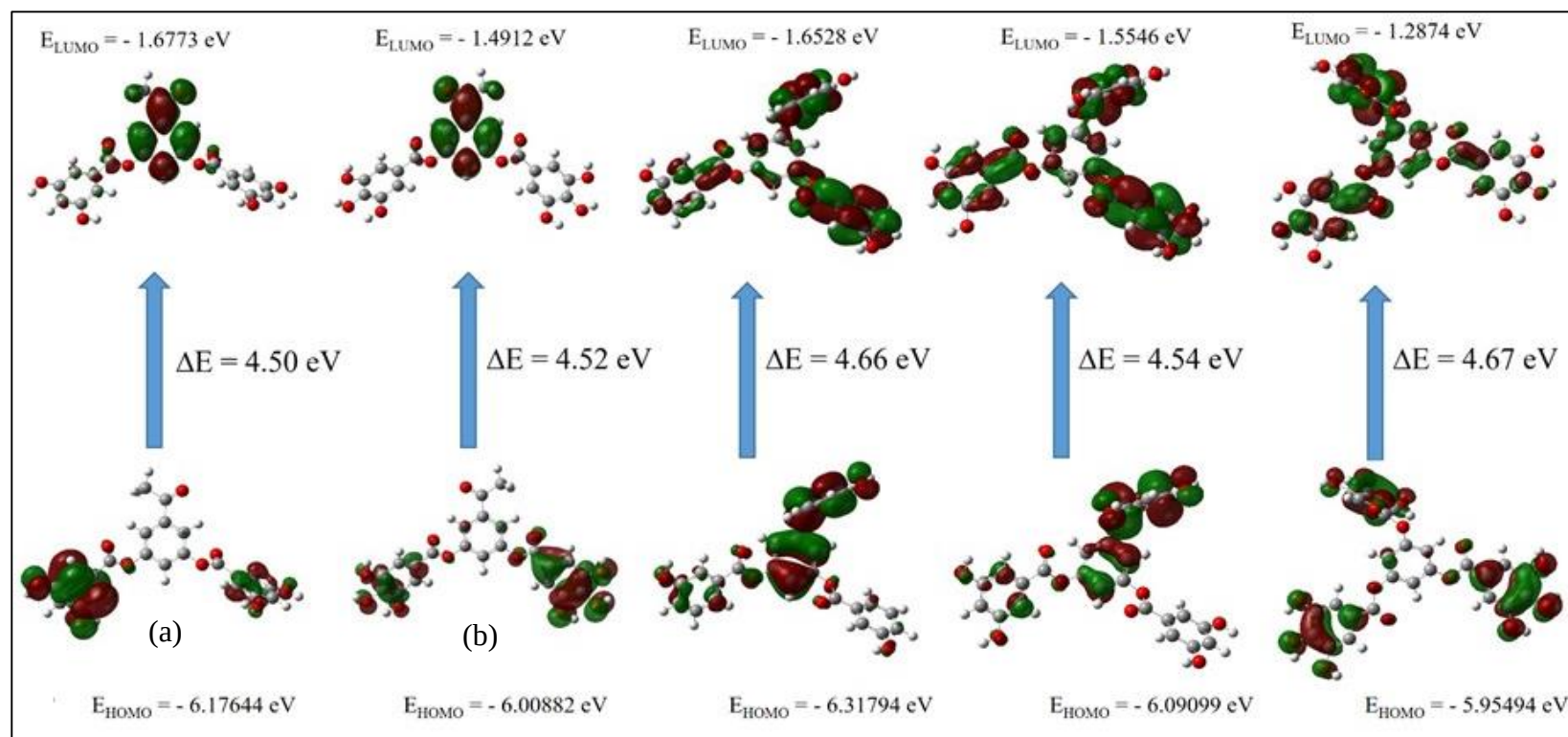
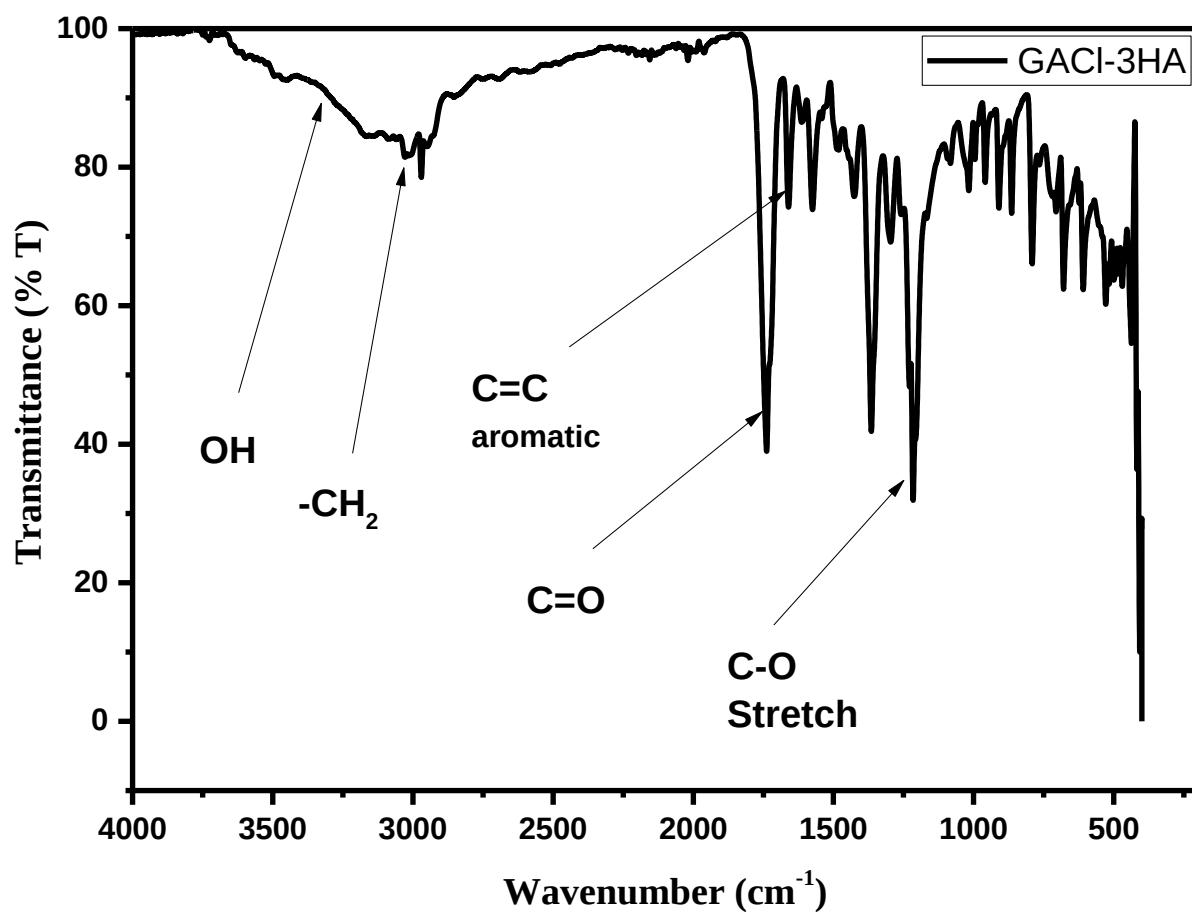
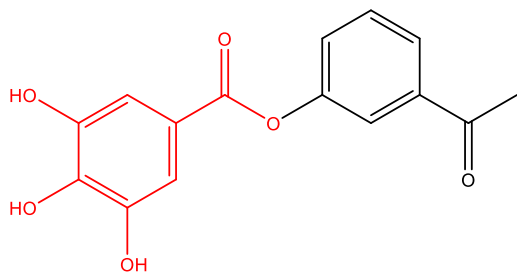


**Figure A6:** Frontier molecular descriptors of selected ester compounds (a) C8 (b) C12 (c) C4 (d) C9 (e) C13

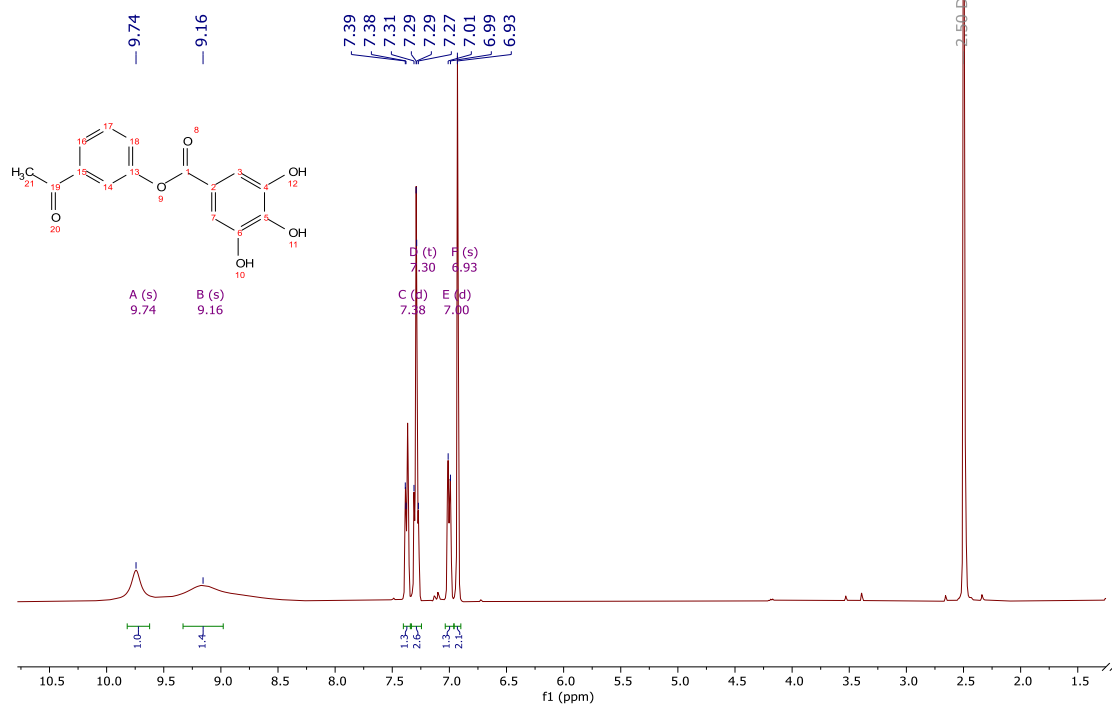


## Appendix B1: Characterization data of mono-esters from 3-hydroxy acetophenone

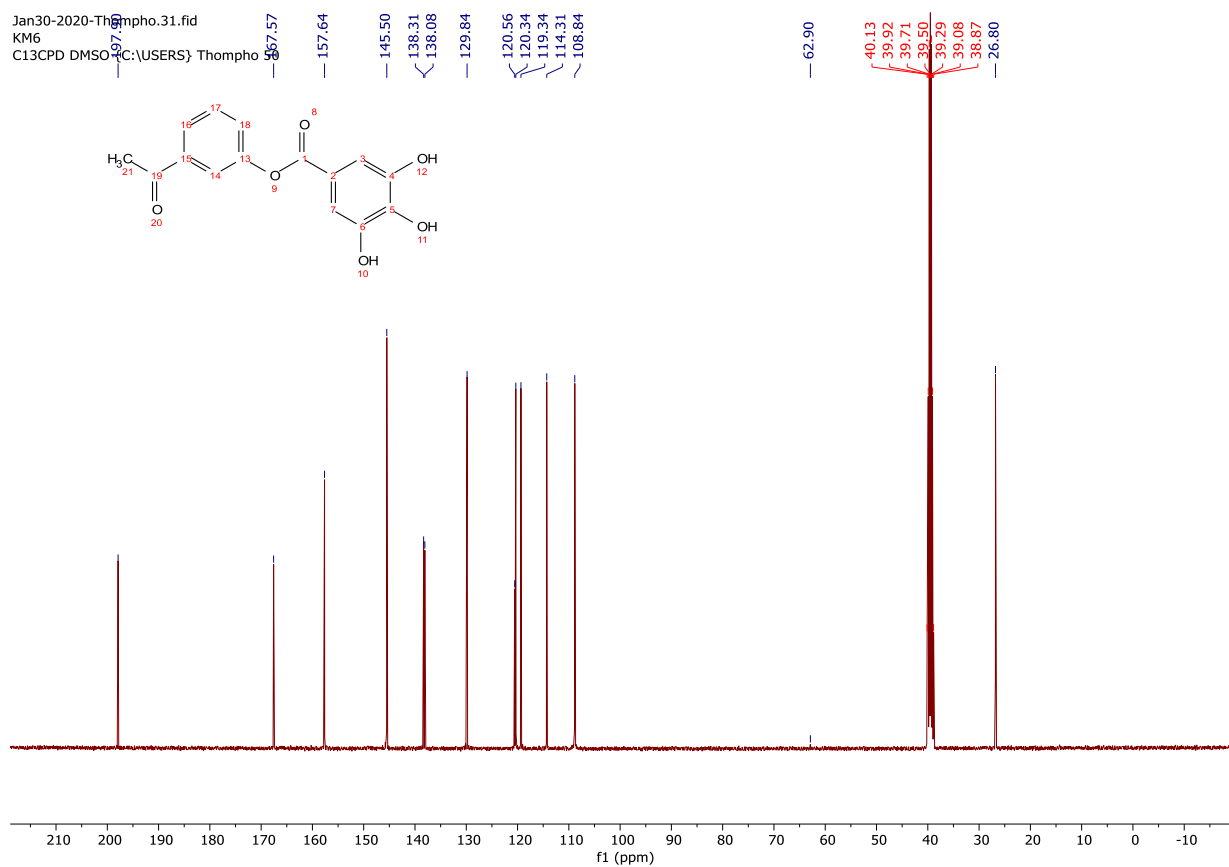


FTIR spectra of 3-acetylphenyl 3,4,5-trihydroxybenzoate (C11)

Jan30-2020-Thompho.30.fid  
KM6  
PROTON DMSO {C:\USERS} Thompho 50

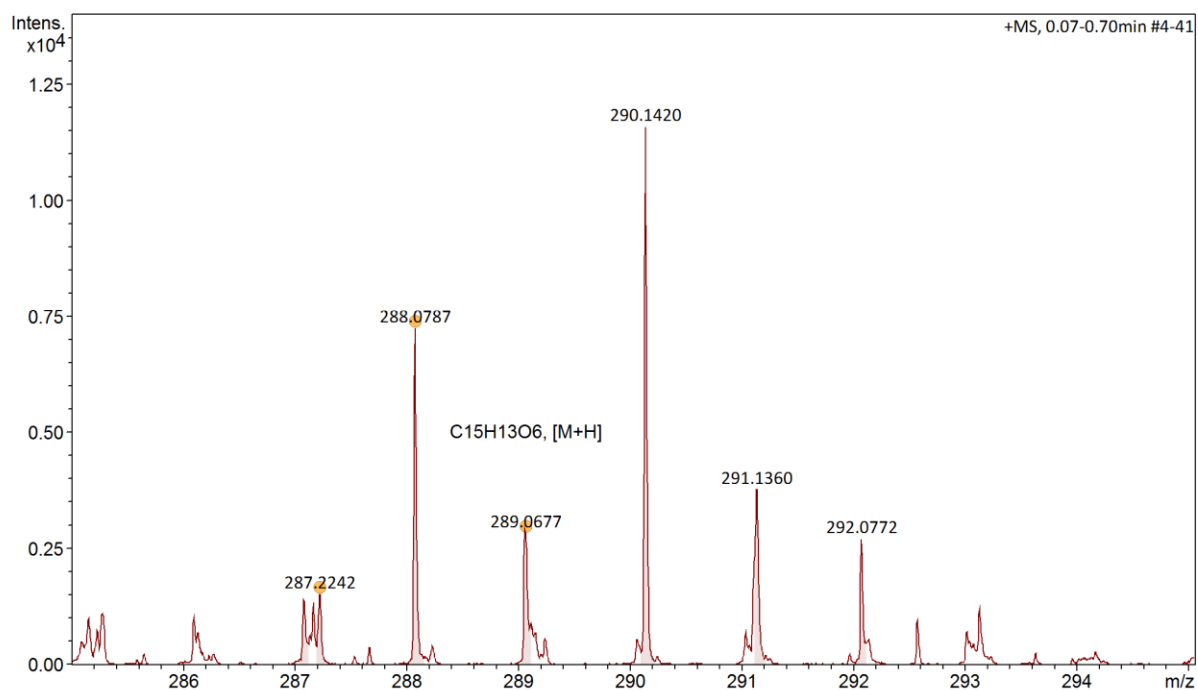
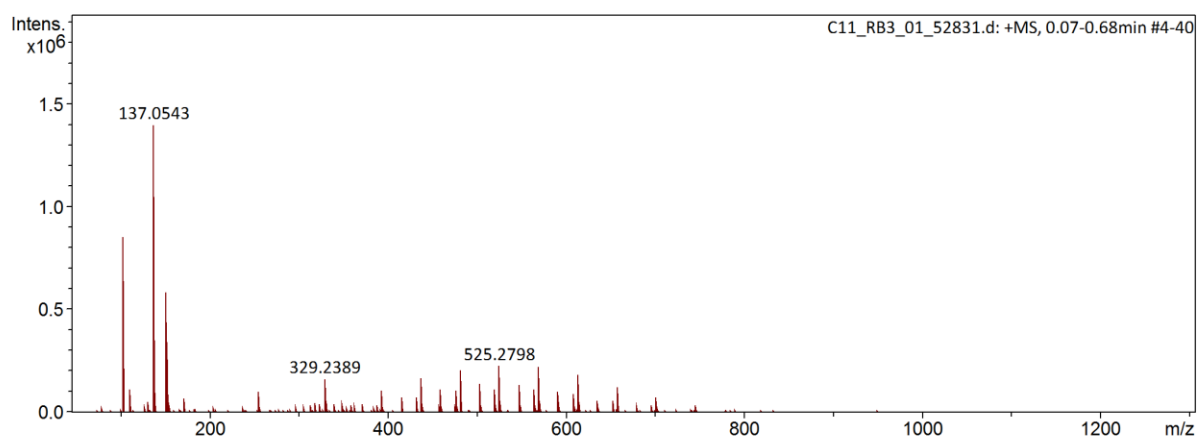


Jan30-2020-Thompho.31.fid  
KM6  
C13CPD DMSO {C:\USERS} Thompho 50

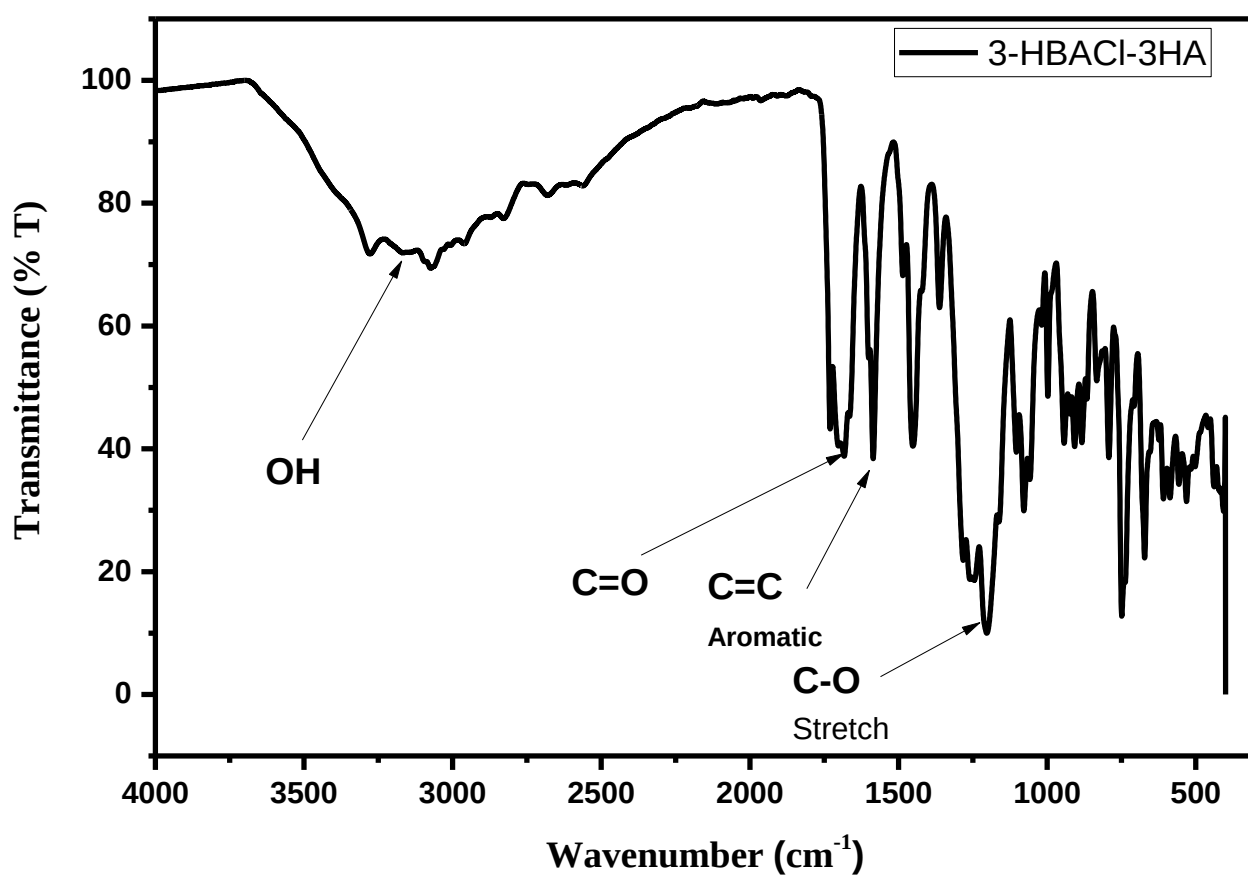
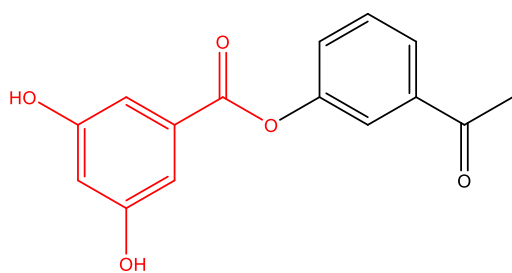


**<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3-acetylphenyl 3,4,5-trihydroxybenzoate (C11)**



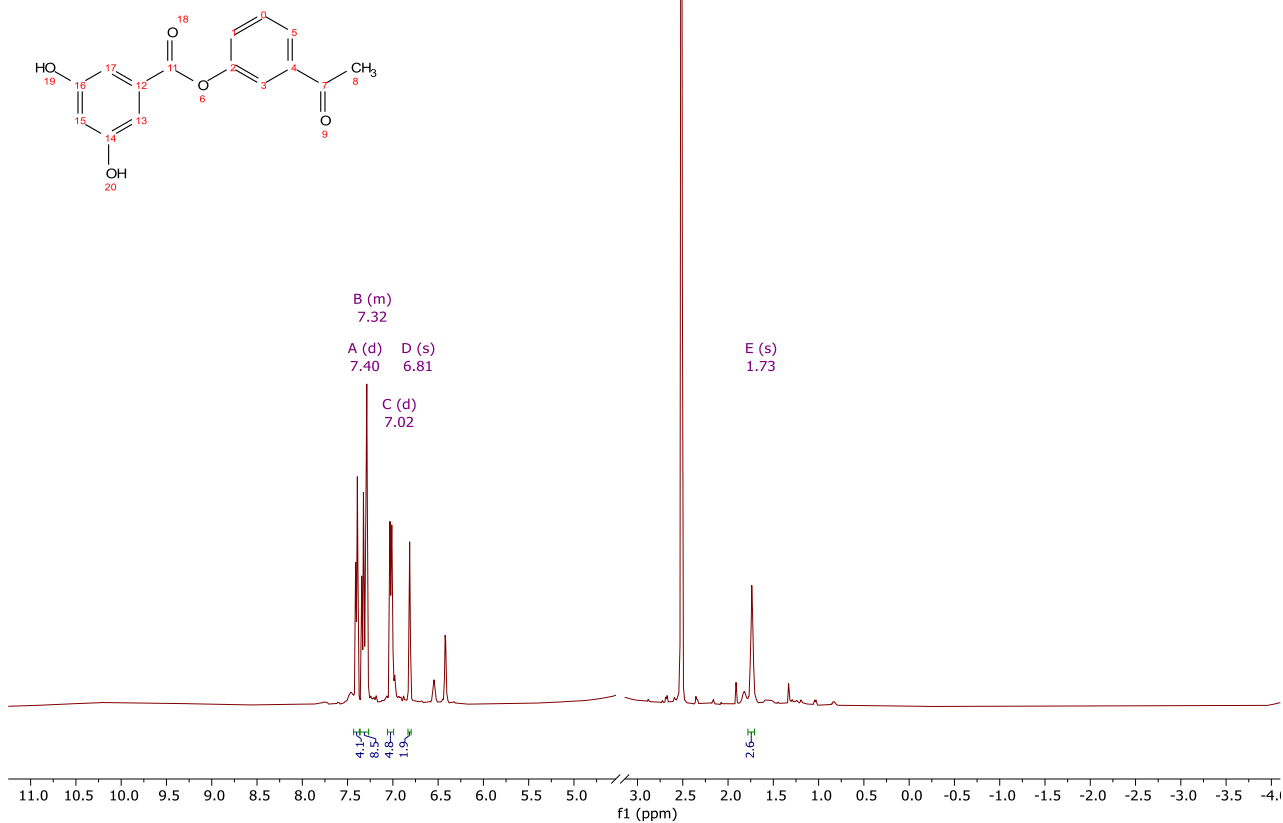


**HRMS of 3-acetylphenyl 3,4,5-trihydroxybenzoate (C11)**

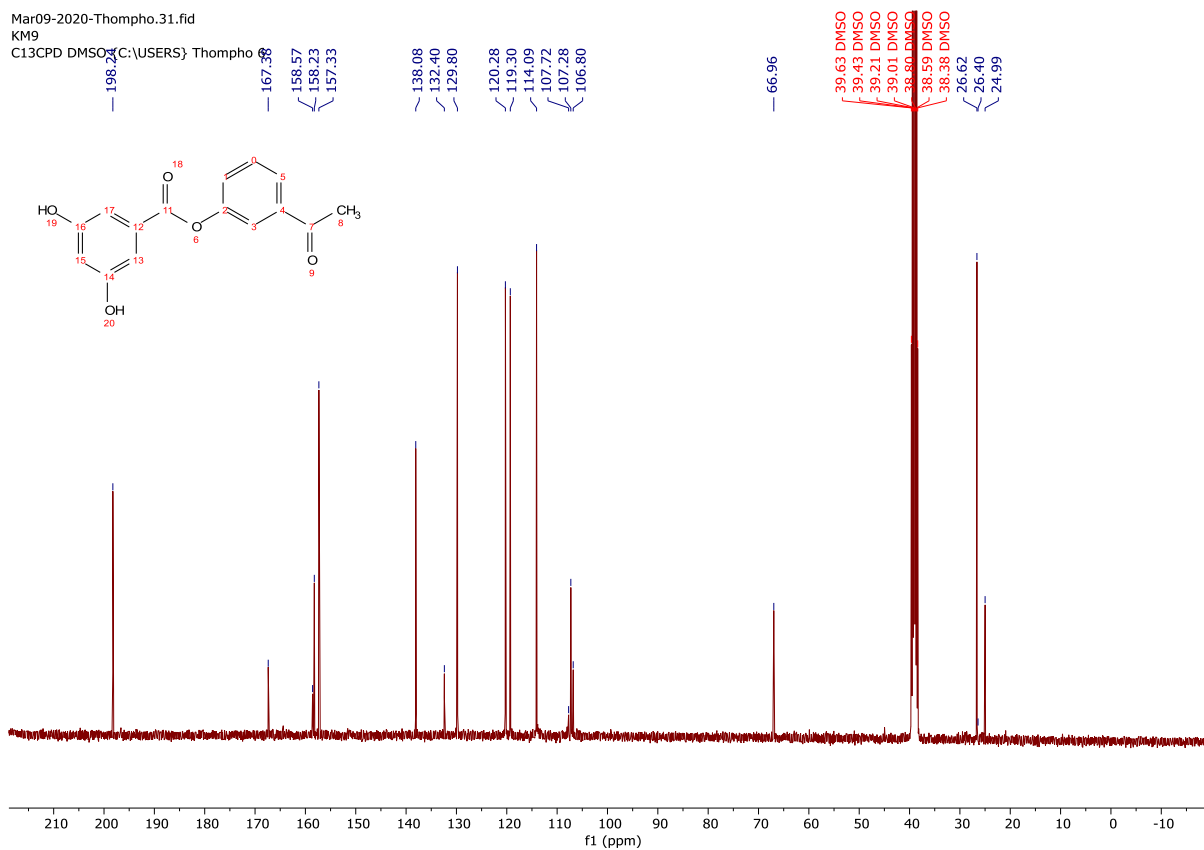


FTIR spectrum of 3-acetylphenyl 3,5-dihydroxybenzoate (C7)

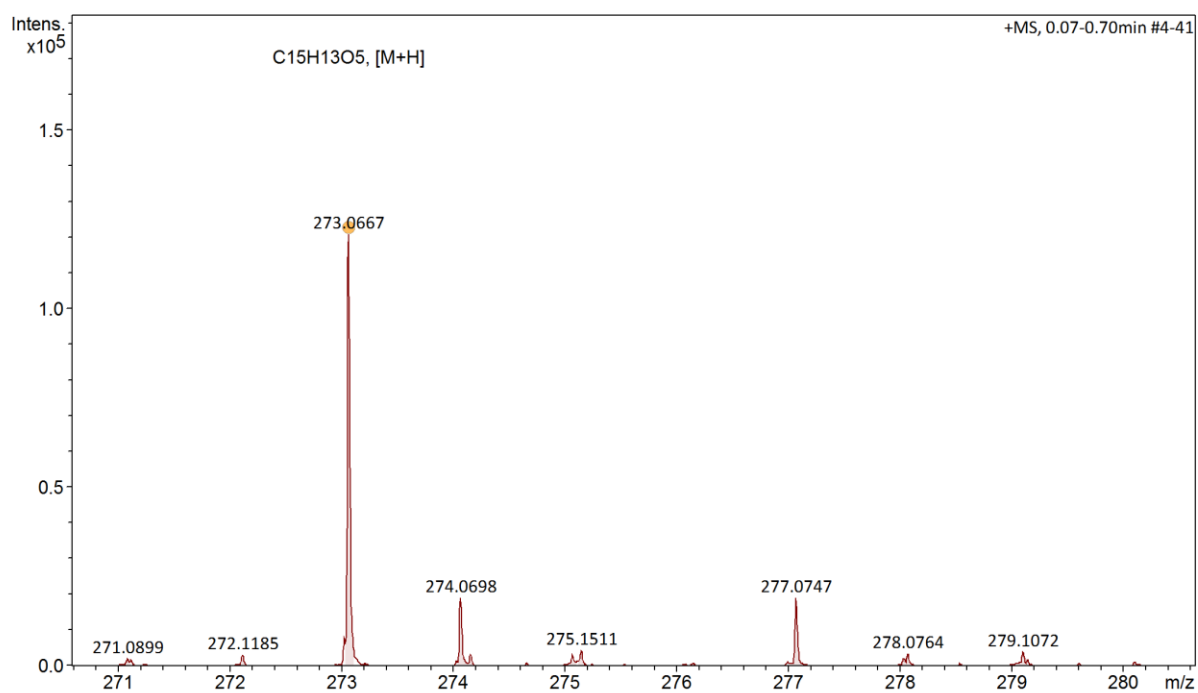
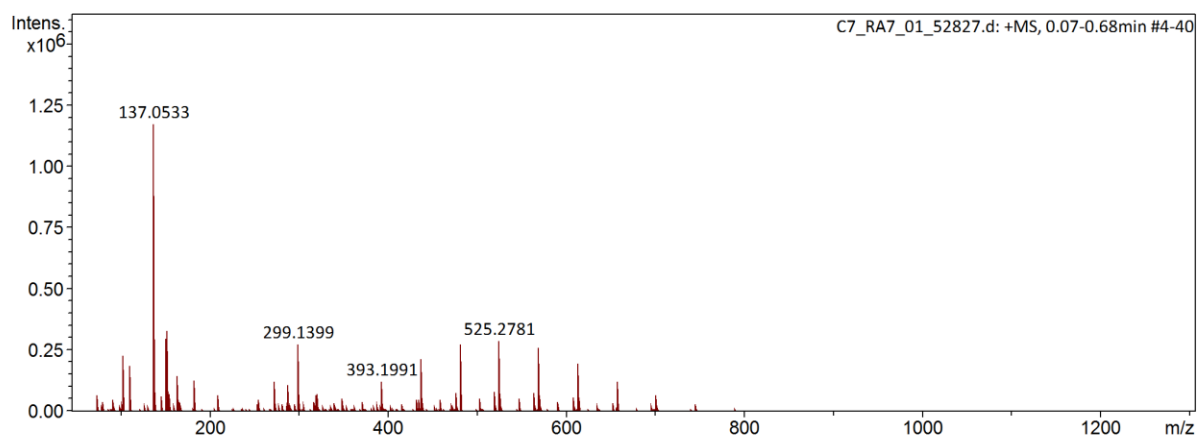
Mar09-2020-Thompho.30.fid  
KM9  
PROTON DMSO {C:\USERS} Thompho 6



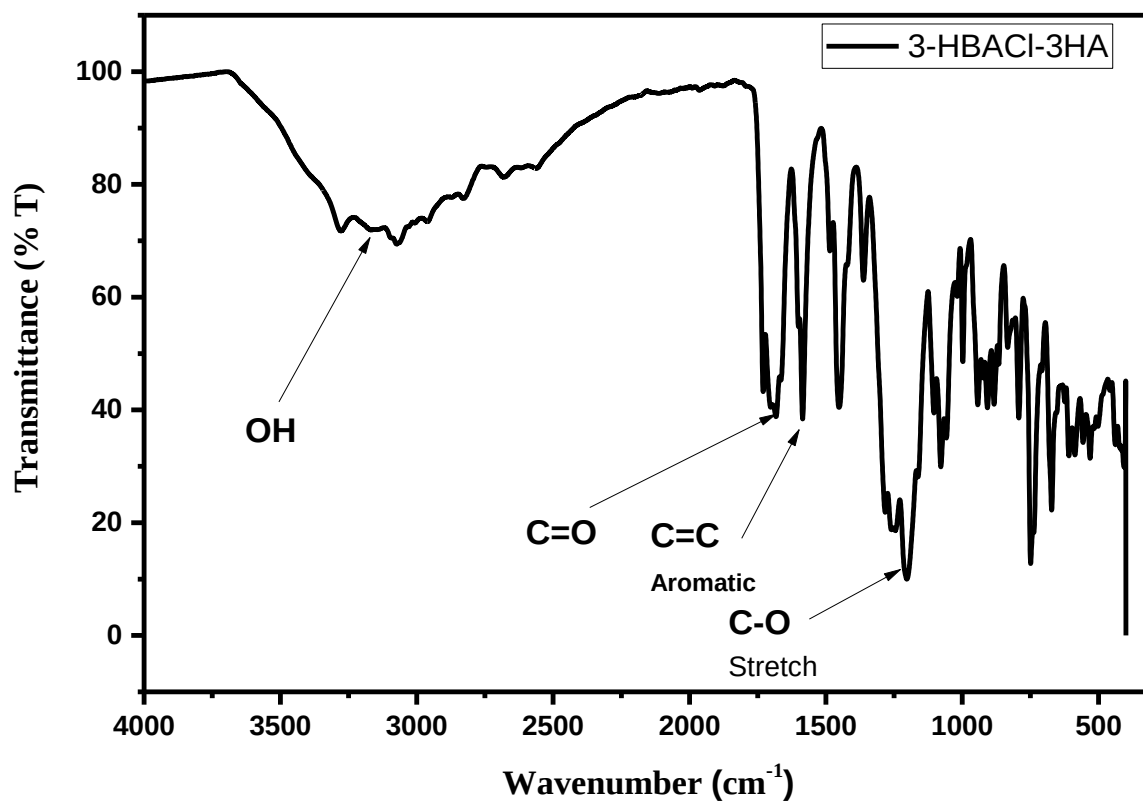
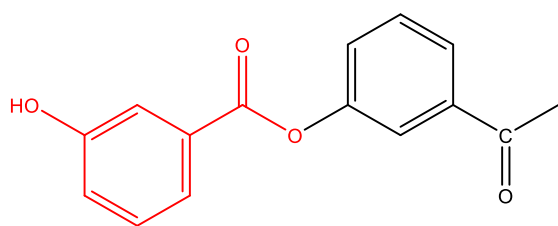
Mar09-2020-Thompho.31.fid  
KM9  
C13CPD DMSO {C:\USERS} Thompho 6



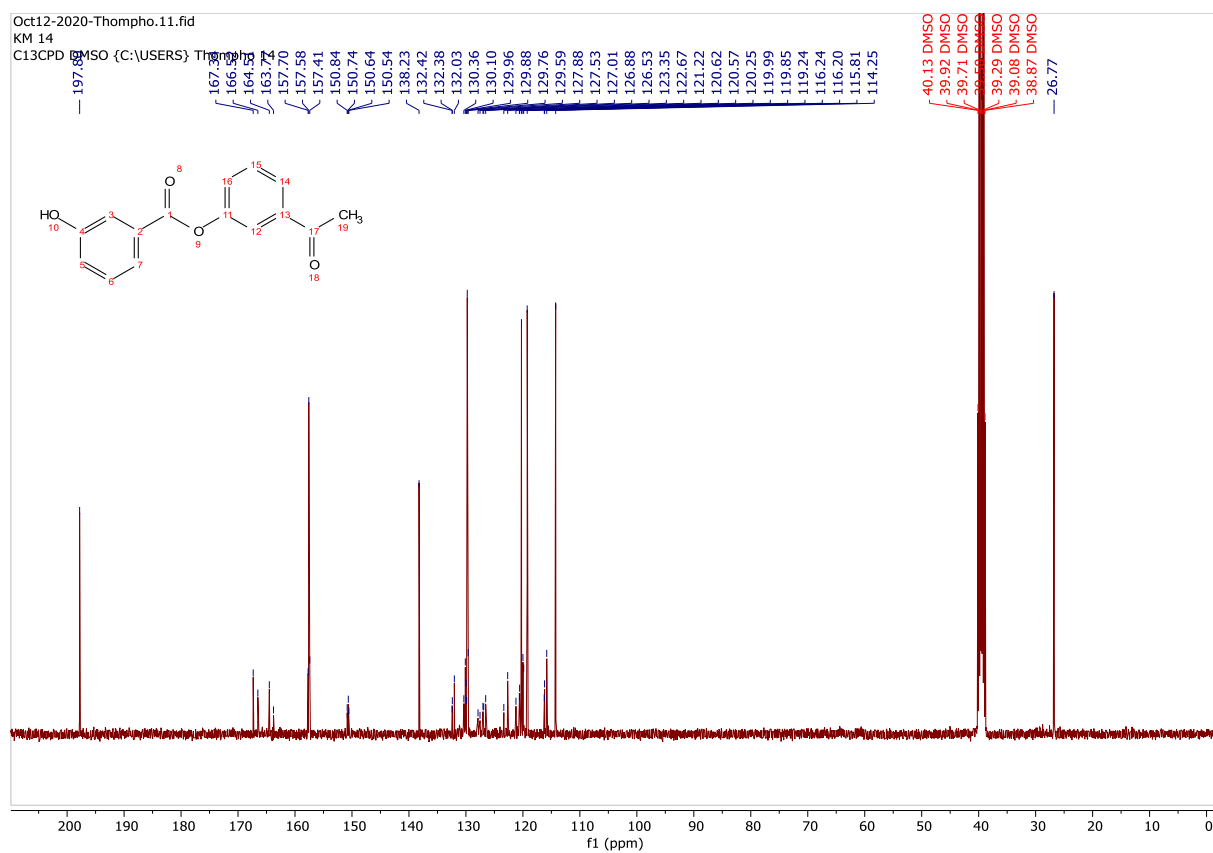
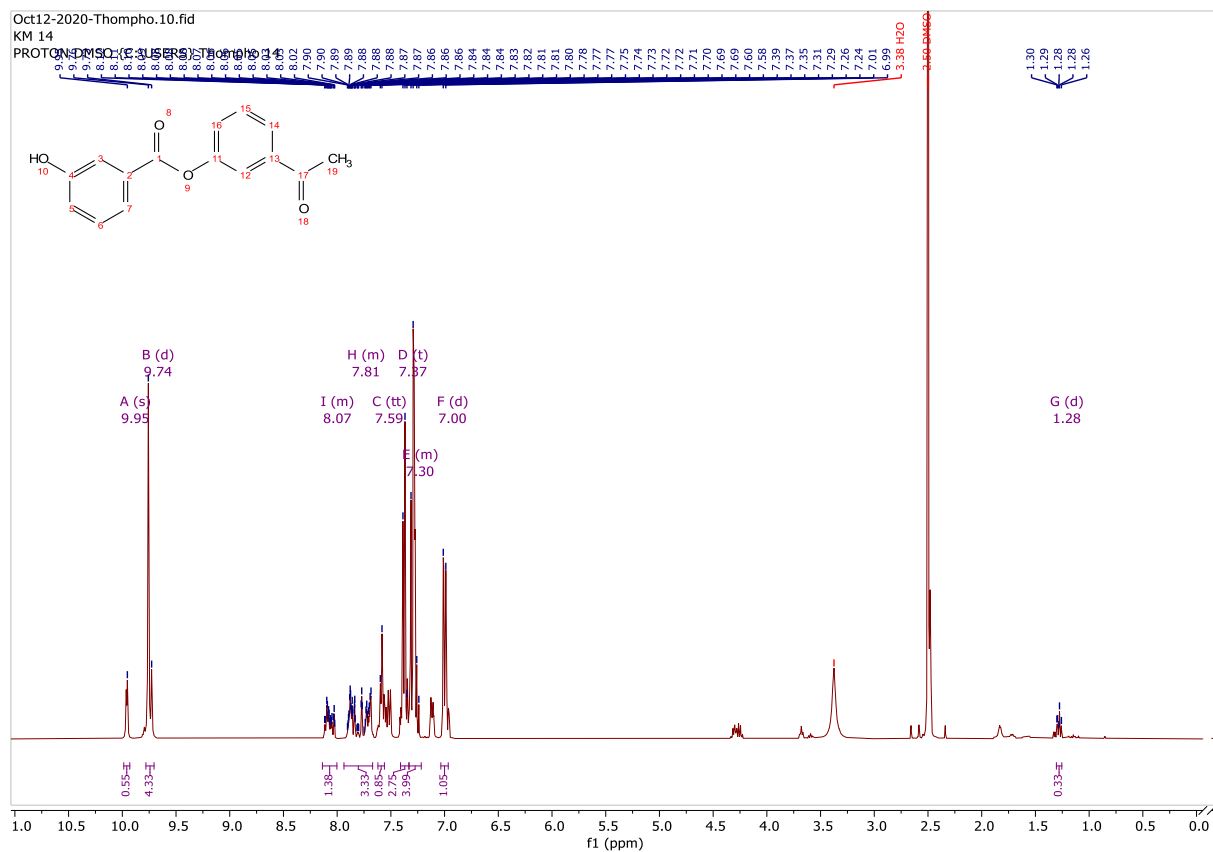
**<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3-acetylphenyl 3,5-dihydroxybenzoate (C7)**



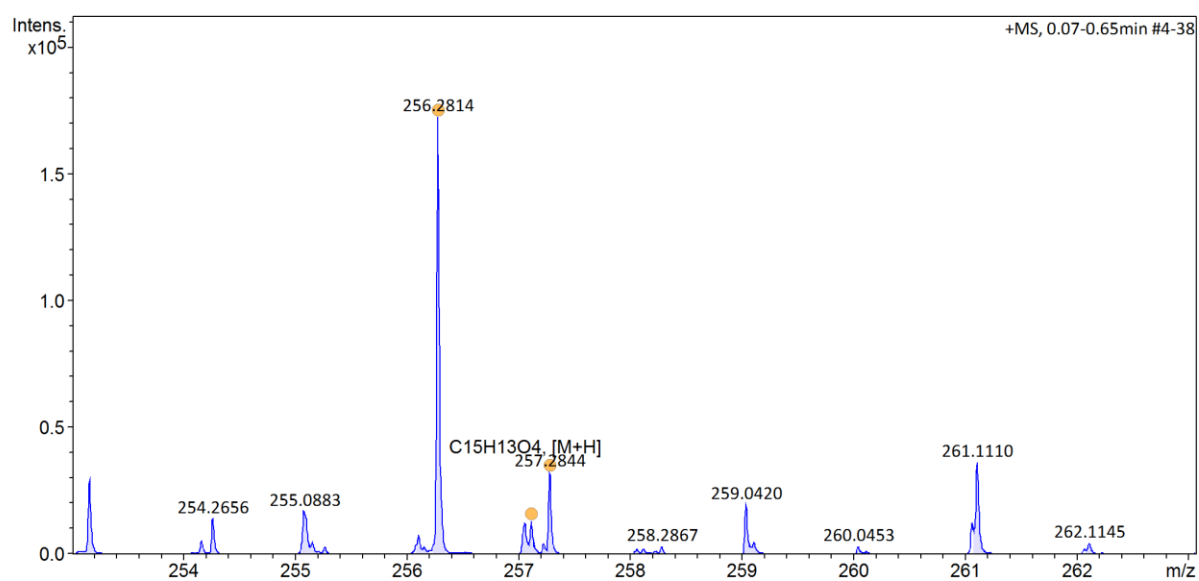
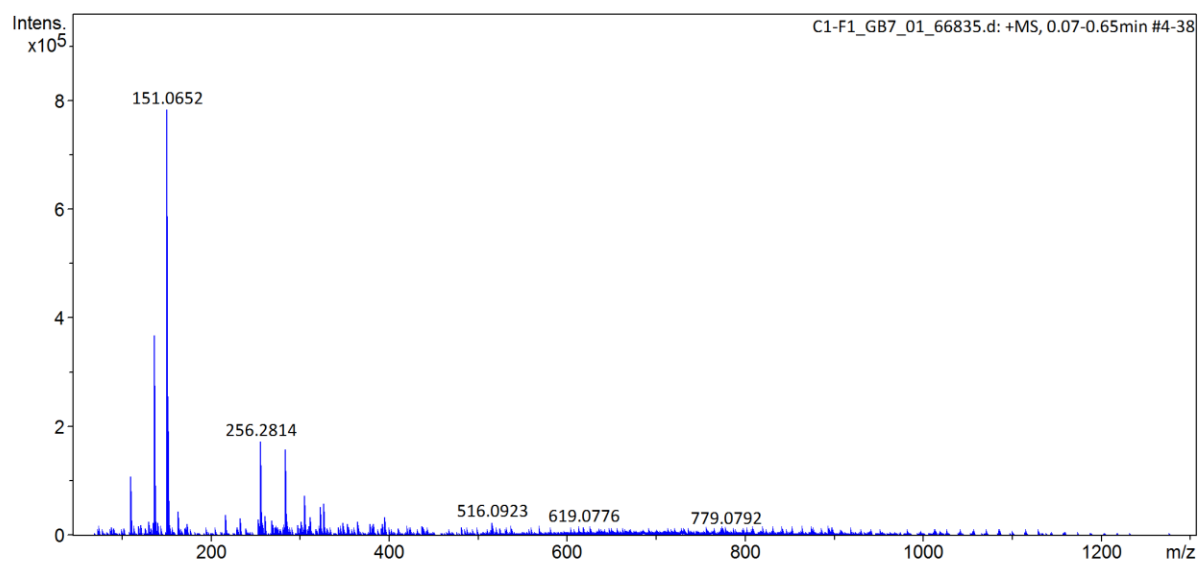
**HRMS of 3-acetylphenyl 3,5-dihydroxybenzoate (C7)**



**FTIR spectrum of 3-acetylphenyl 3-hydroxybenzoate (C1)**

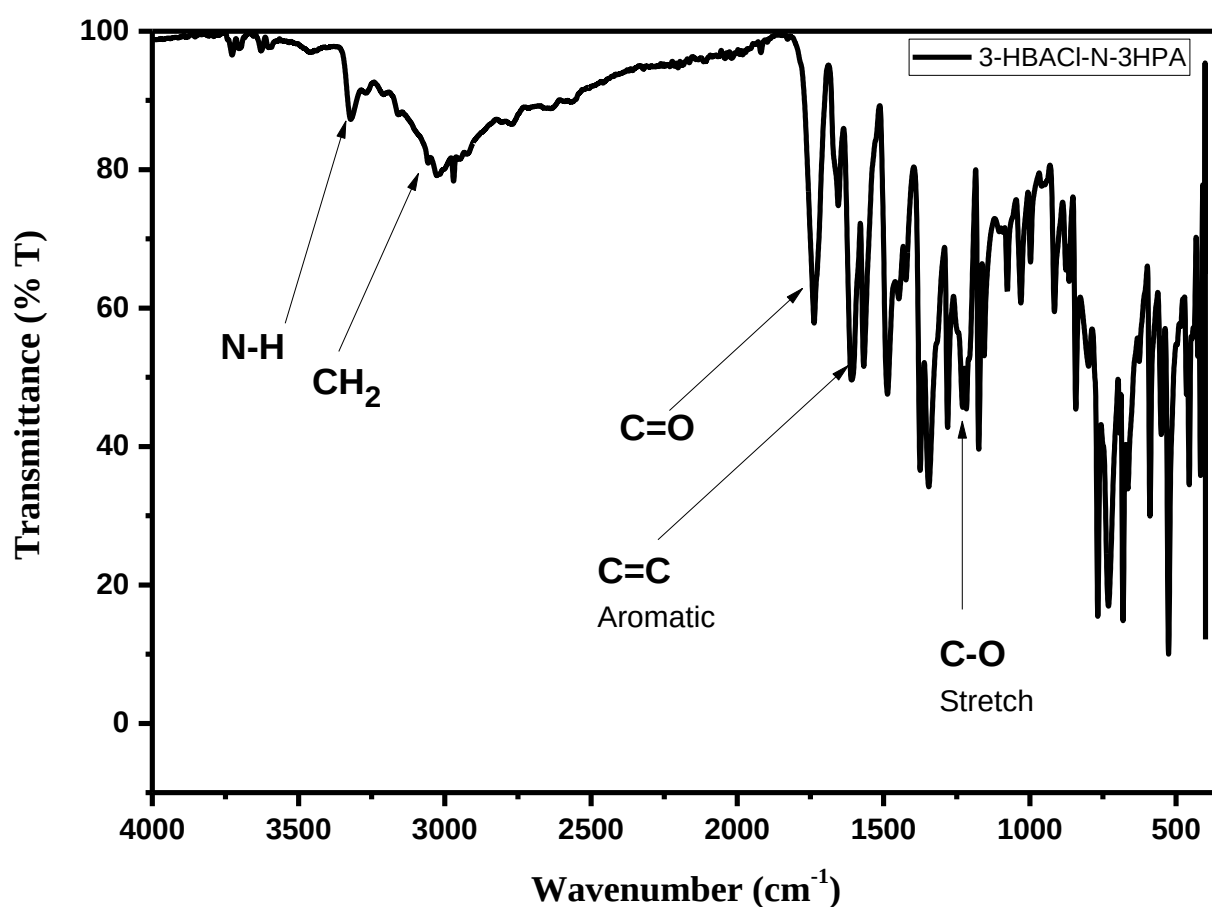
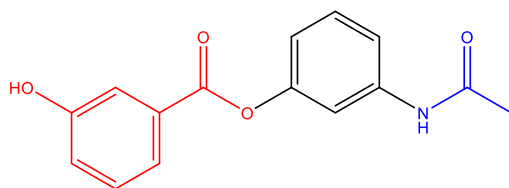


**<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3-acetylphenyl 3-hydroxybenzoate (C1)**



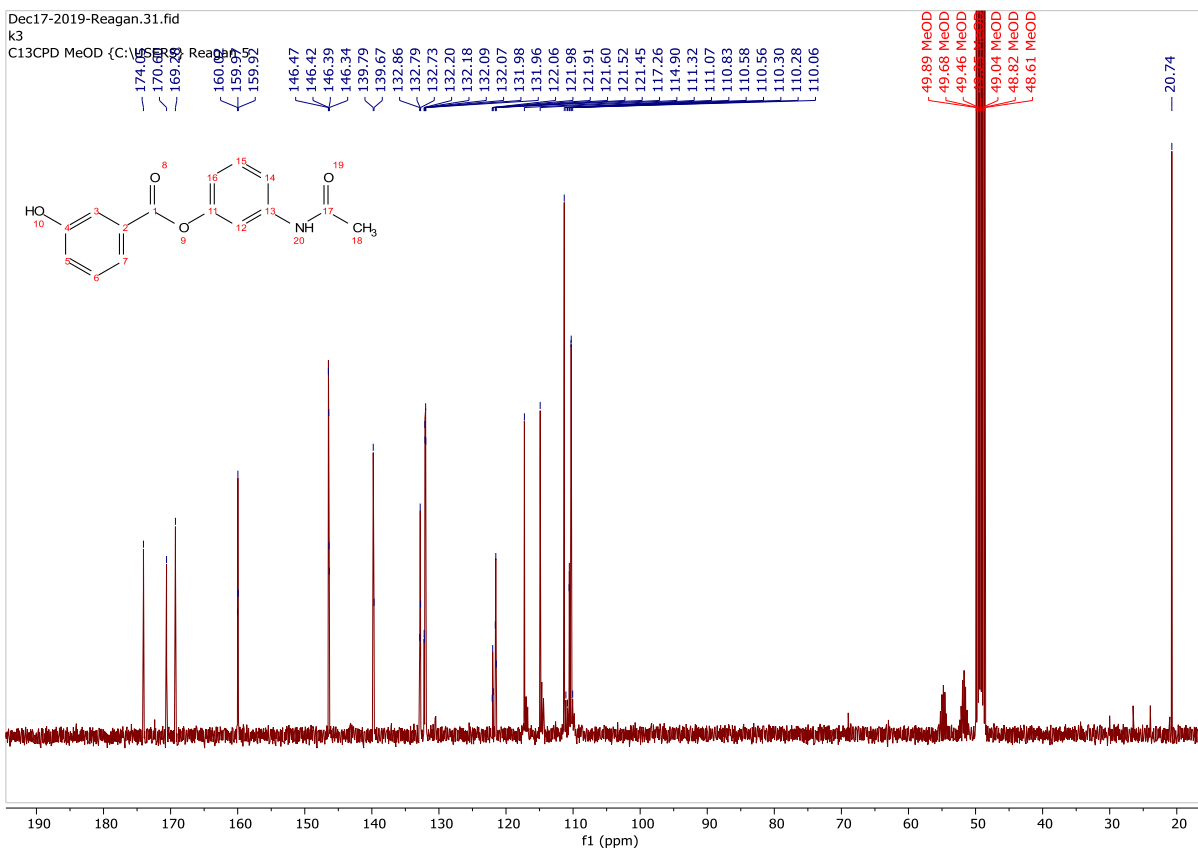
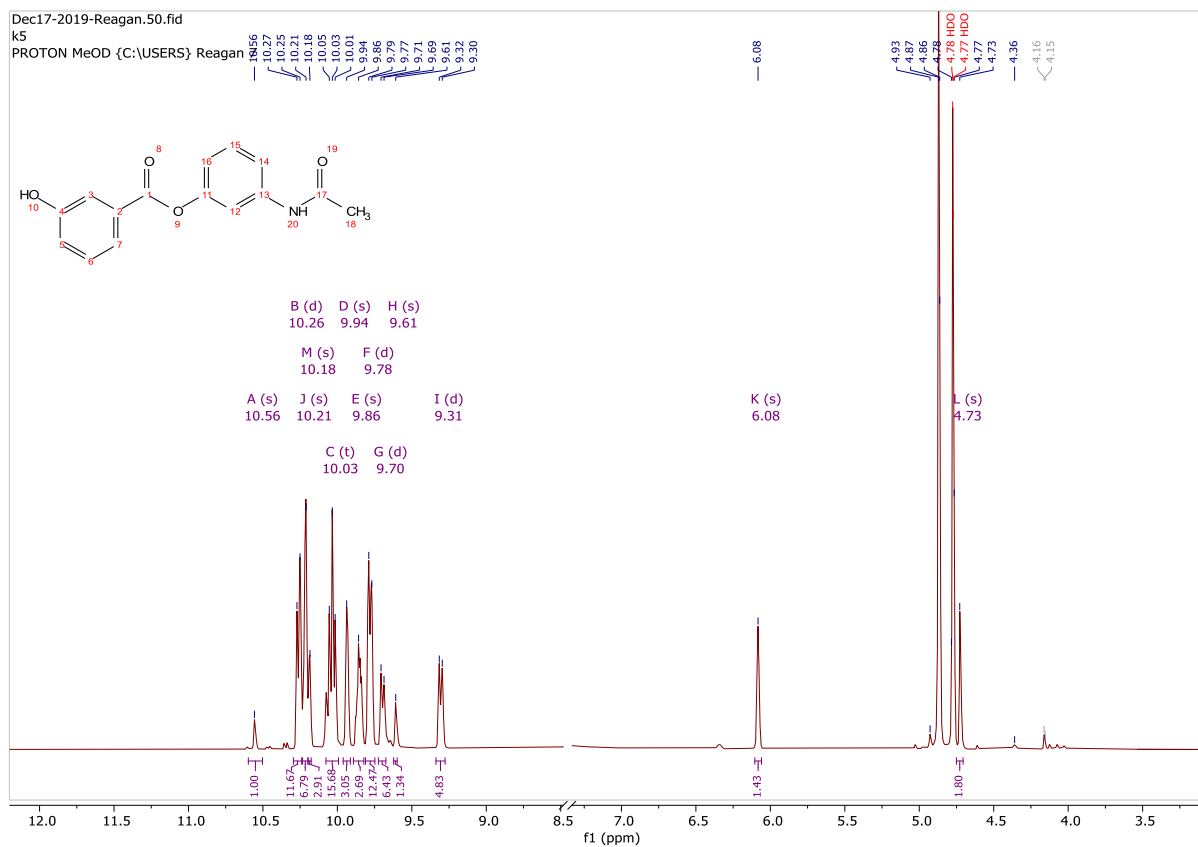
**HRMS of 3-acetylphenyl 3-hydroxybenzoate (C1)**

## Appendix B2: Characterization data of mono-esters from *N*-acetamide

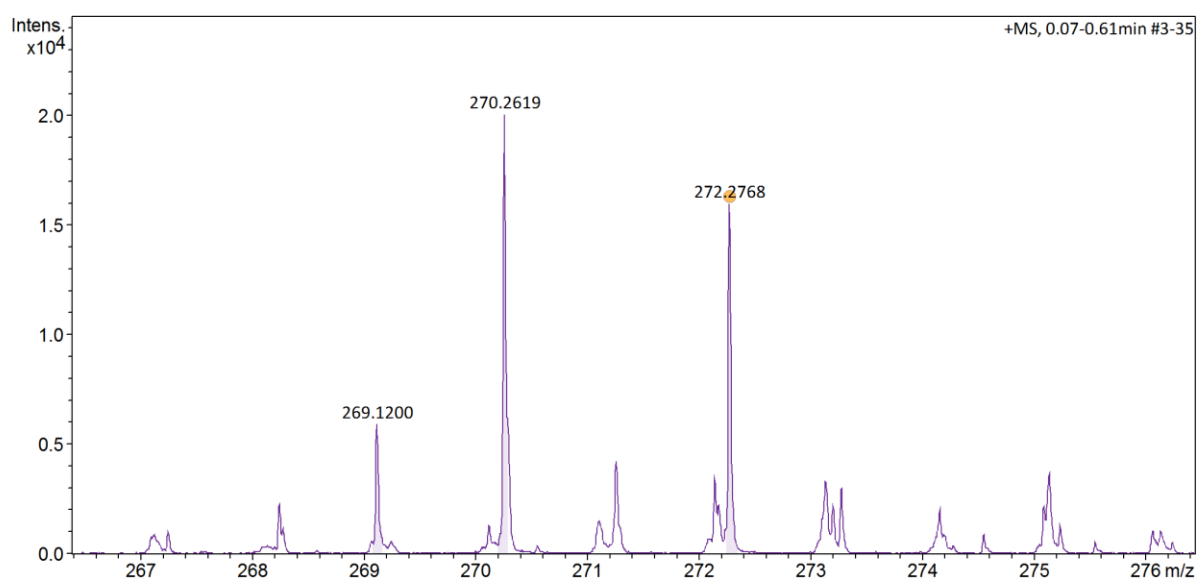
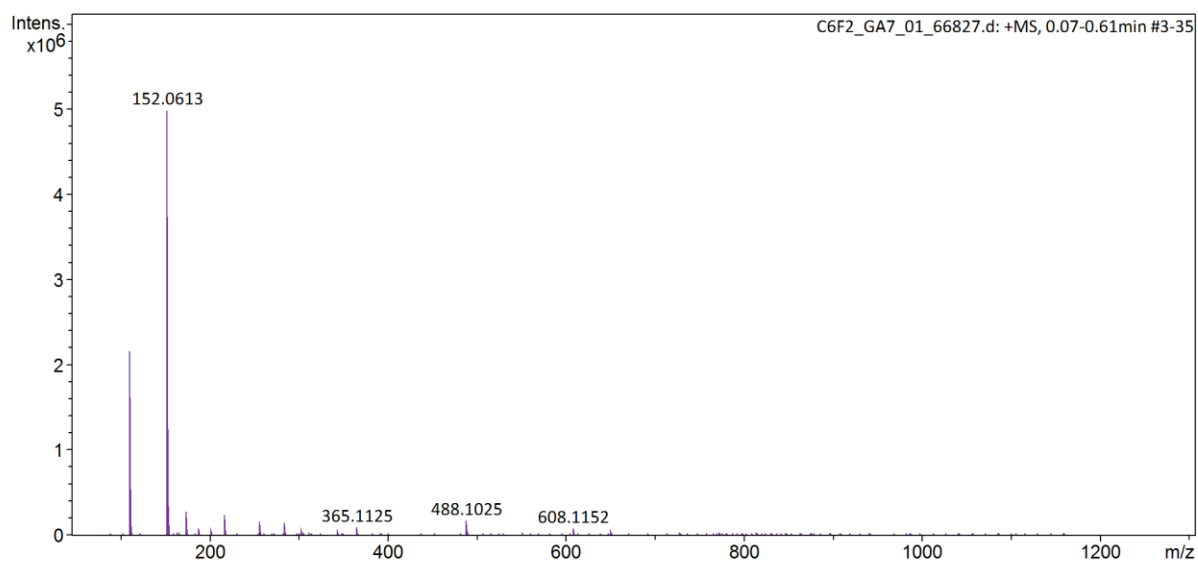


FTIR spectrum of 3-acetamidophenyl 3-hydroxybenzoate (C6)

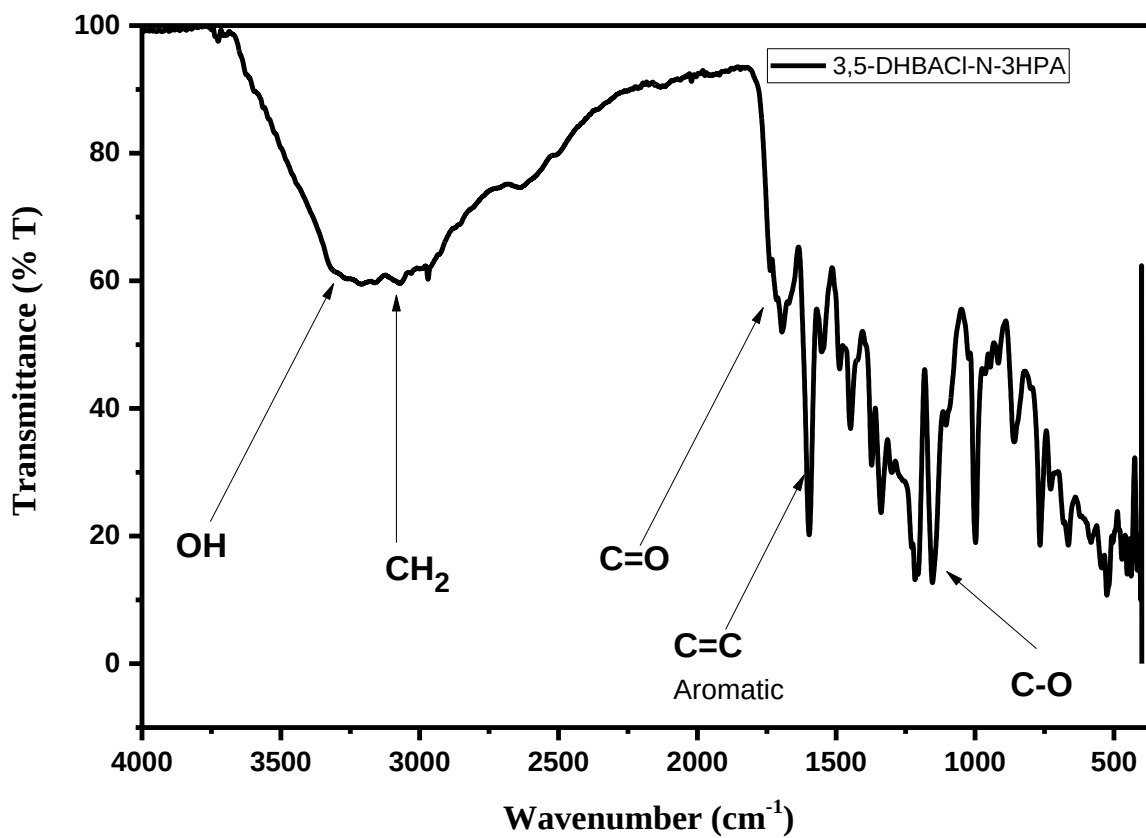
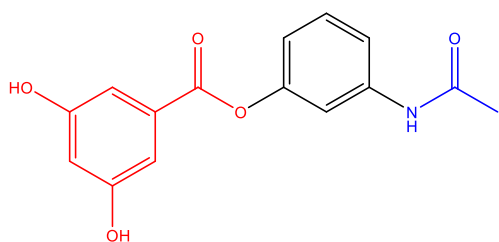




**<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3-acetamidophenyl 3-hydroxybenzoate (C6)**

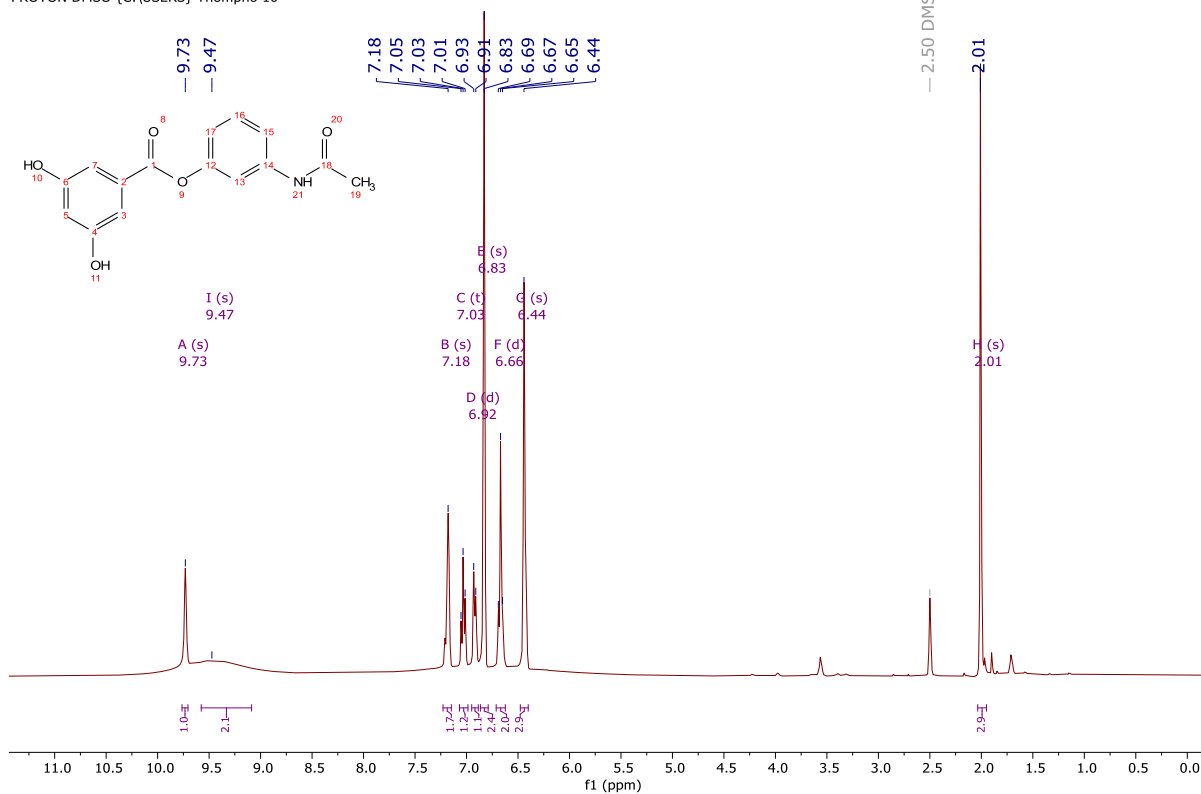


**HRMS of 3-acetamidophenyl 3-hydroxybenzoate (C6)**

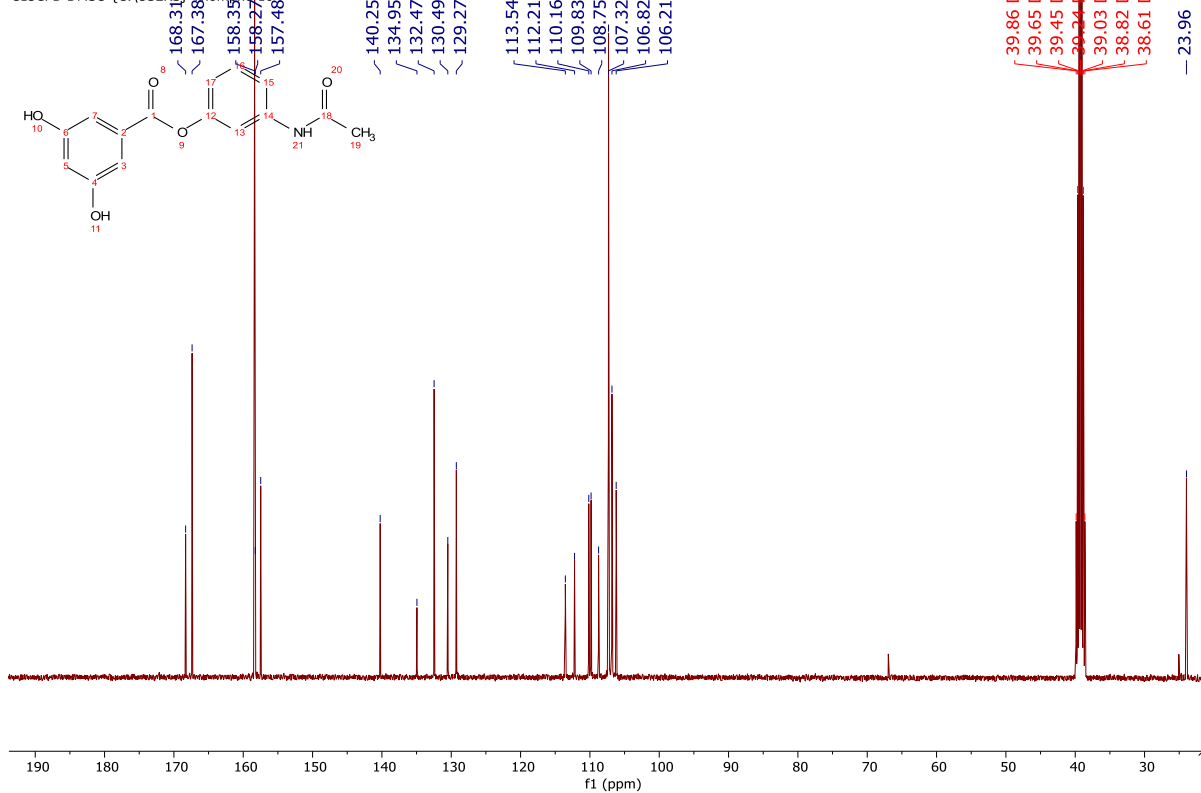


FTIR spectrum of 3-acetamidophenyl 3,5-dihydroxybenzoate (C10)

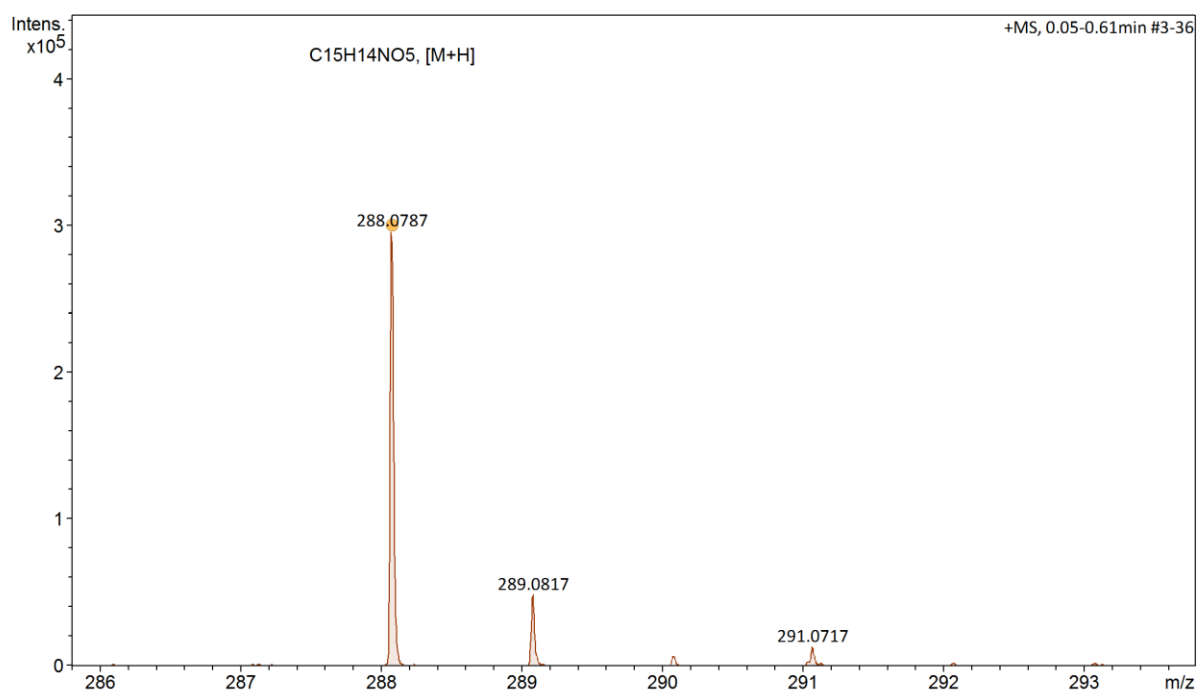
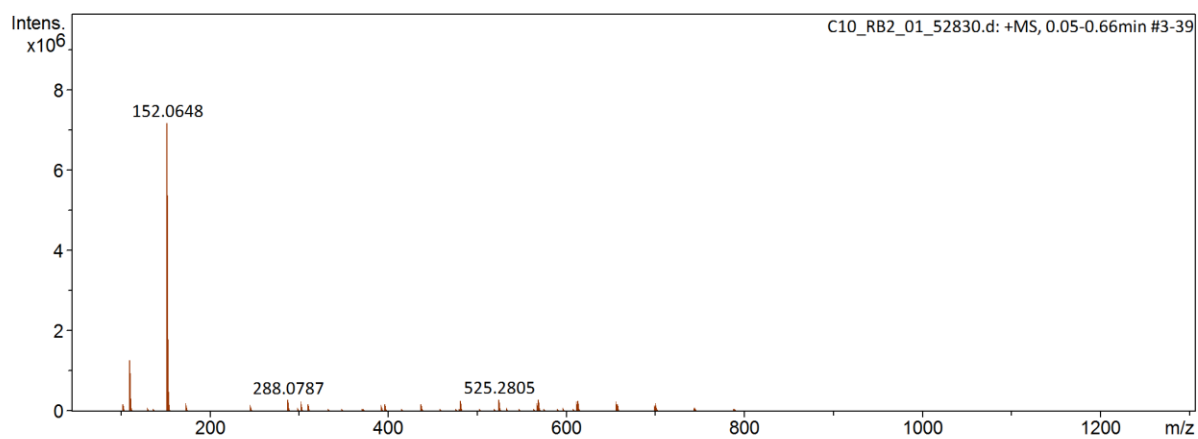
Feb25-2020-Thompho.20.fid  
Km10  
PROTON DMSO {C:\USERS} Thompho 10



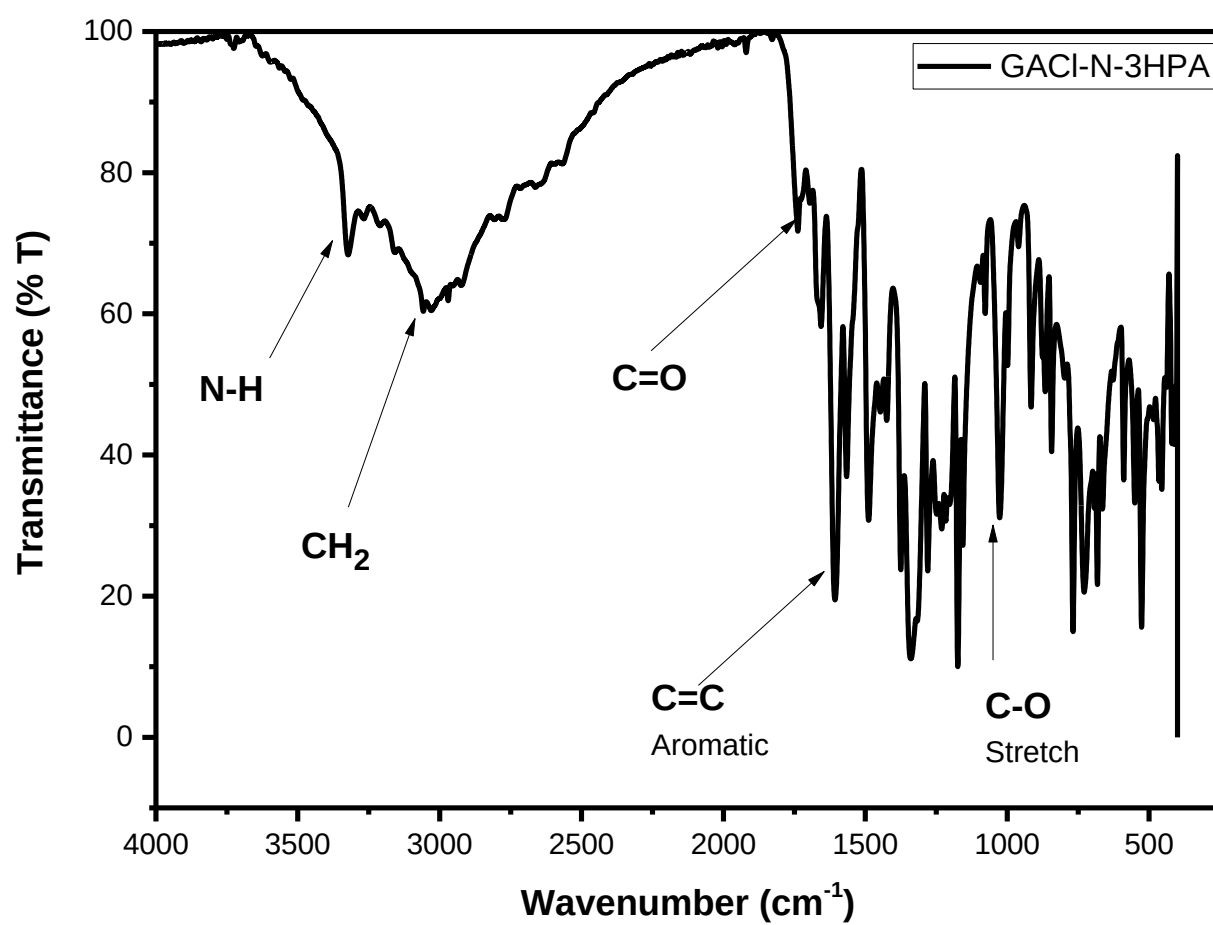
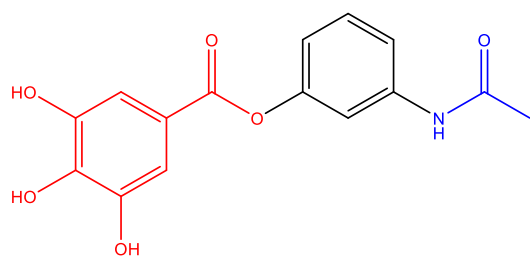
Feb25-2020-Thompho.21.fid  
Km10  
C13CPD DMSO {C:\USERS} Thompho 10



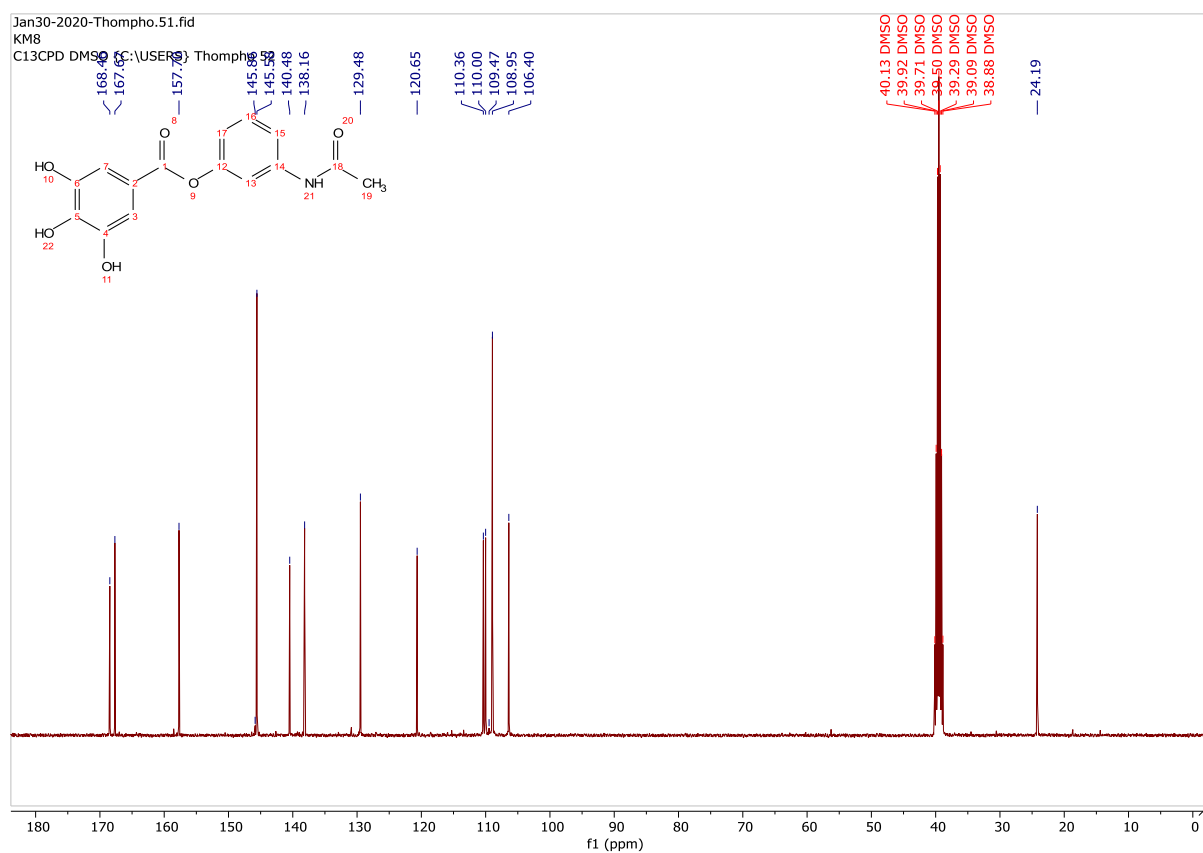
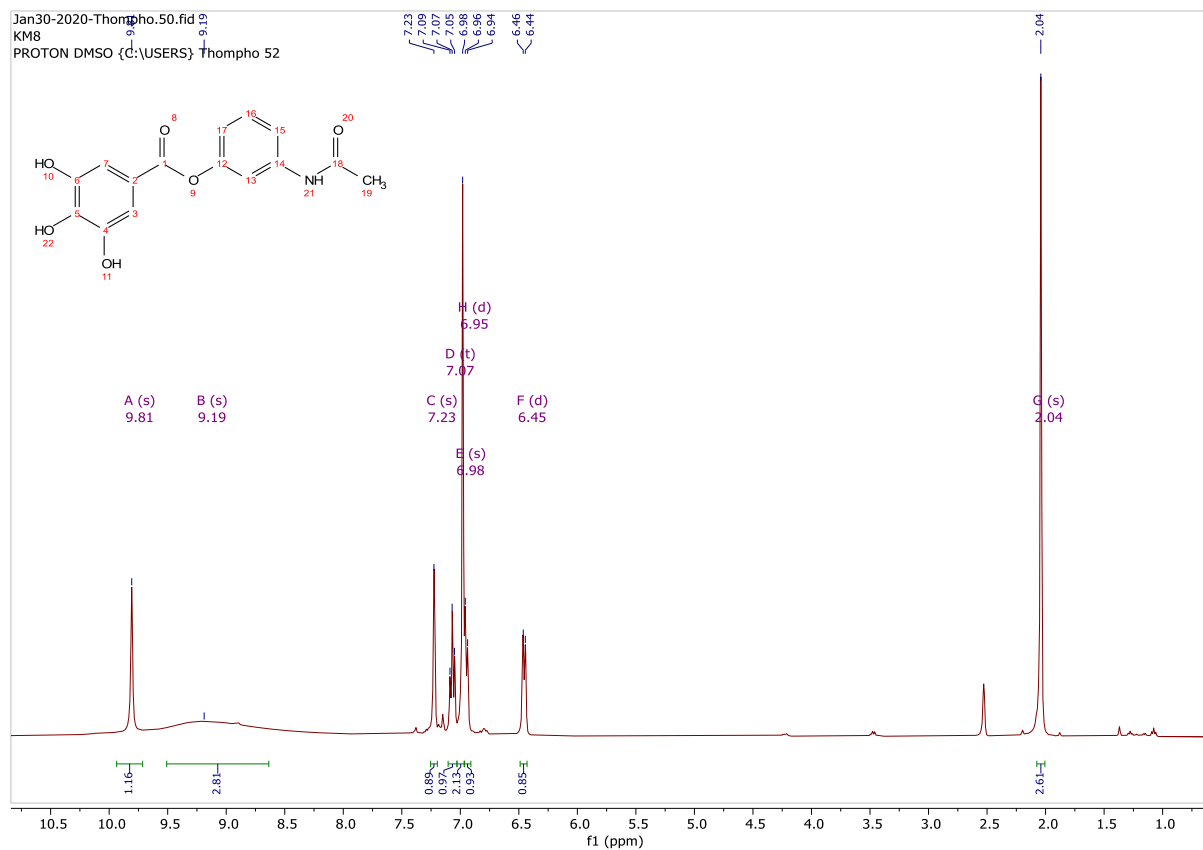
**<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3-acetamidophenyl 3,5-dihydroxybenzoate (C10)**



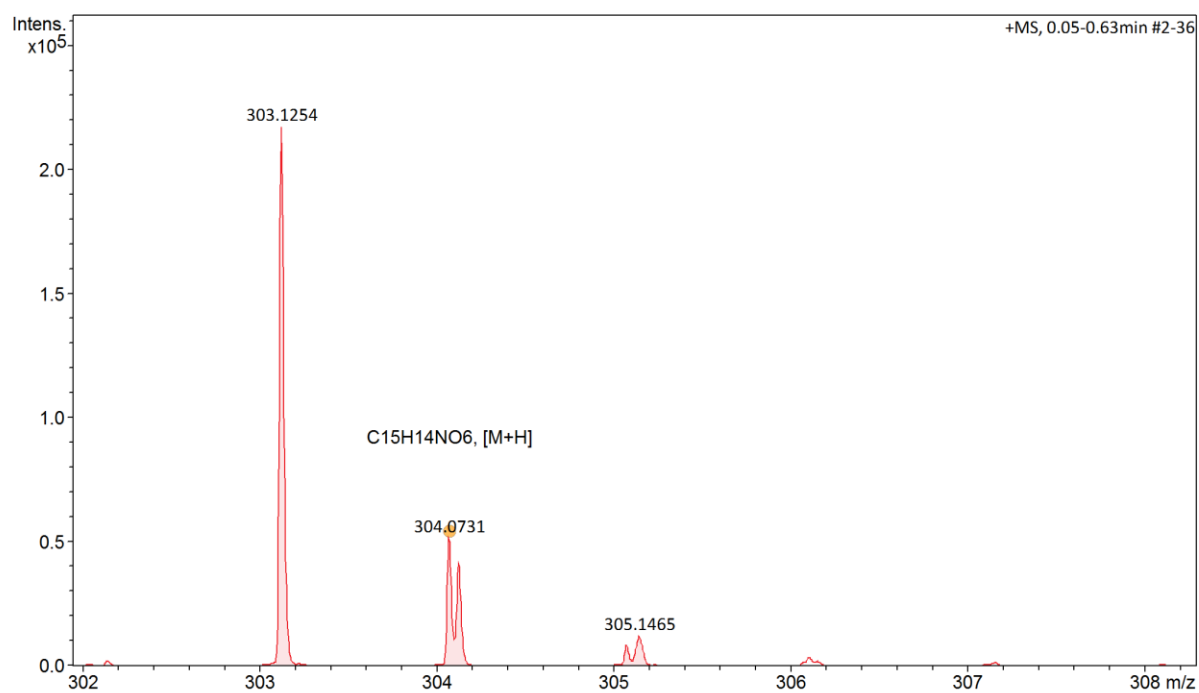
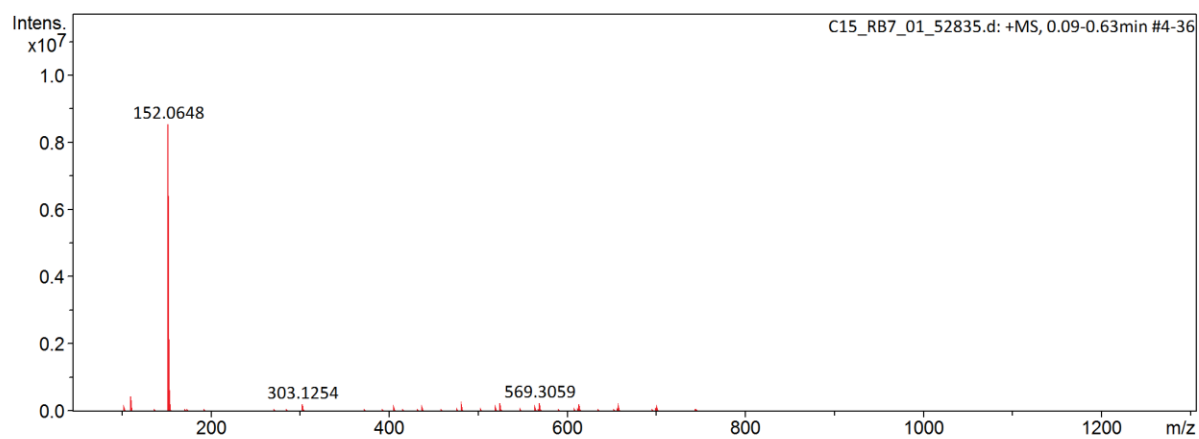
**HRMS of 3-acetamidophenyl 3,5-dihydroxybenzoate (C10)**



FTIR spectrum of 3-acetamidophenyl 3,4,5-trihydroxybenzoate (C15)



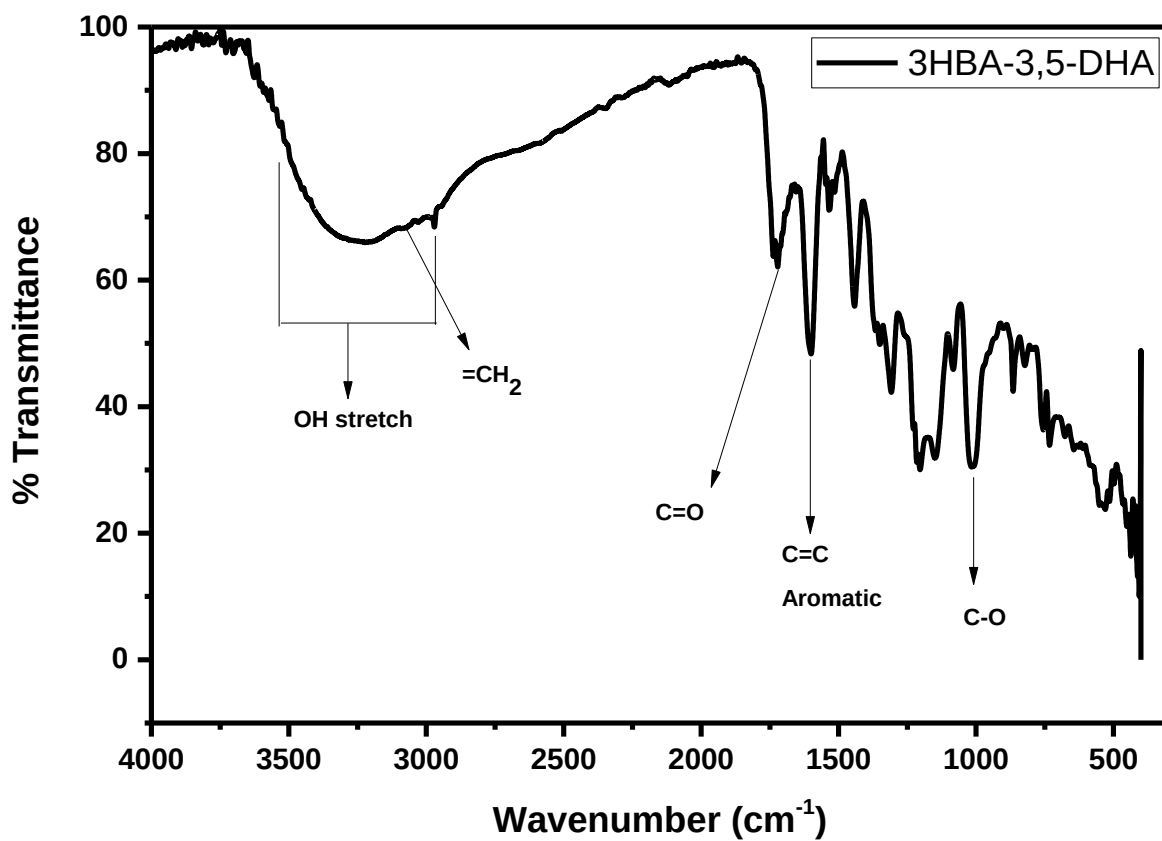
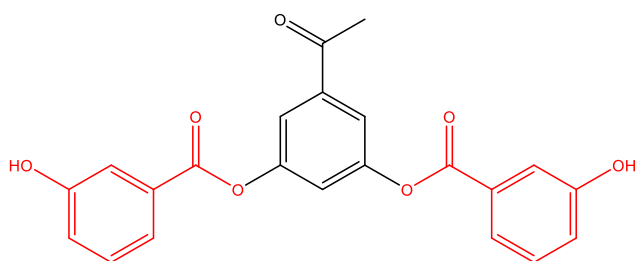
**<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3-acetamidophenyl 3,4,5-trihydroxybenzoate (C15)**



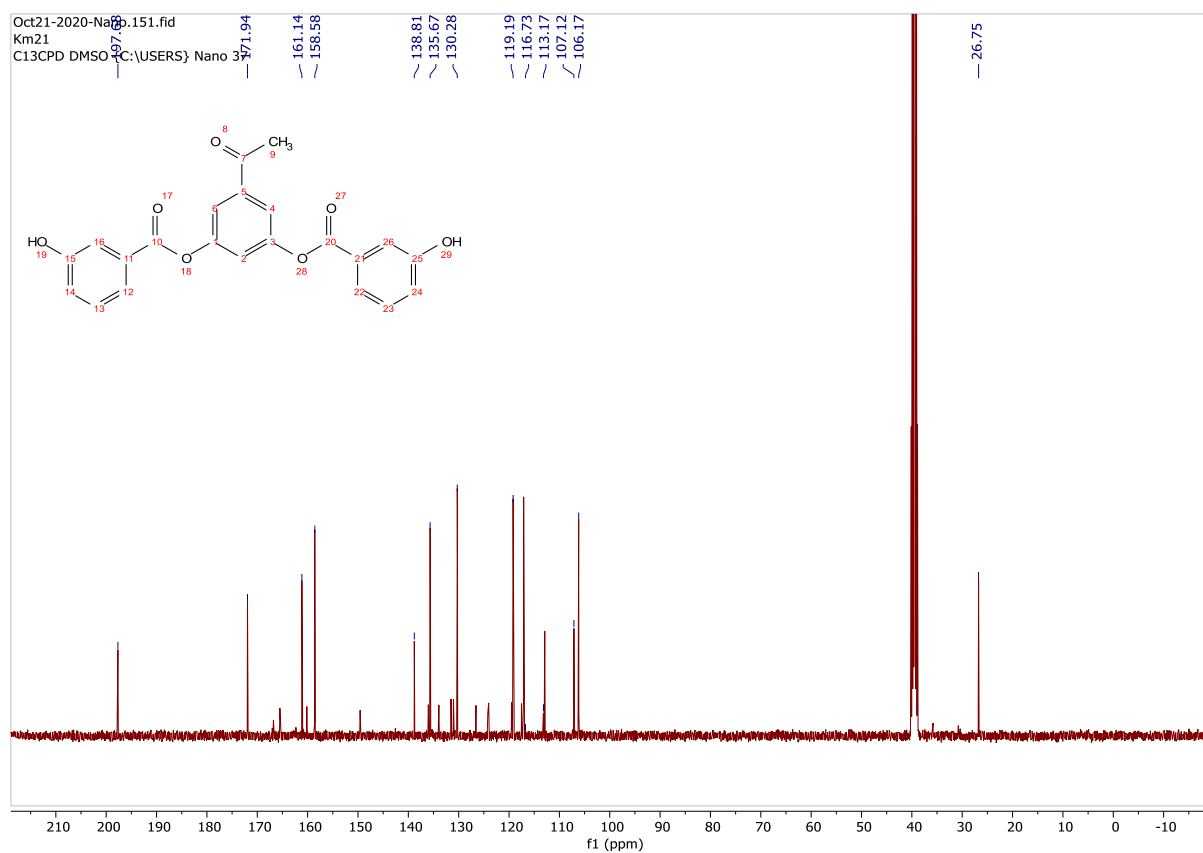
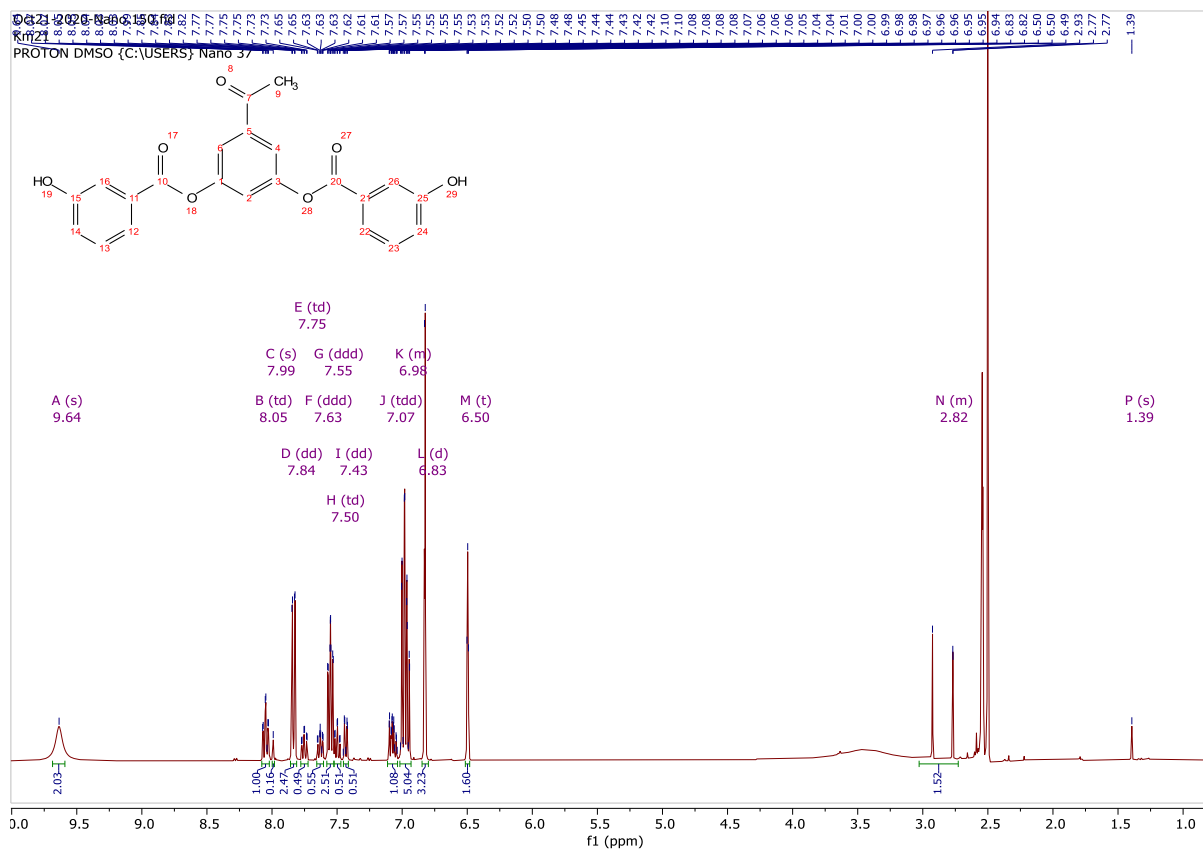
**HRMS of 3-acetamidophenyl 3,4,5-trihydroxybenzoate (C15)**



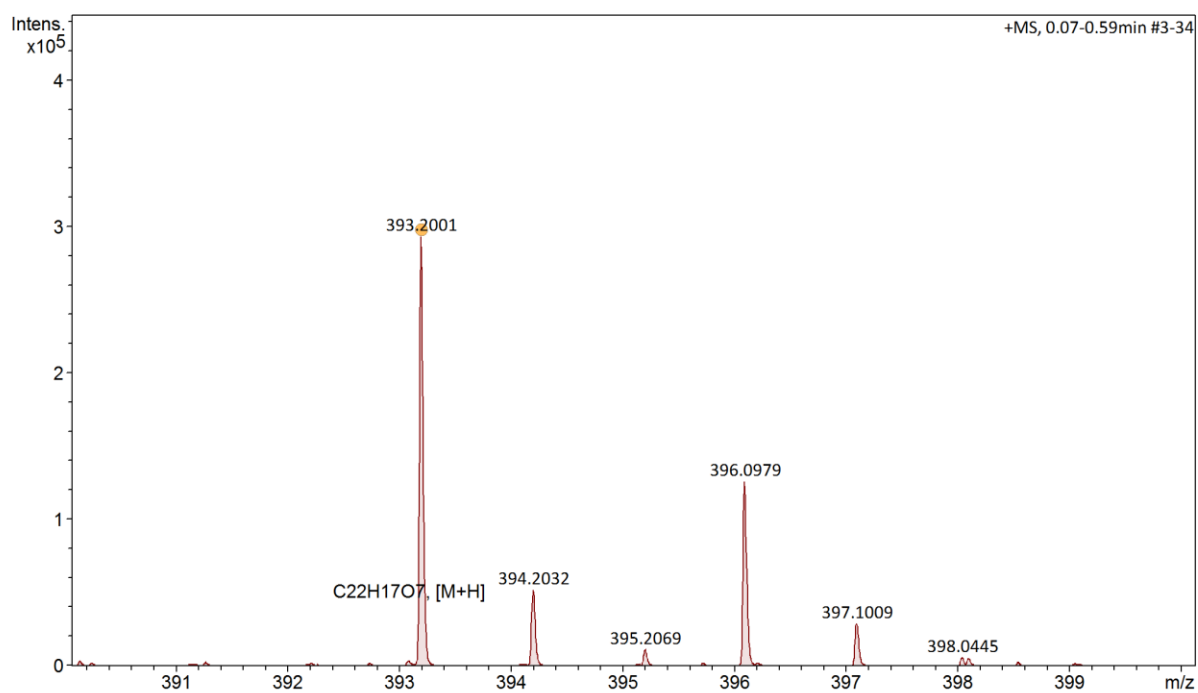
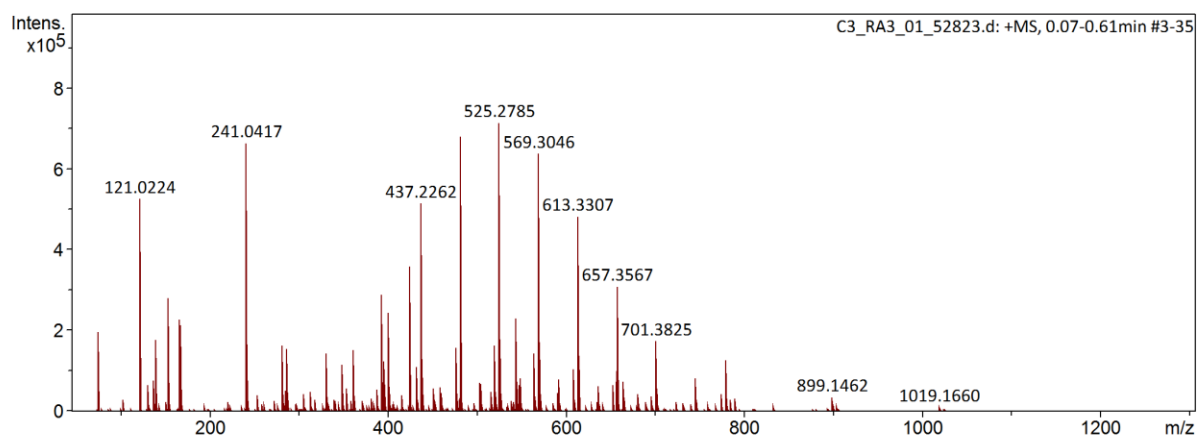
## Appendix B3: Characterization data of di-esters



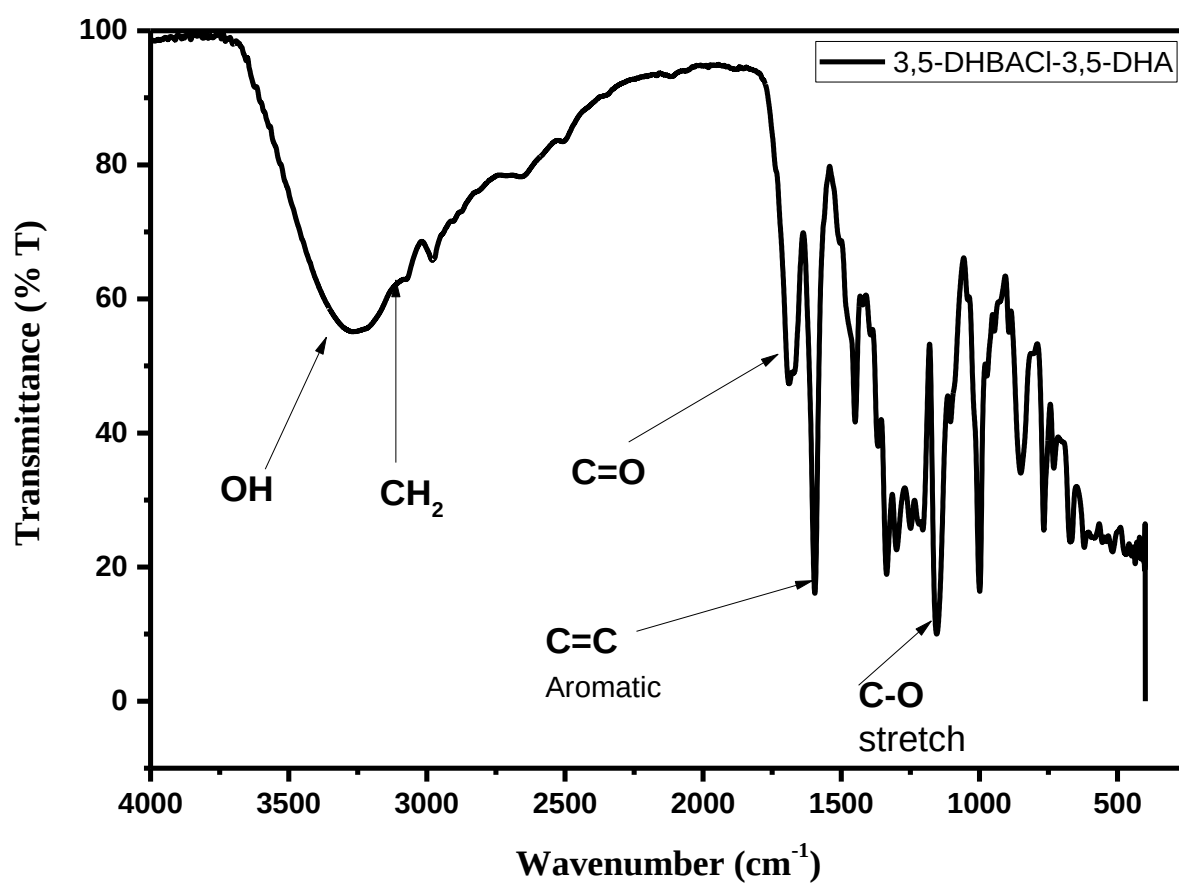
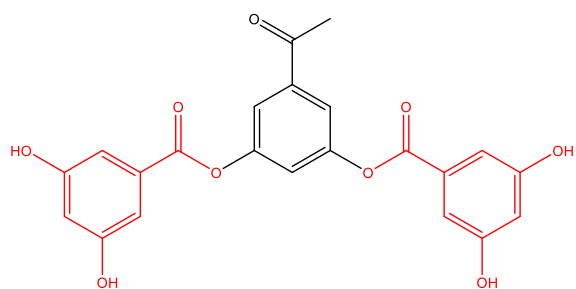
FTIR spectrum of 5-acetyl-1,3-phenylene bis(3-hydroxybenzoate) (C3)



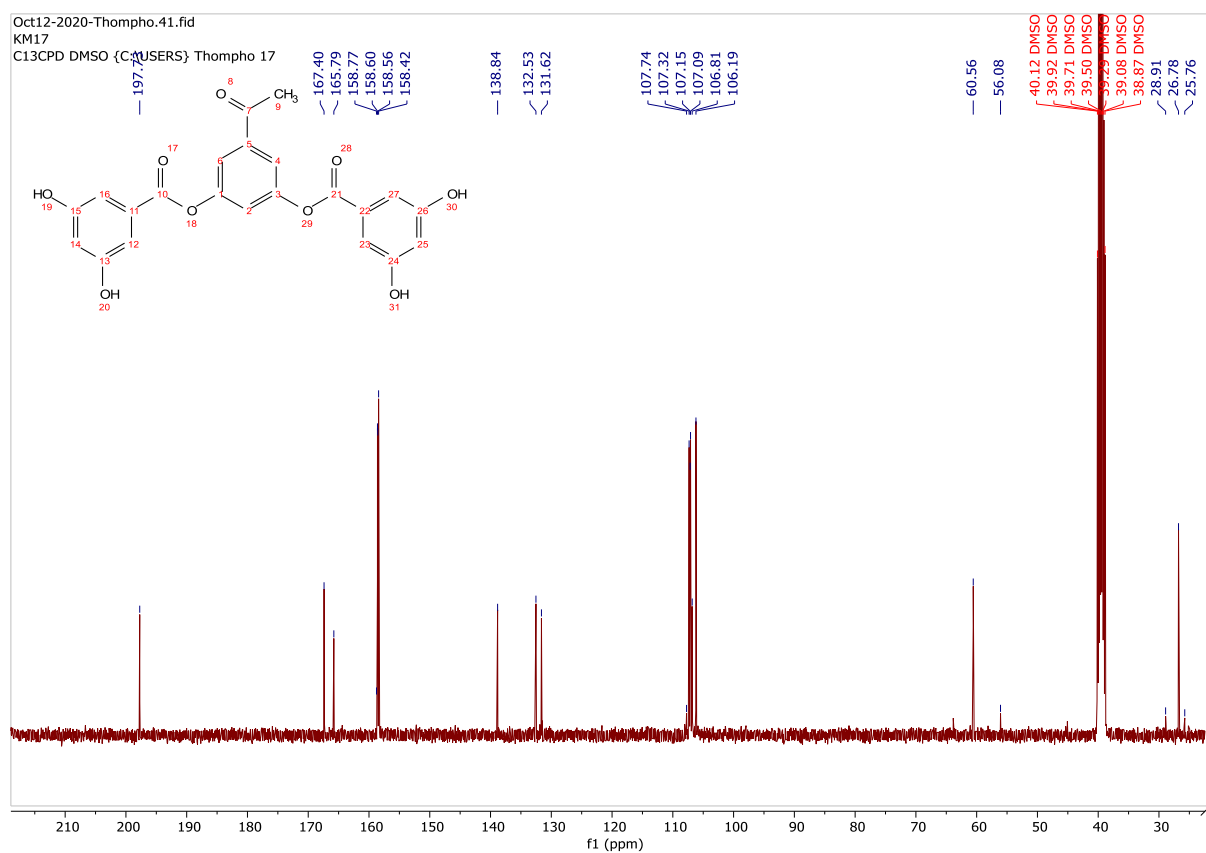
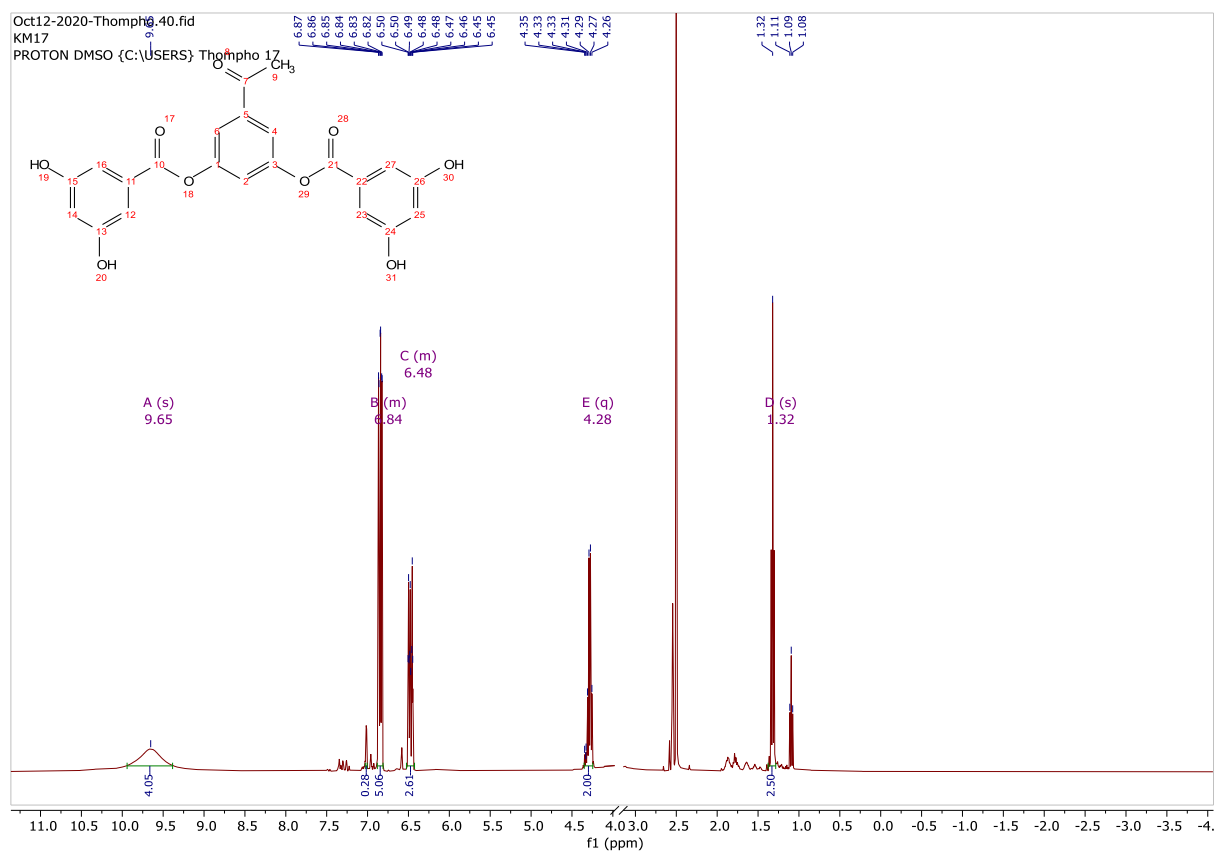
**<sup>1</sup>H and <sup>13</sup>C NMR of 5-acetyl-1,3-phenylene bis(3-hydroxybenzoate) (C3)**



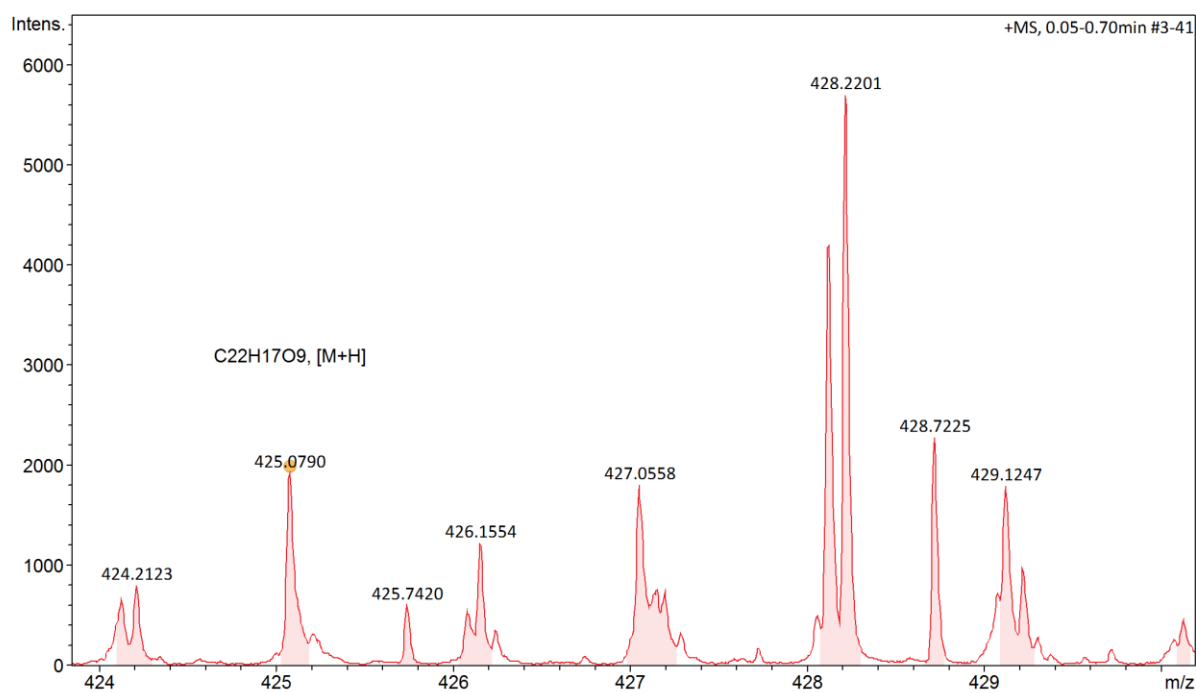
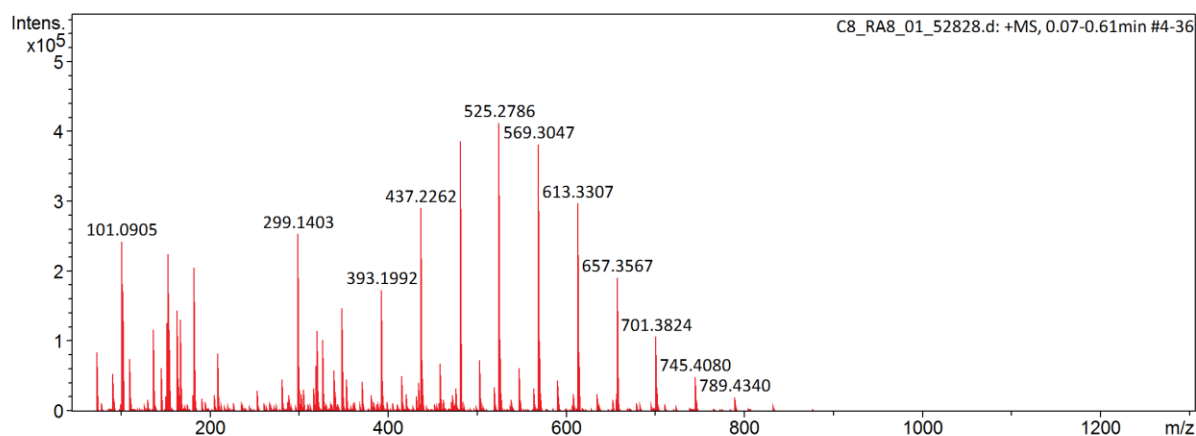
**HRMS of 5-acetyl-1,3-phenylene bis(3-hydroxybenzoate) (C3)**



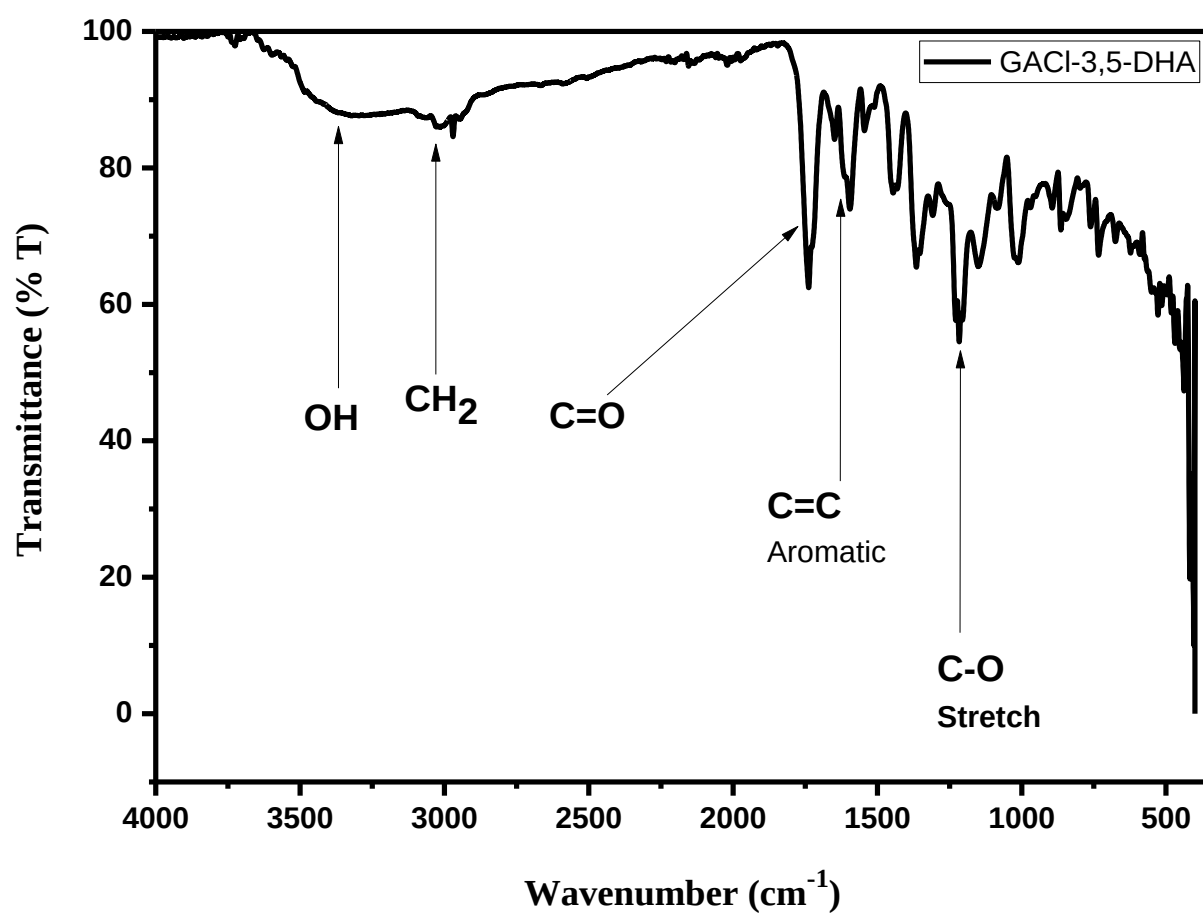
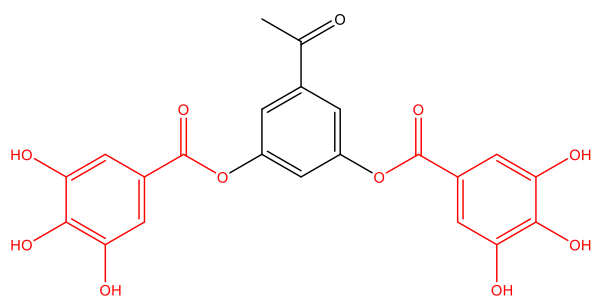
FTIR spectrum of 5-acetyl-1,3-phenylene bis(3,5-dihydroxybenzoate) (C8)



### <sup>1</sup>H and <sup>13</sup>C NMR of 5-acetyl-1,3-phenylene bis(3,5-dihydroxybenzoate) (C8)

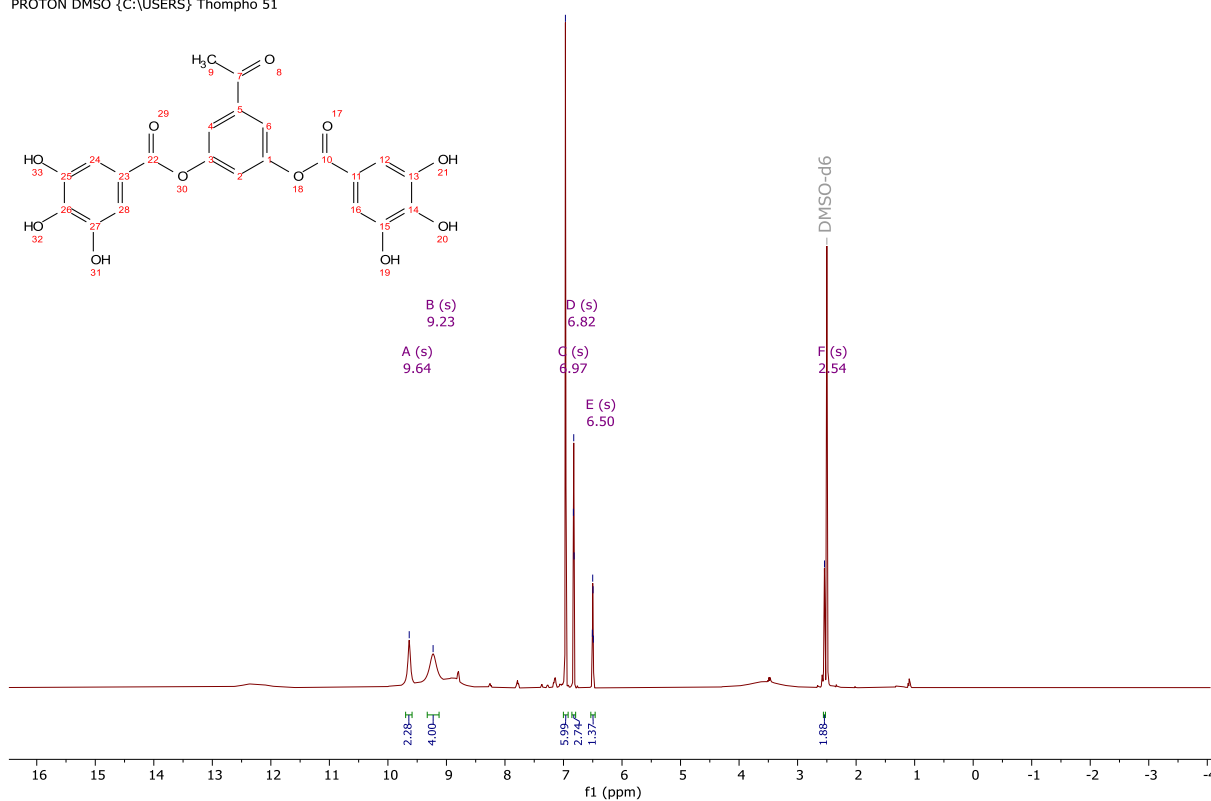


**HRMS of 5-acetyl-1,3-phenylene bis(3,5-dihydroxybenzoate) (C8)**

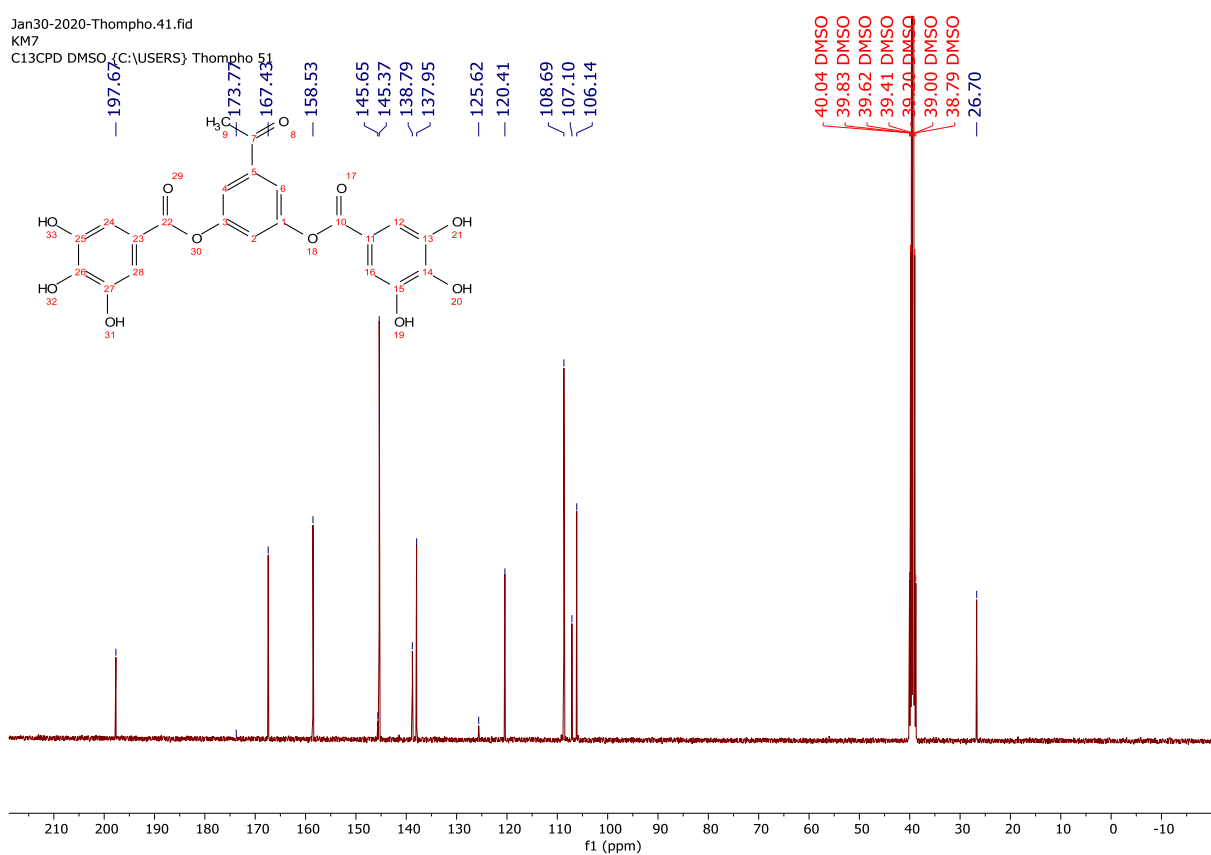


FTIR spectrum of 5-acetyl-1,3-phenylene bis(3,4,5-trihydroxybenzoate) (C12)

Jan30-2020-Thompho.40.fid  
KM7  
PROTON DMSO {C:\USERS} Thompho 51

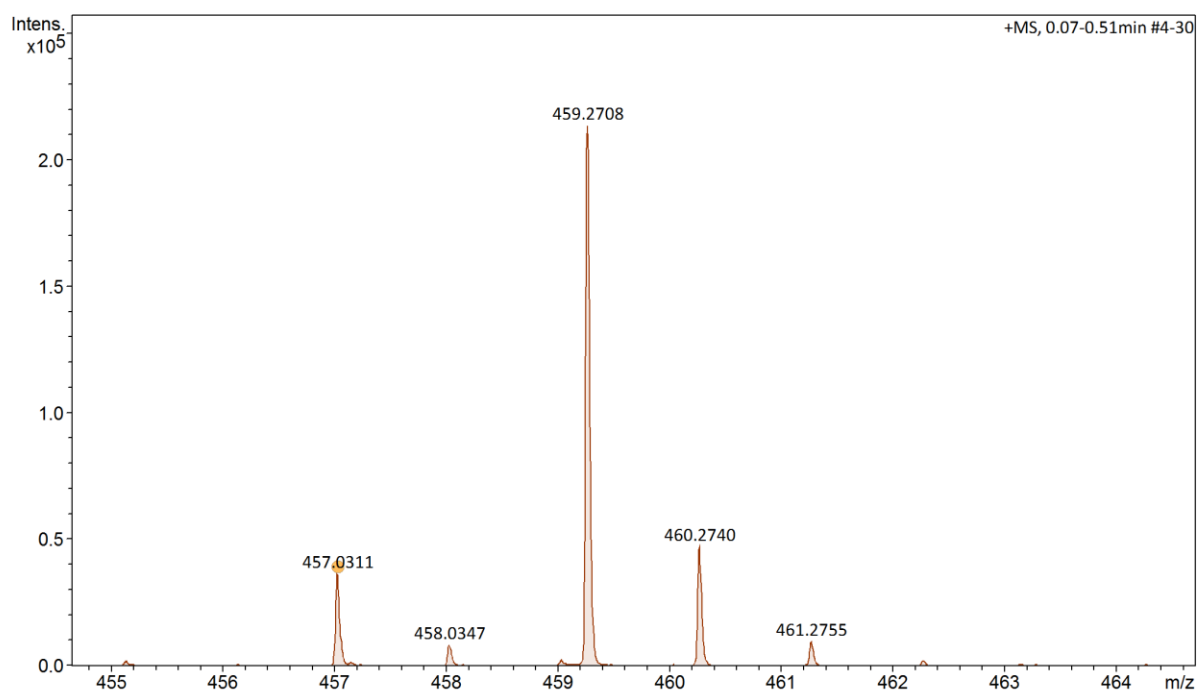
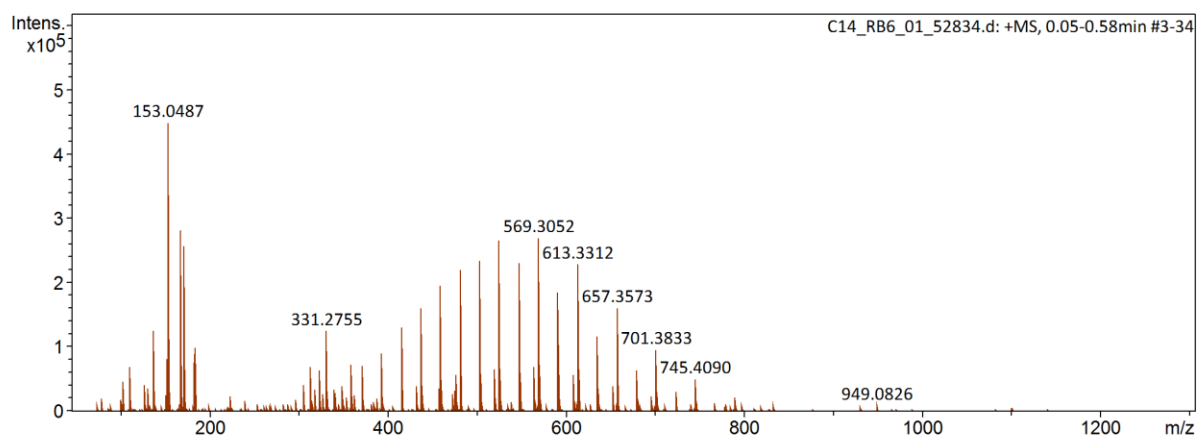


Jan30-2020-Thompho.41.fid  
KM7  
C13CPD DMSO {C:\USERS} Thompho 51



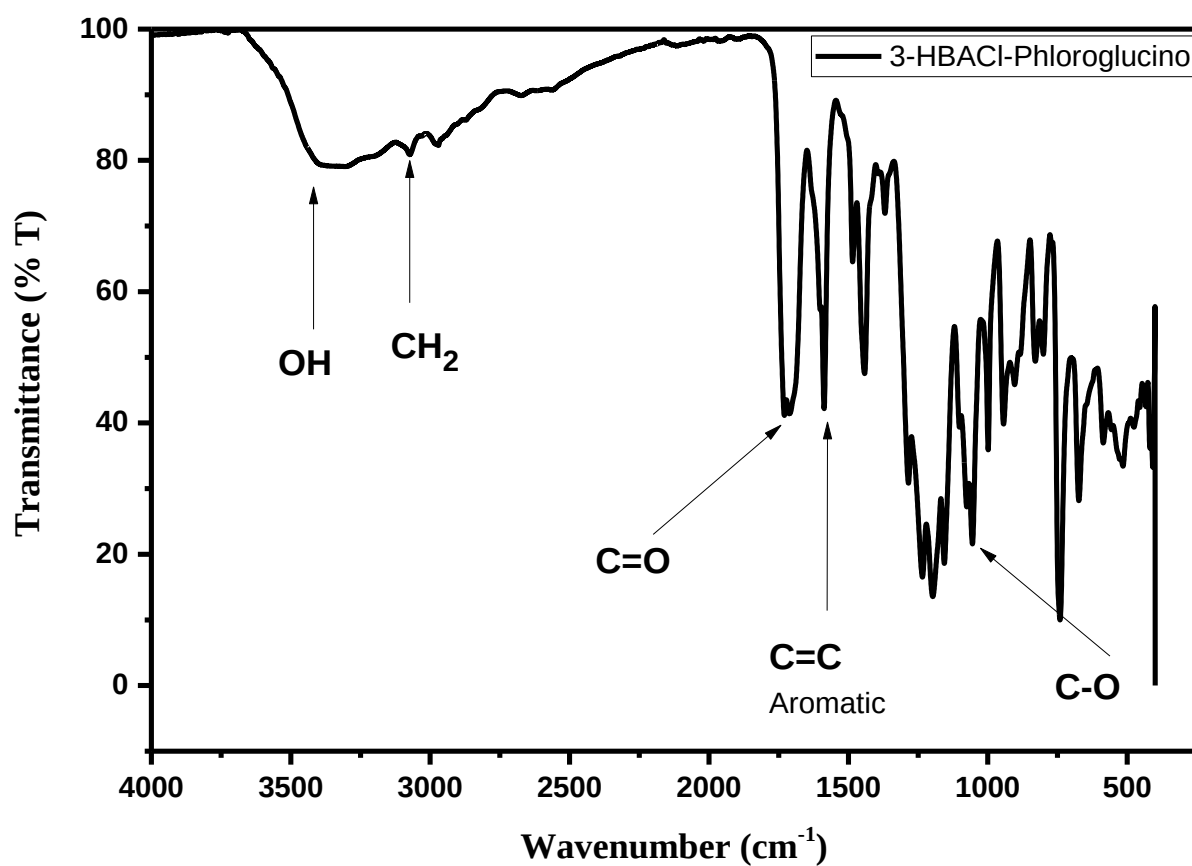
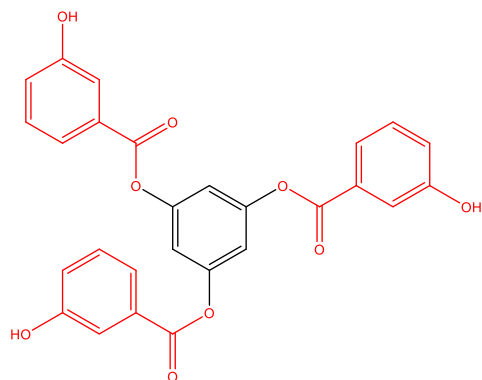
**<sup>1</sup>H and <sup>13</sup>C NMR of 5-acetyl-1,3-phenylene bis(3,4,5-trihydroxybenzoate) (C12)**



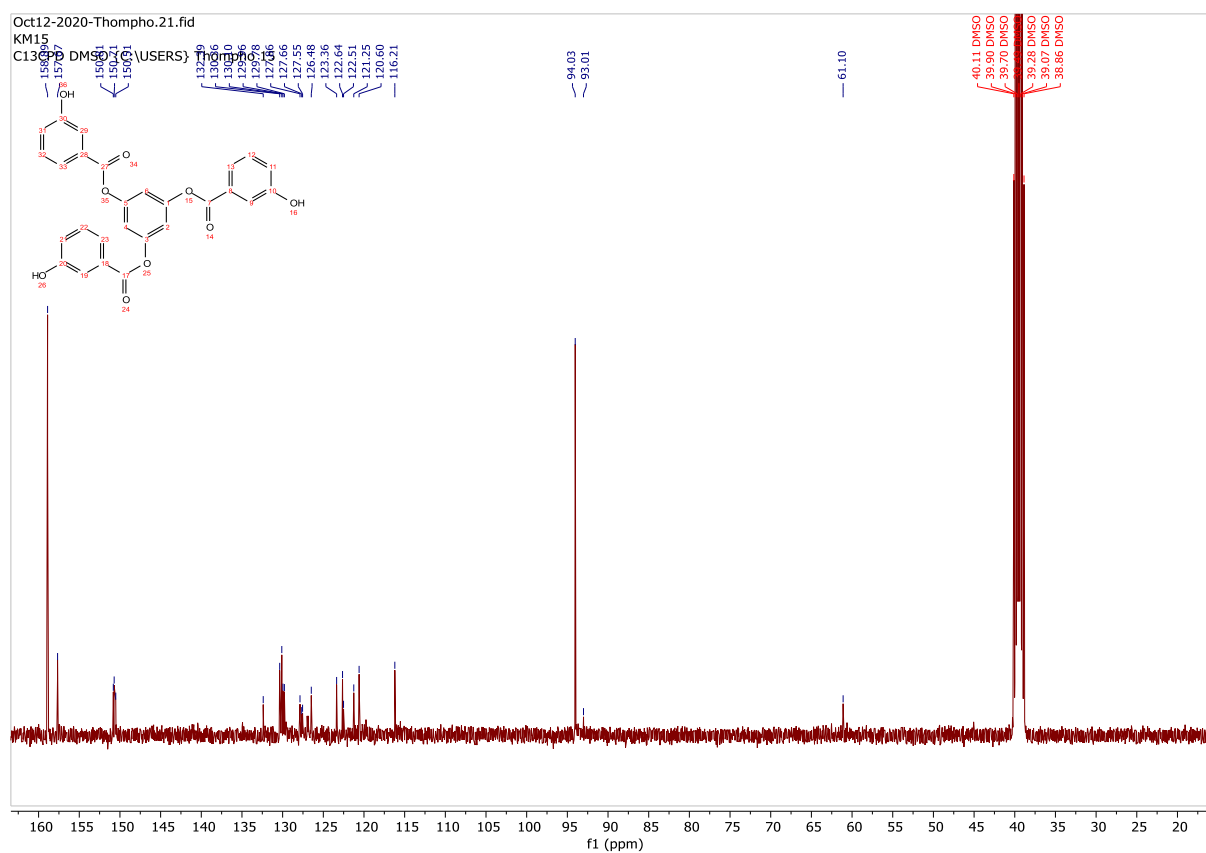
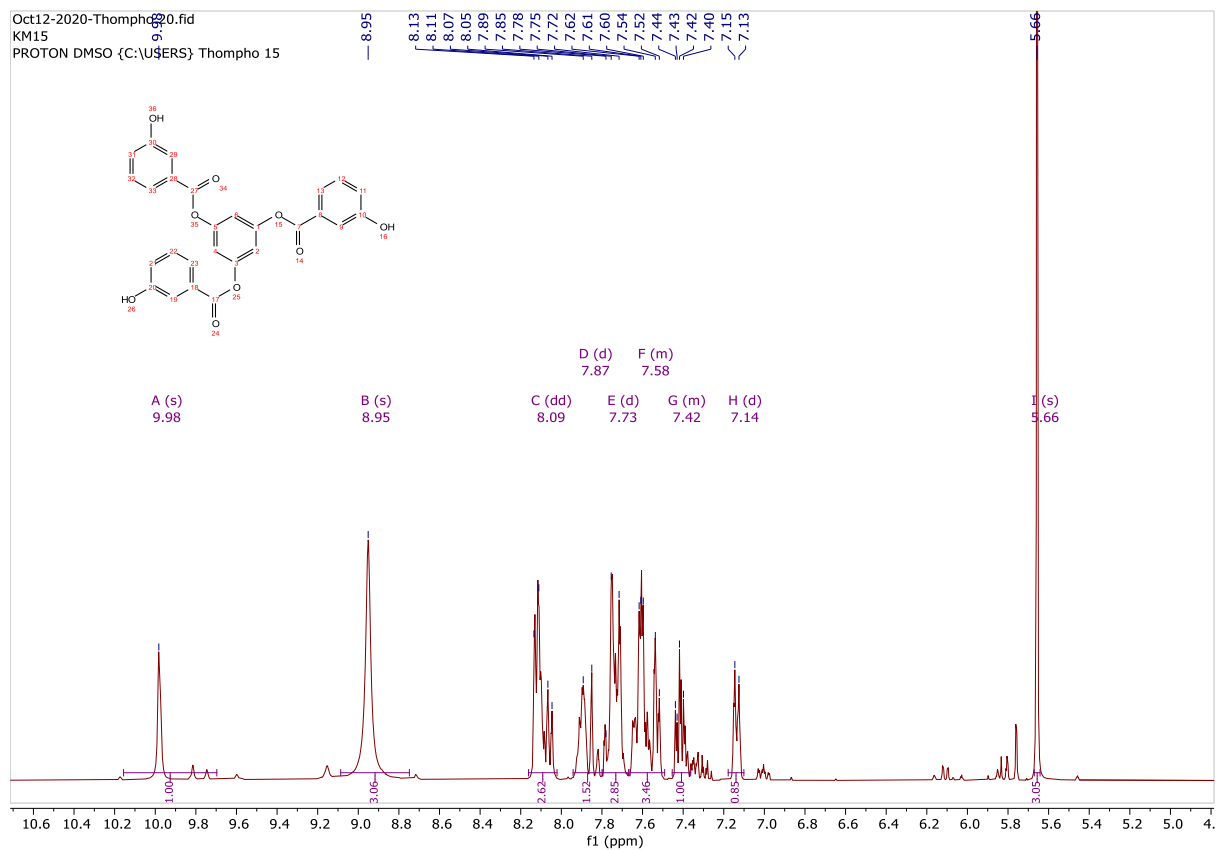


**HRMS of 5-acetyl-1,3-phenylene bis(3,4,5-trihydroxybenzoate) (C12)**

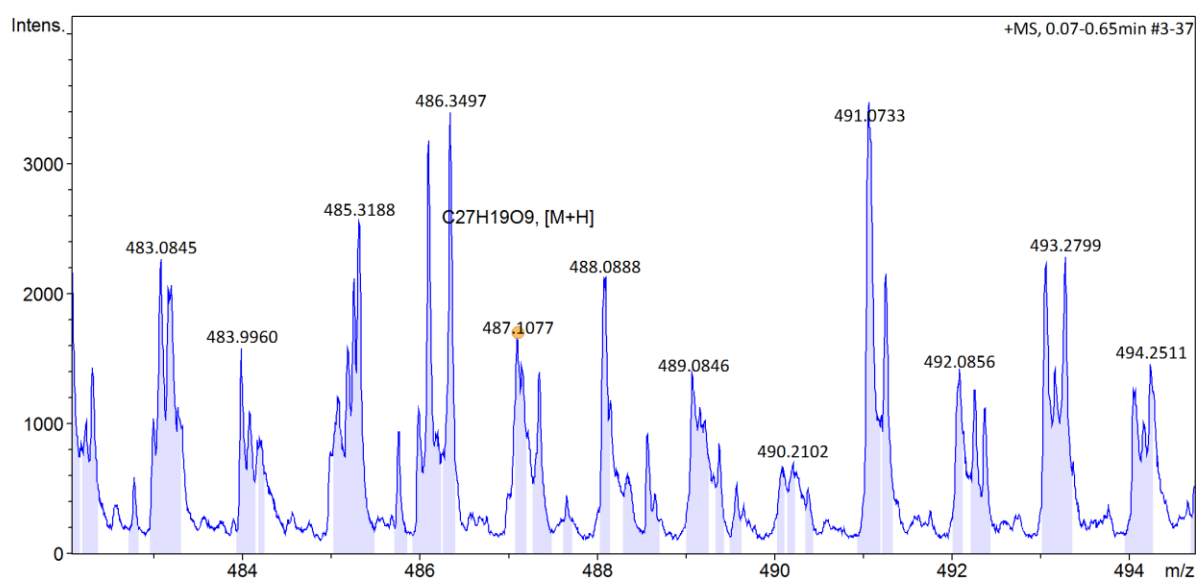
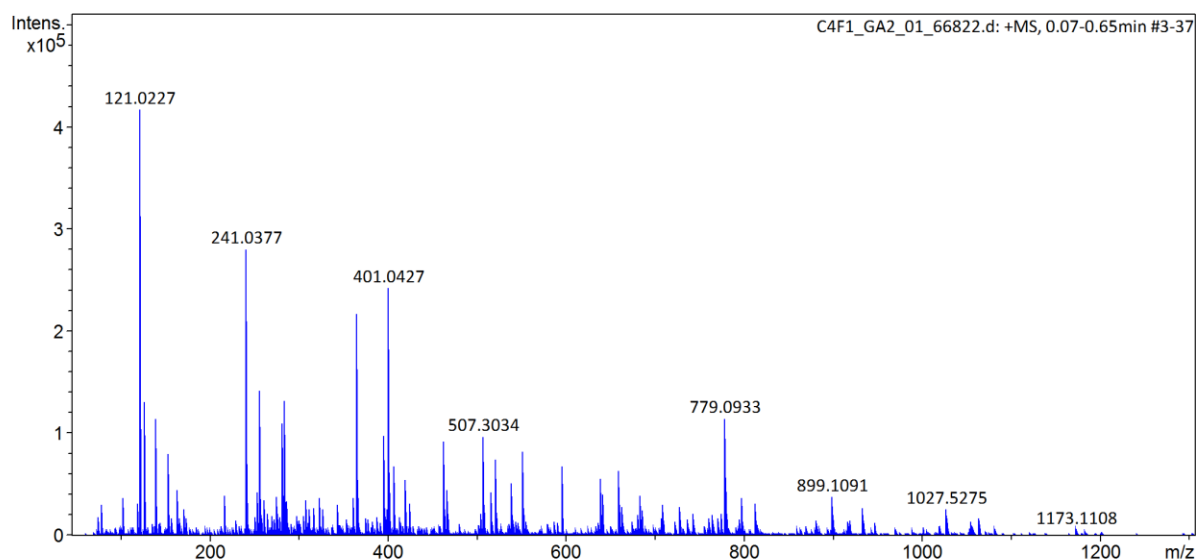
## Appendix B4: Characterization data of tri-substituted esters



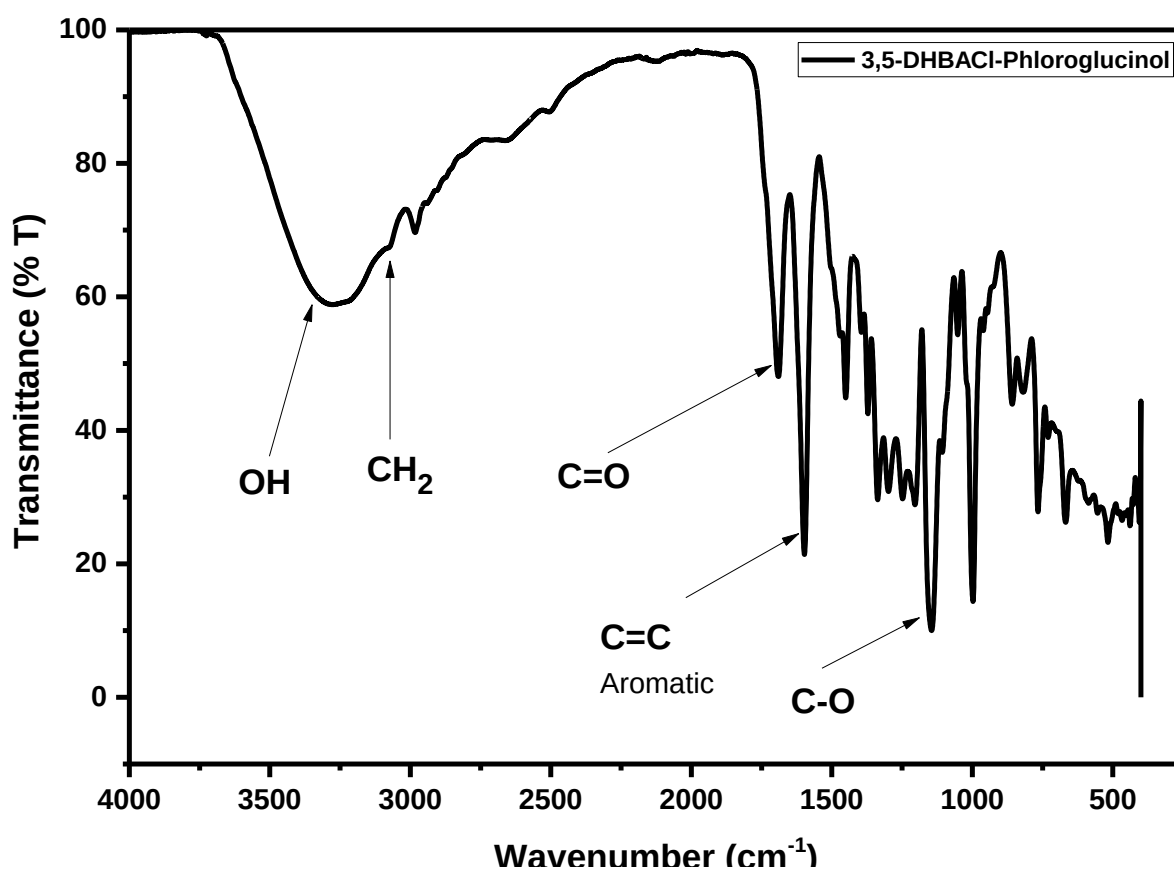
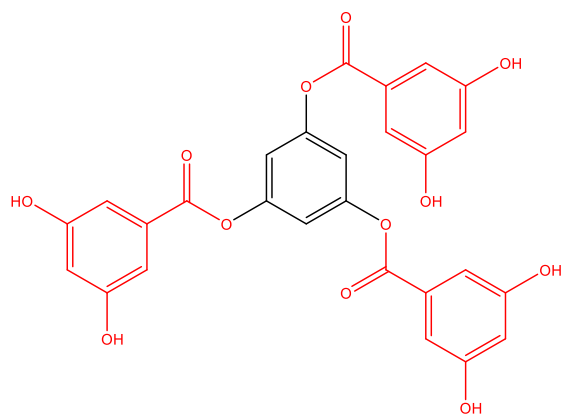
FTIR spectrum of benzene-1,3,5-triyl tris(3-hydroxybenzoate) (C4)



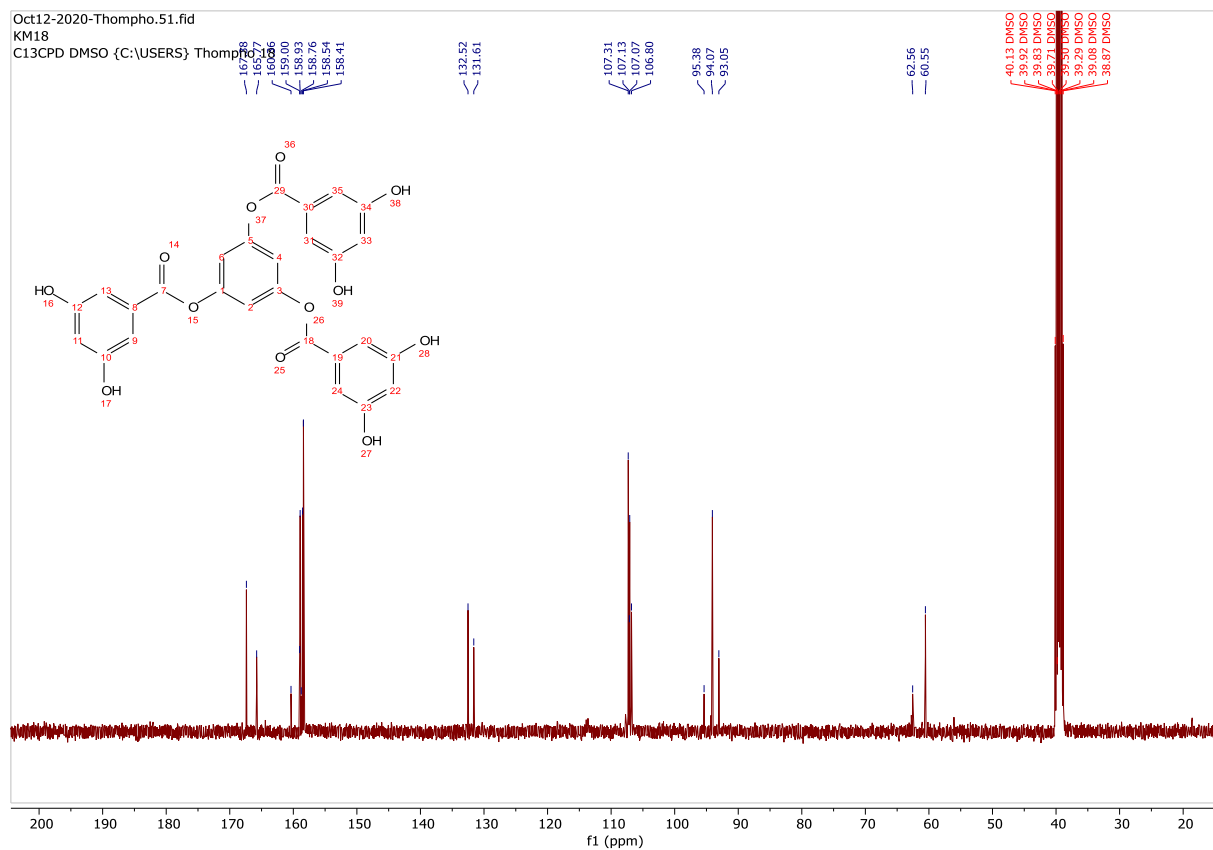
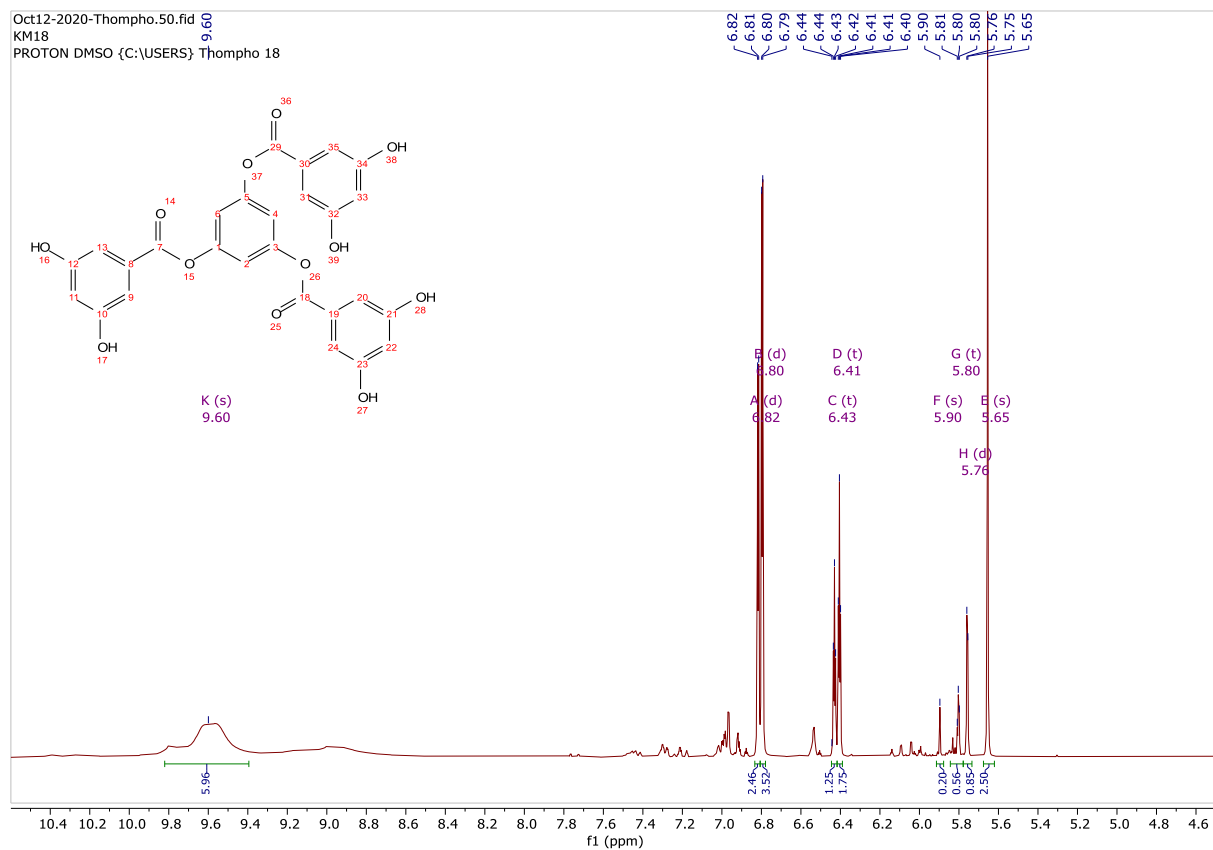
**<sup>1</sup>H and <sup>13</sup>C NMR of benzene-1,3,5-triyl tris(3-hydroxybenzoate) (C4)**



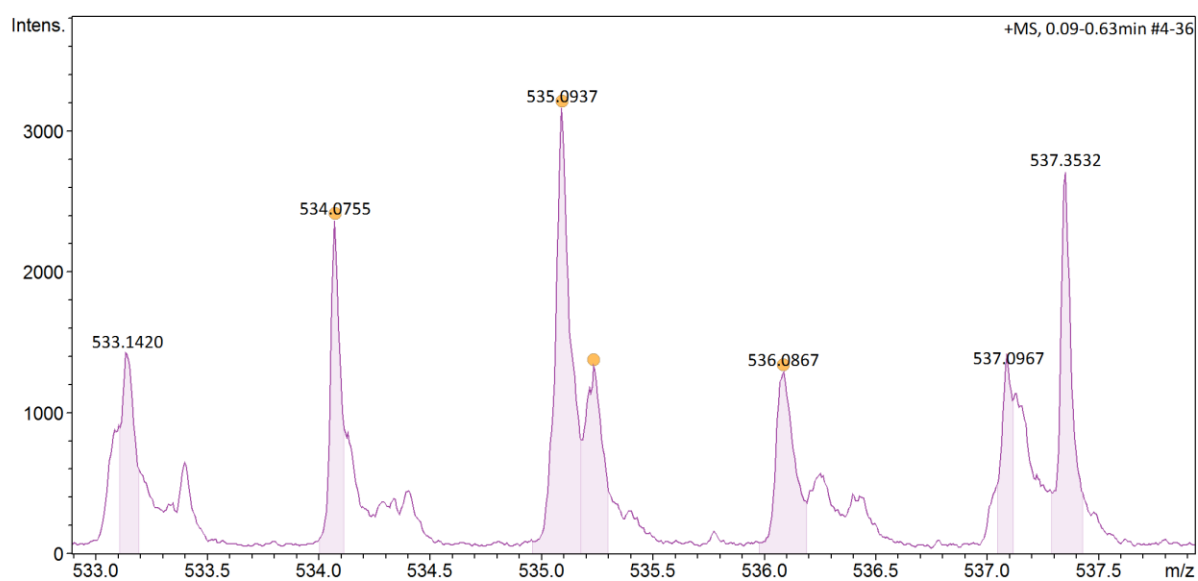
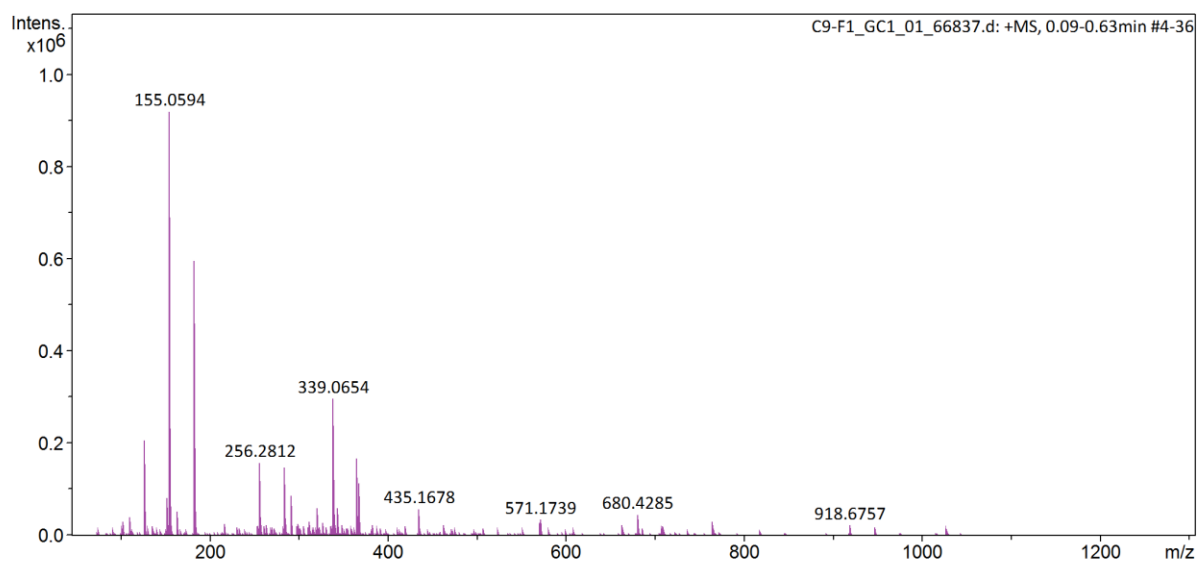
**HRMS of benzene-1,3,5-triyl tris(3-hydroxybenzoate) (C4)**



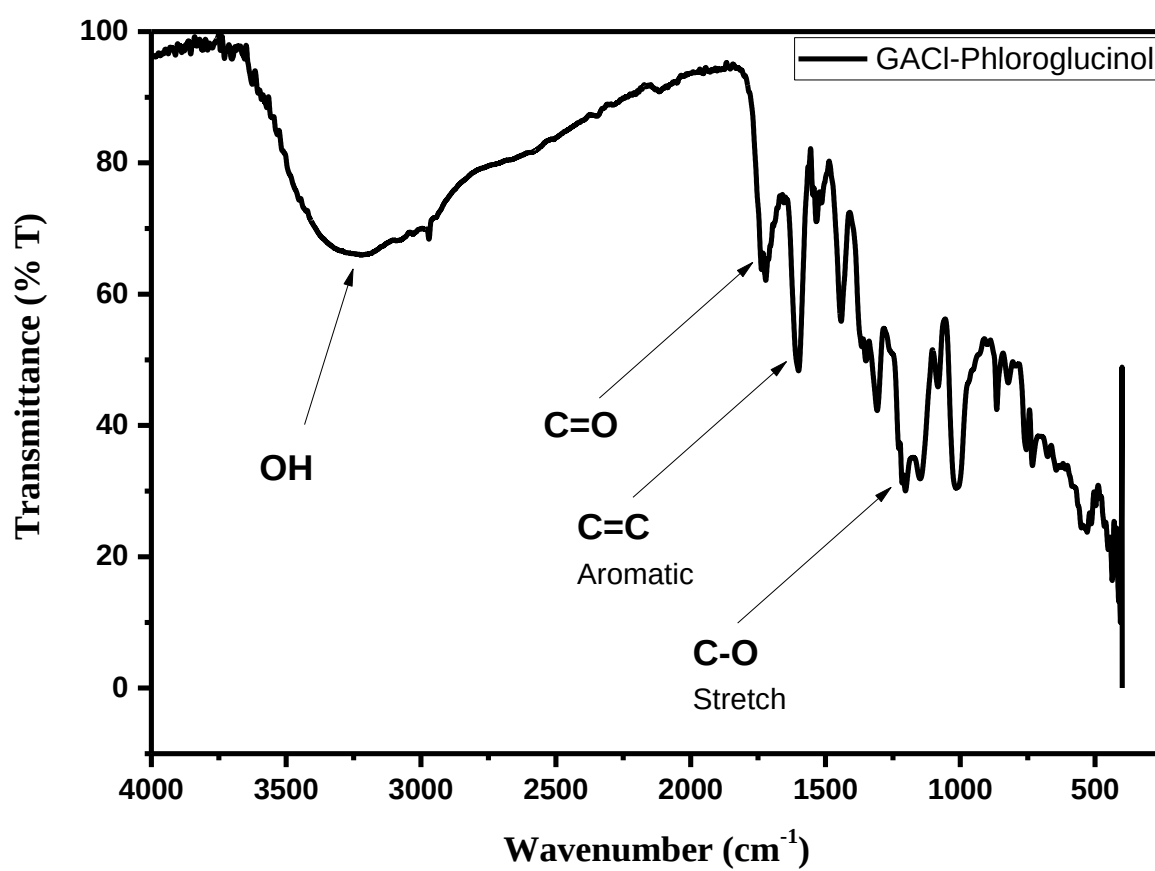
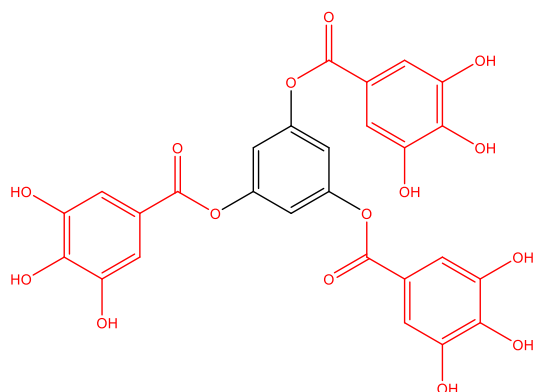
FTIR spectrum of Benzene-1,3,5-triyl tris(3,5-dihydroxybenzoate) (C9)



$^1\text{H}$  and  $^{13}\text{C}$  NMR of Benzene-1,3,5-triyl tris(3,5-dihydroxybenzoate) (C9)

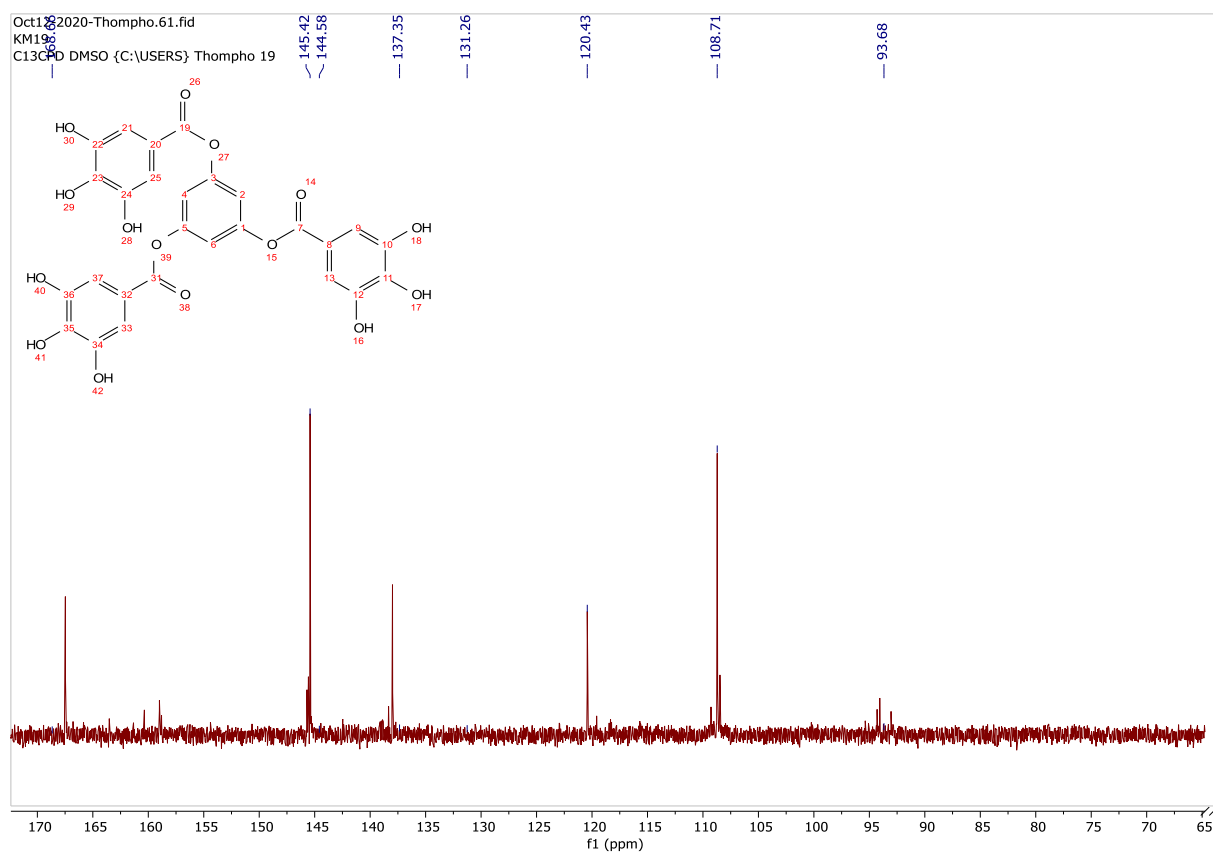
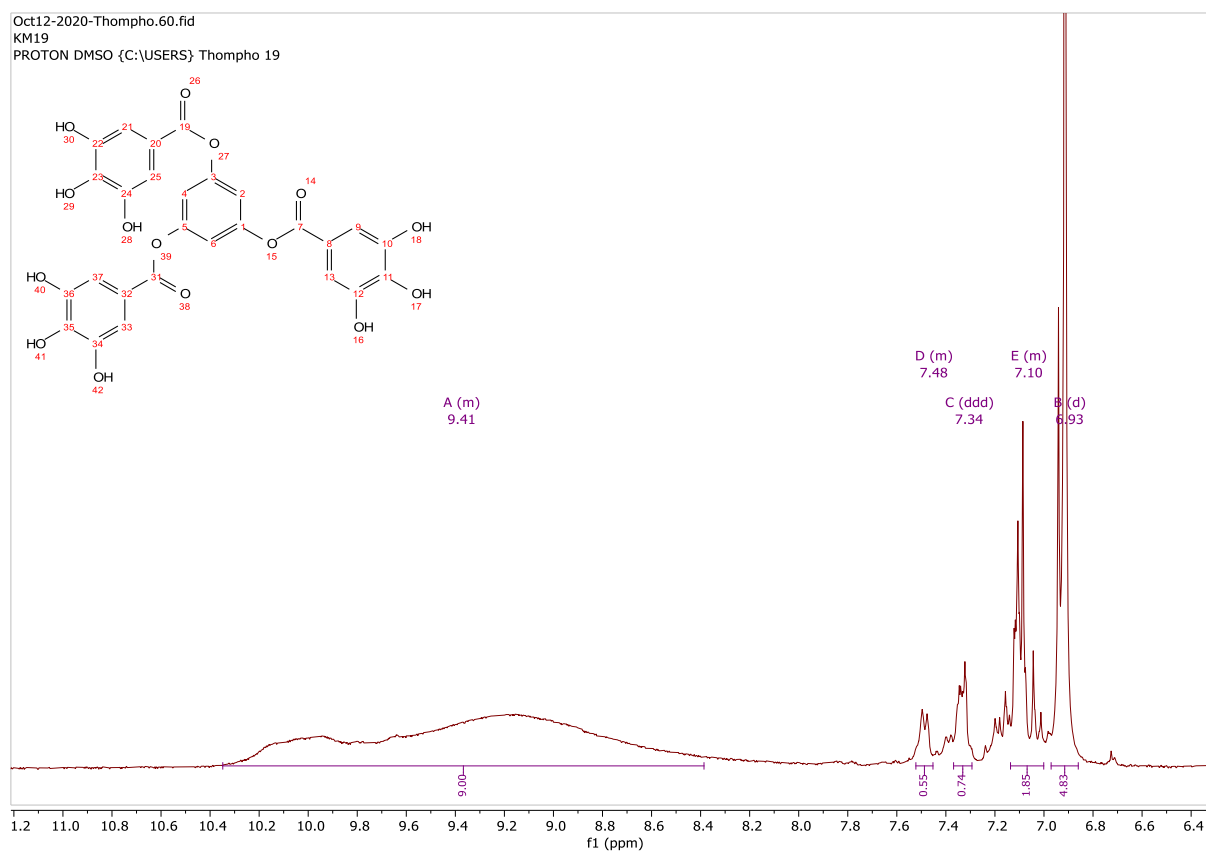


**HRMS of Benzene-1,3,5-triyl tris(3,5-dihydroxybenzoate) (C9)**

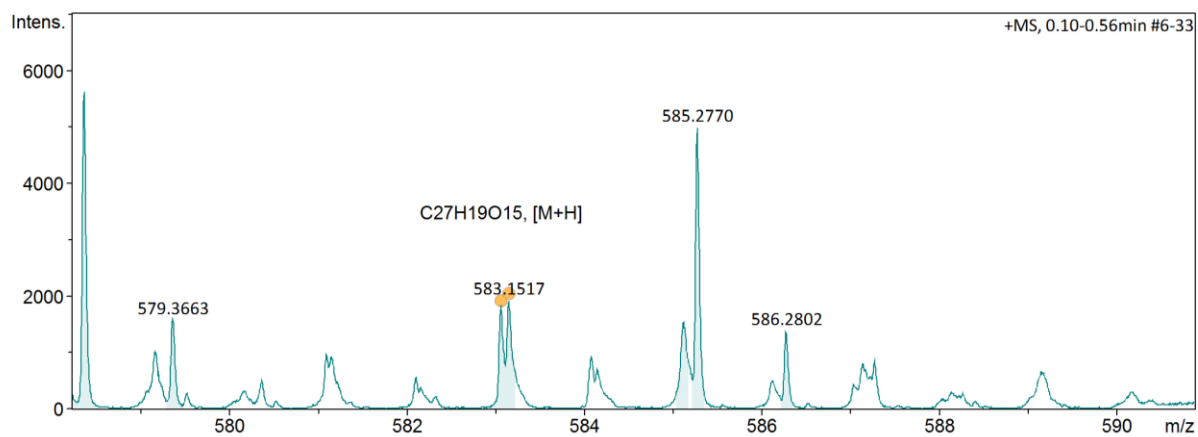
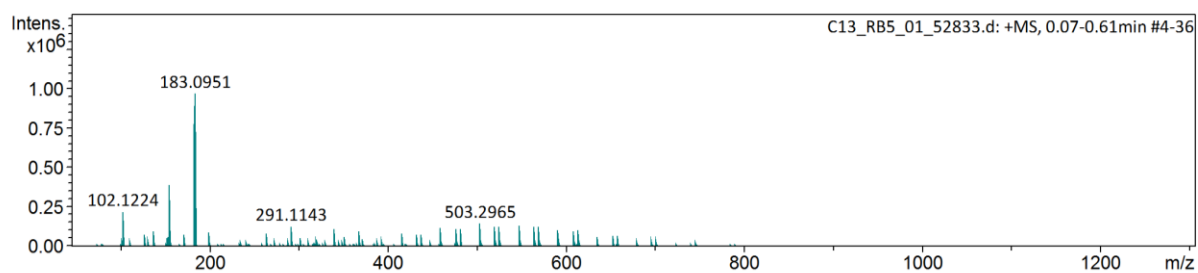


FTIR spectrum of benzene-1,3,5-triyl tris(3,4,5-trihydroxybenzoate) (C13)



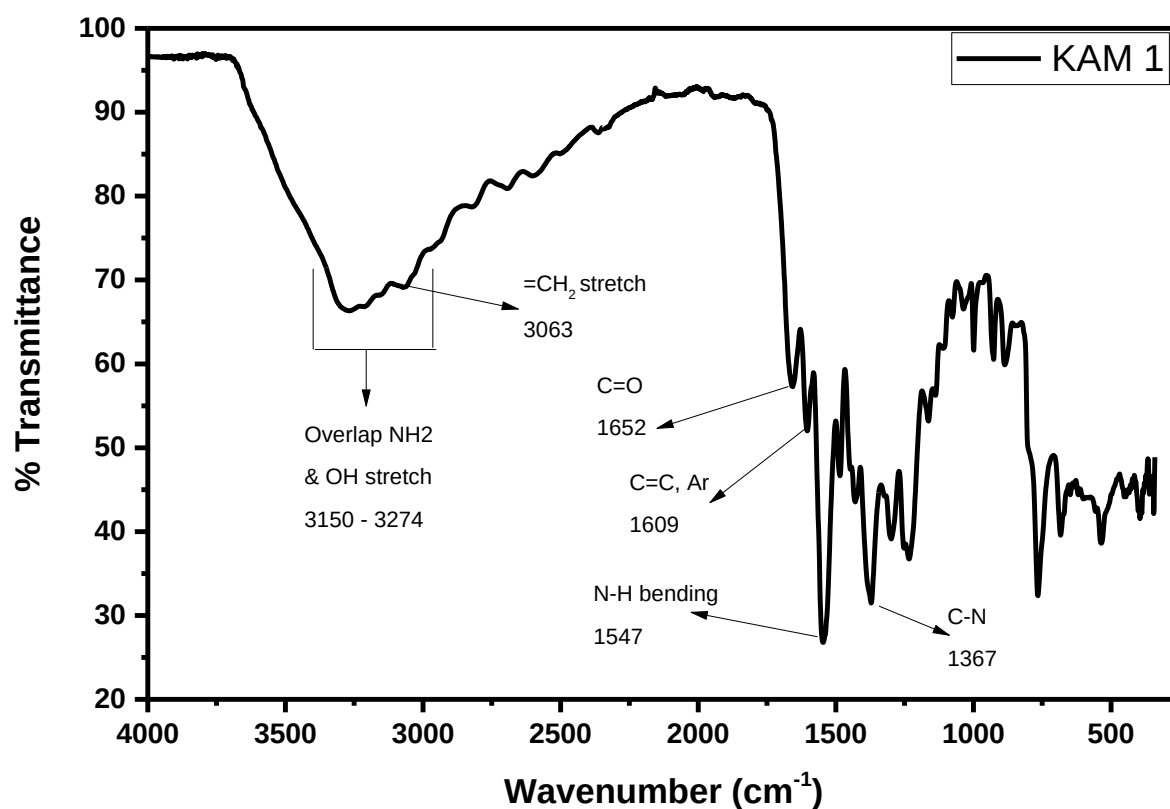
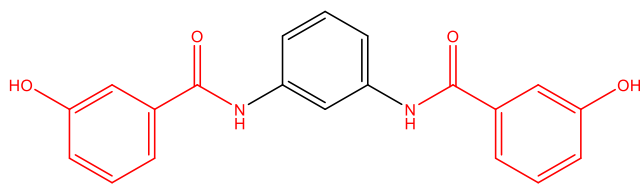


**$^1\text{H}$  and  $^{13}\text{C}$  NMR of benzene-1,3,5-triyl tris(3,4,5-trihydroxybenzoate) (C13)**

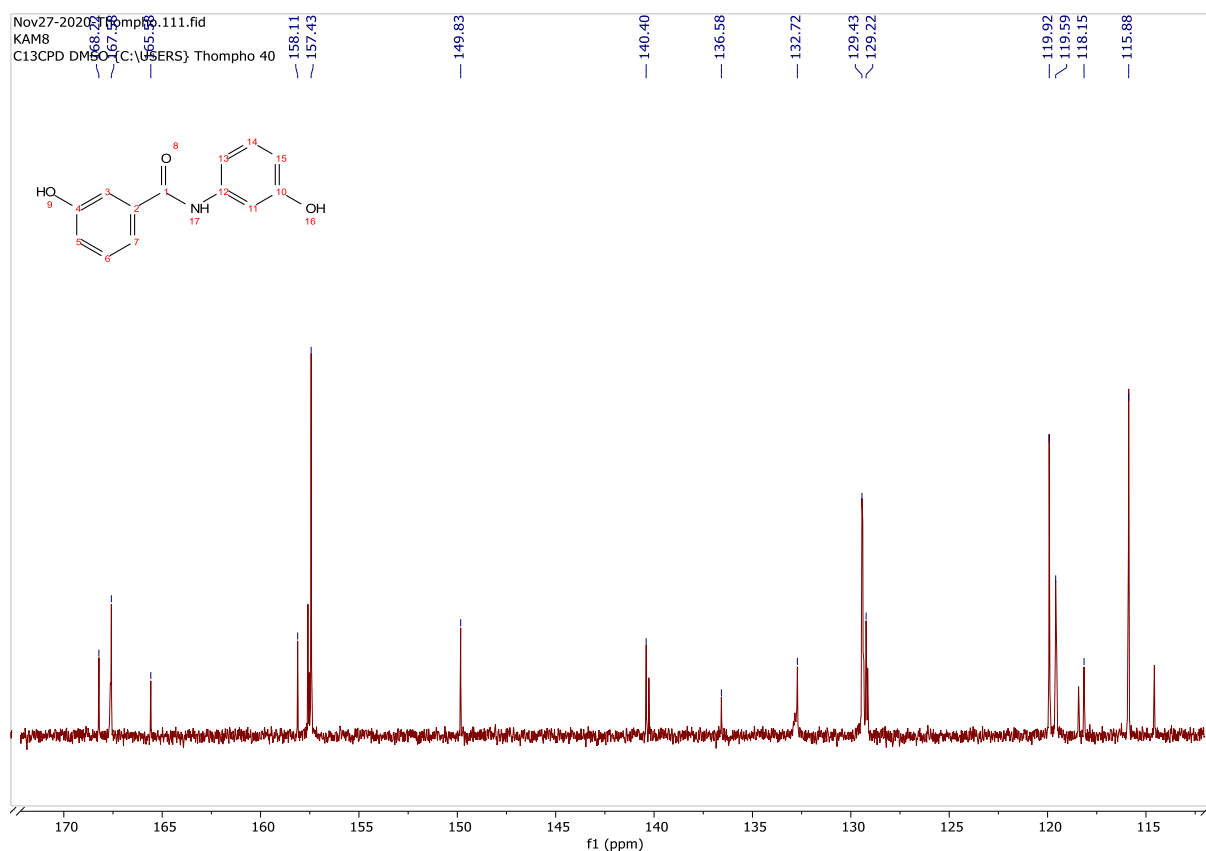
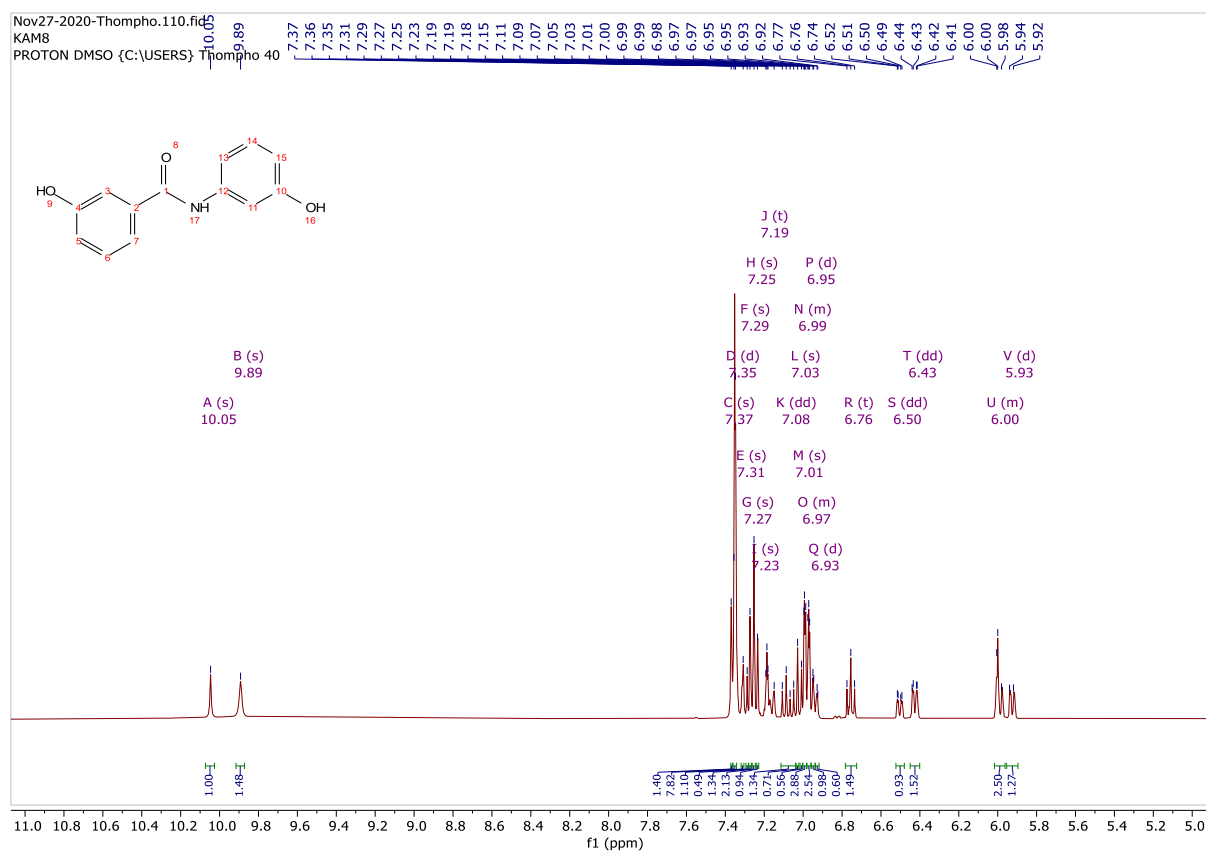


**HRMS of benzene-1,3,5-triyl tris(3,4,5-trihydroxybenzoate) (C13)**

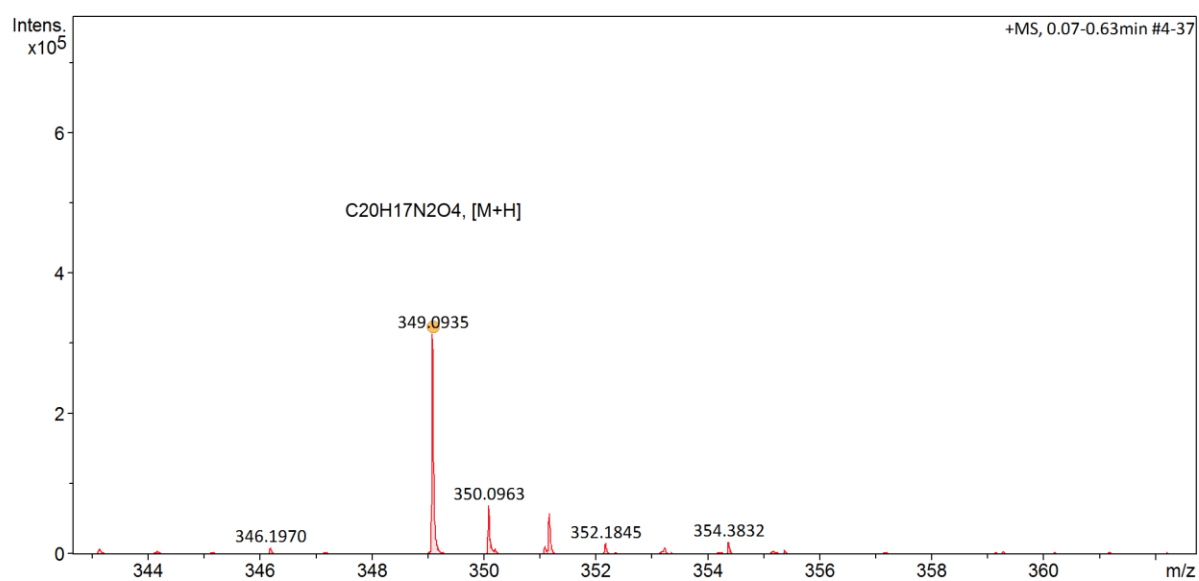
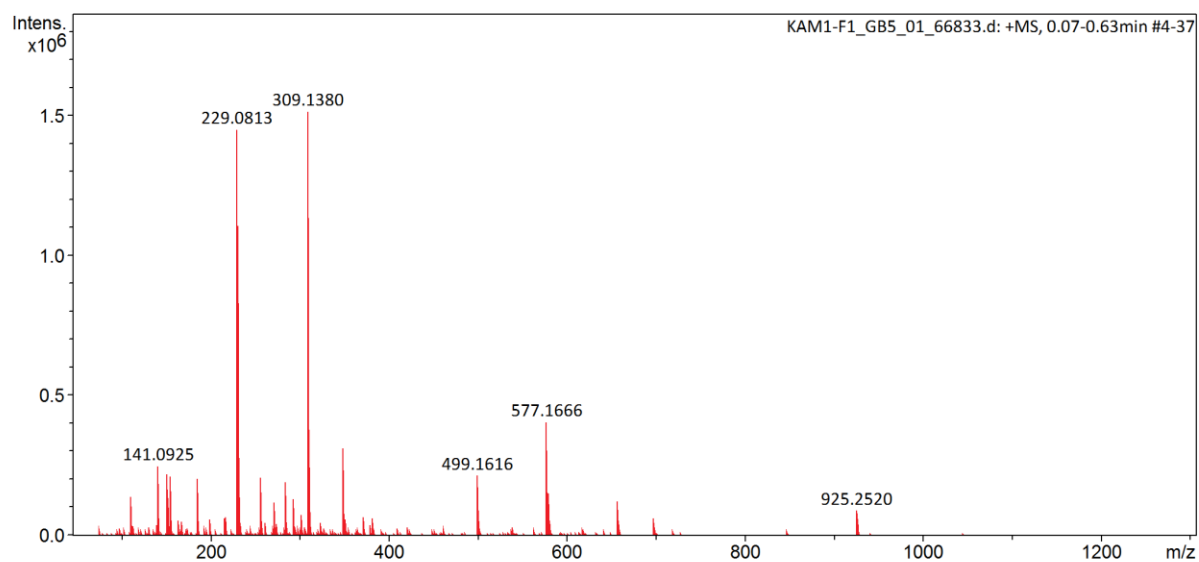
## Appendix C: Characterization data for amide compounds



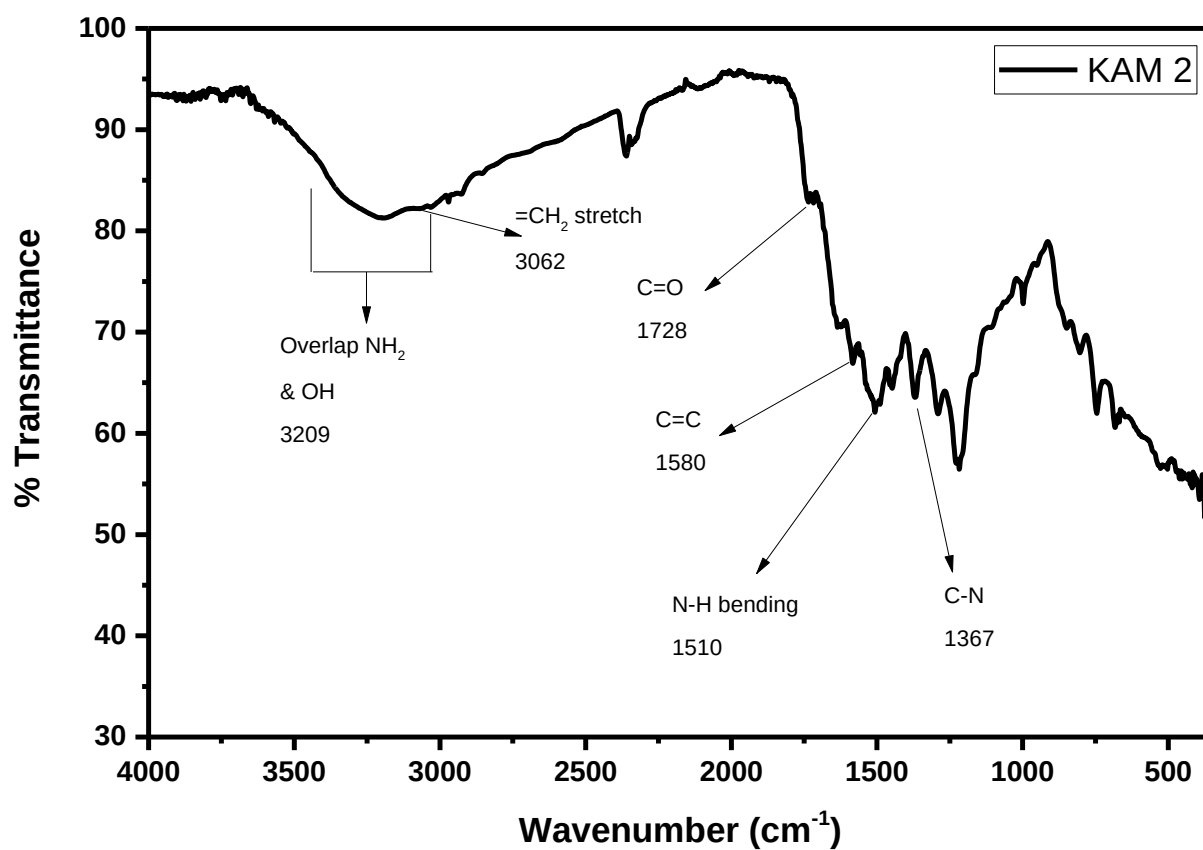
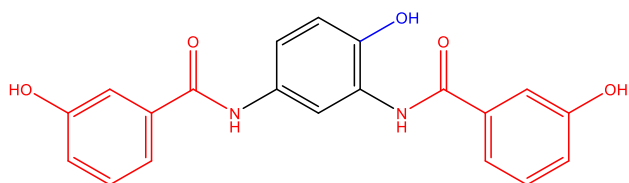
FTIR spectrum of *N,N'*-(1,3-phenylene)bis(3-hydroxybenzamide) (KAM 1)



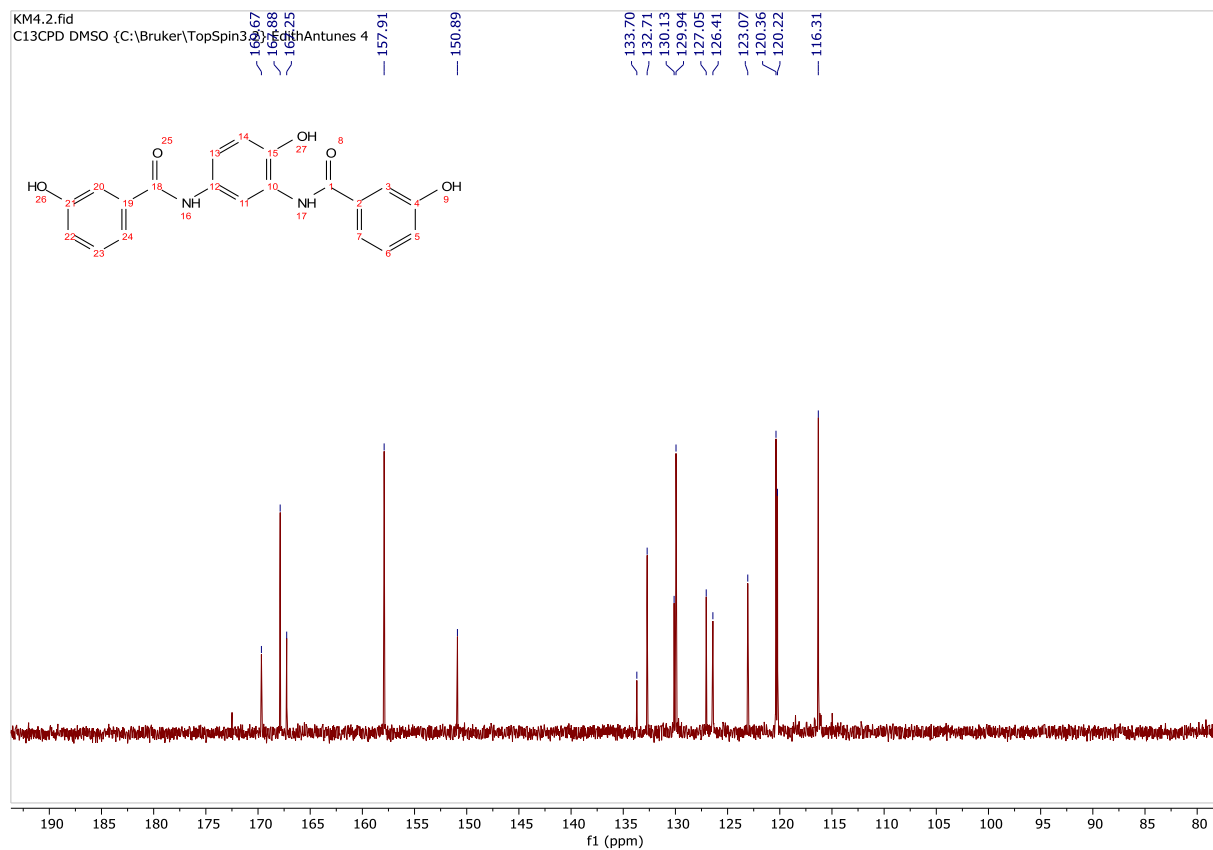
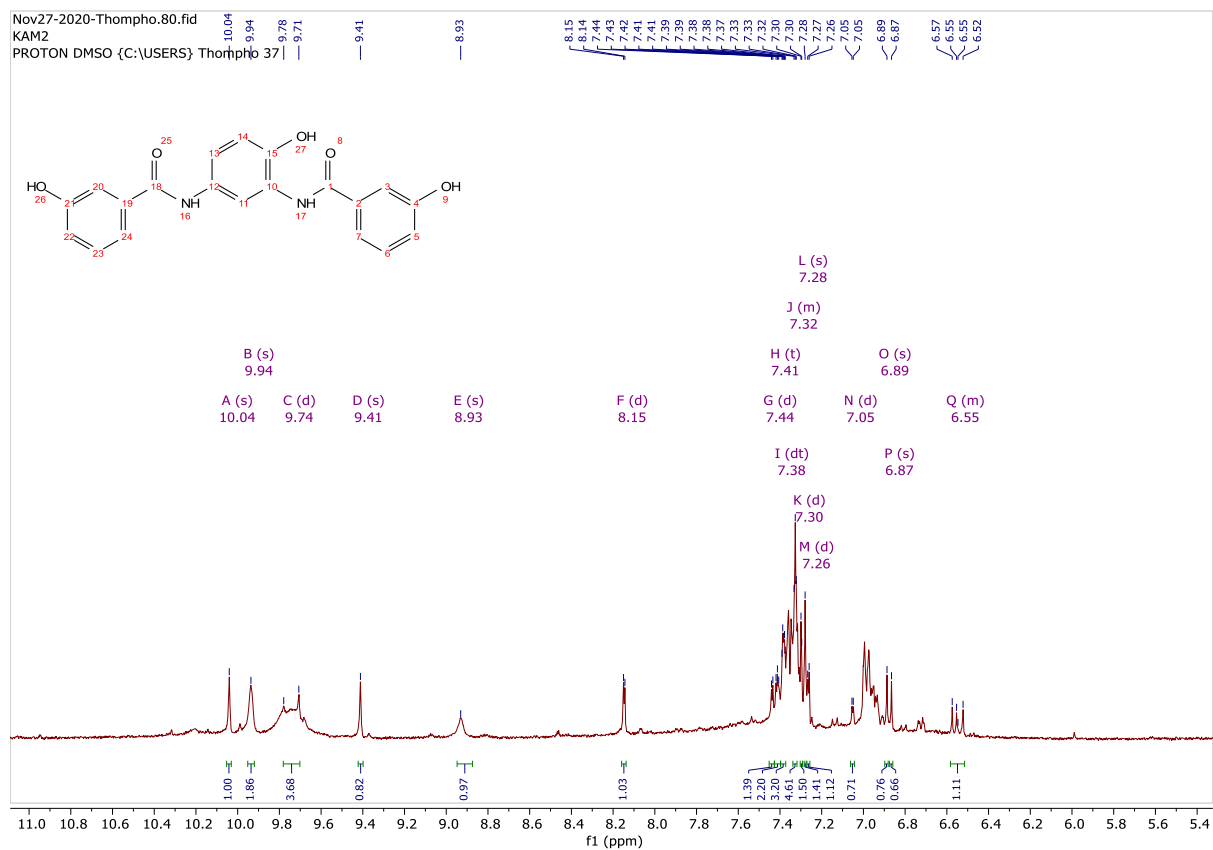
**<sup>1</sup>H and <sup>13</sup>C NMR spectra of *N,N'*-(1,3-phenylene)bis(3-hydroxybenzamide) (KAM 1)**



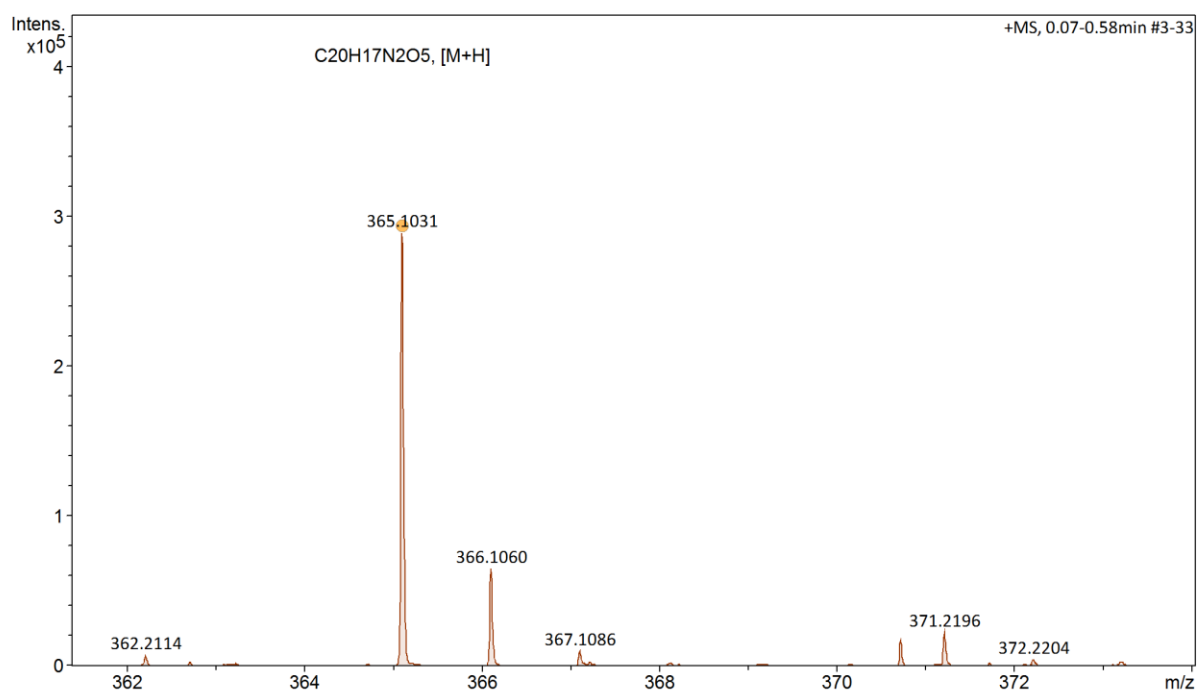
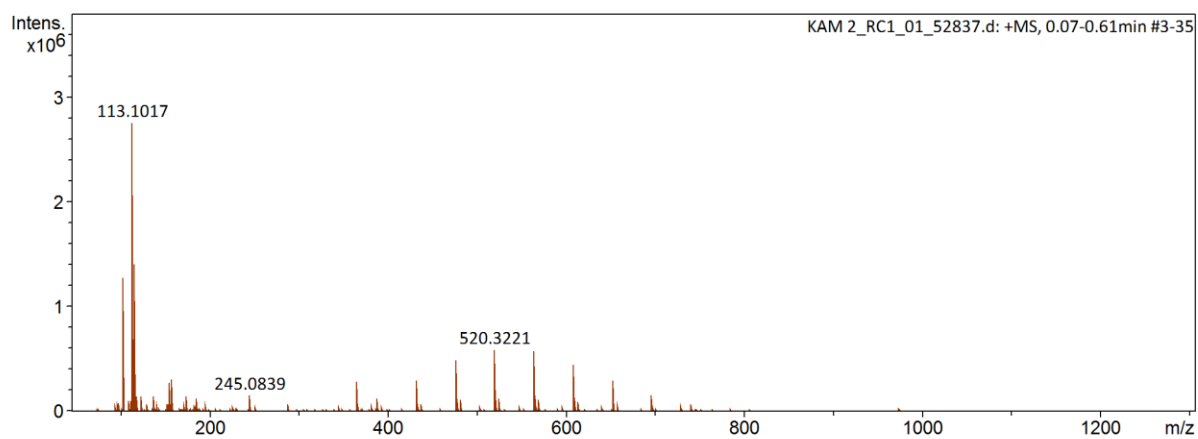
**HRMS of *N,N'*-(1,3-phenylene)bis(3-hydroxybenzamide) (KAM 1)**



FTIR spectrum of *N,N'*-(4-hydroxy-1,3-phenylene)bis(3-hydroxybenzamide) (KAM 2)

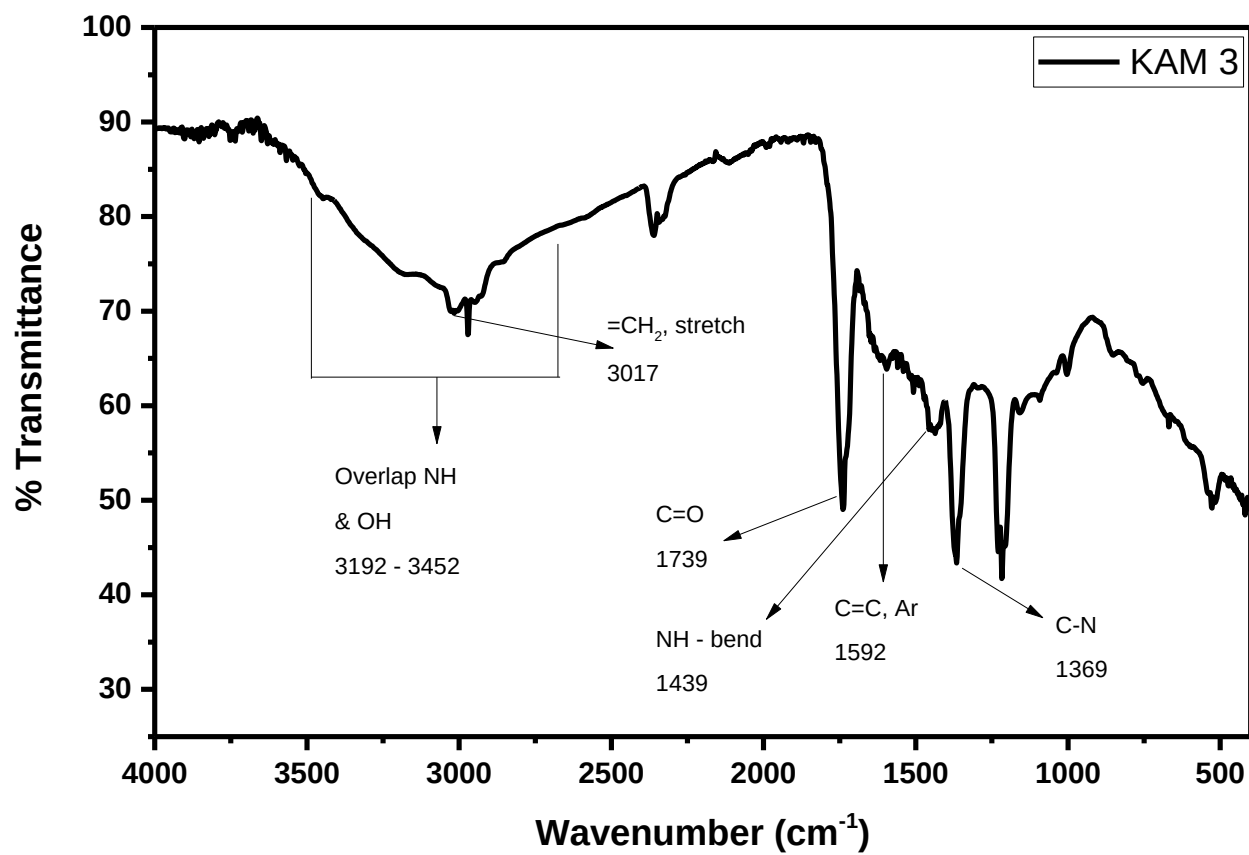
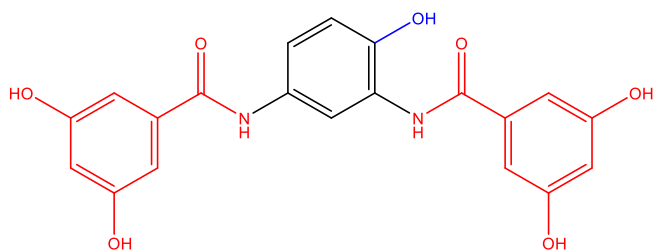


**$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of  $N,N'$ -(4-hydroxy-1,3-phenylene)bis(3-hydroxybenzamide) (KAM 2)**

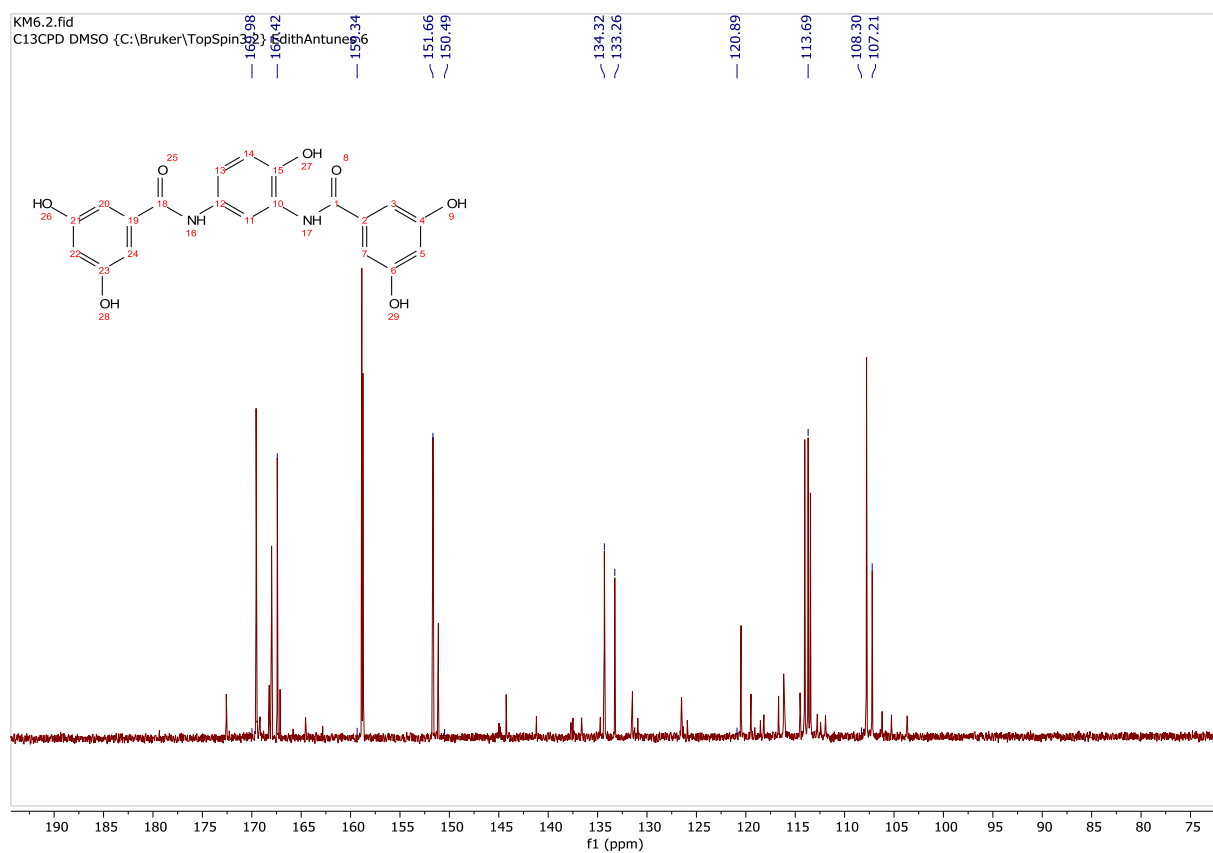
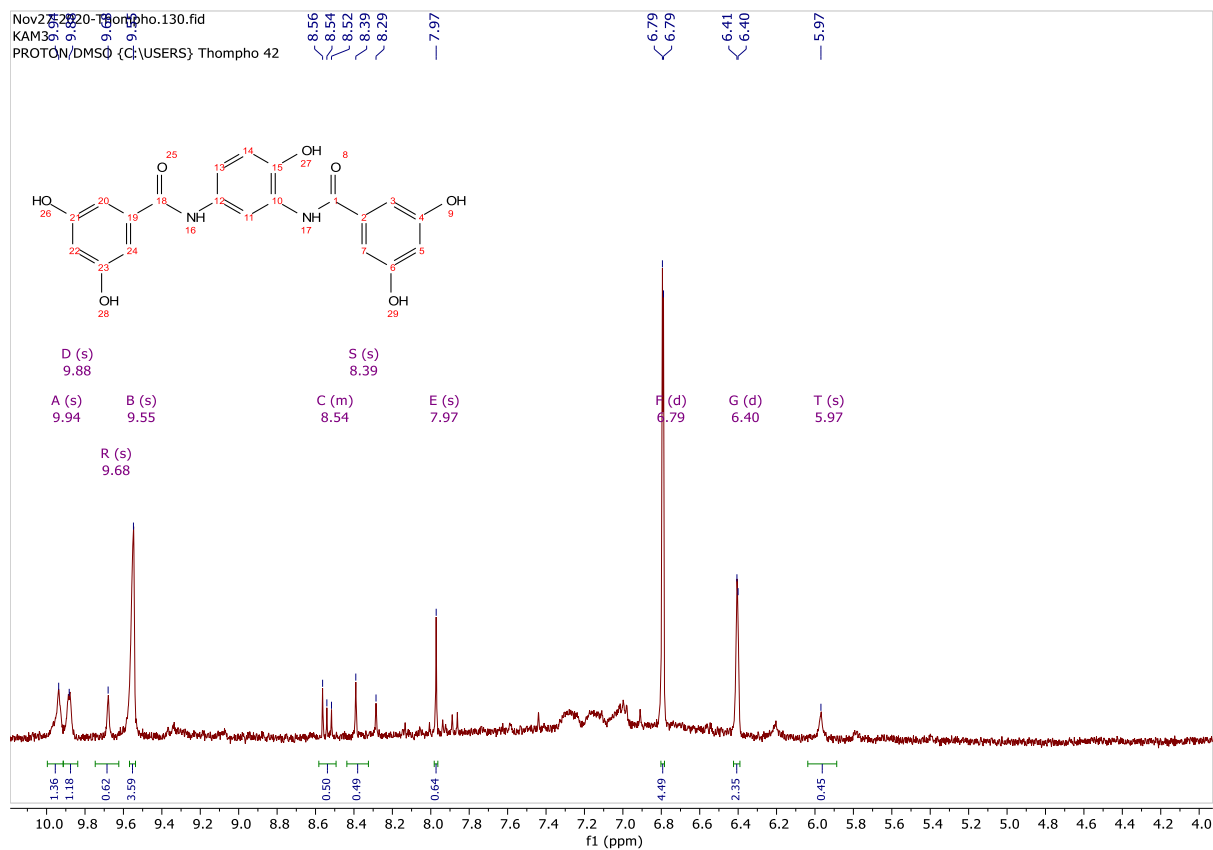


**HRMS of *N,N'*-(4-hydroxy-1,3-phenylene)bis(3-hydroxybenzamide) (KAM 2)**



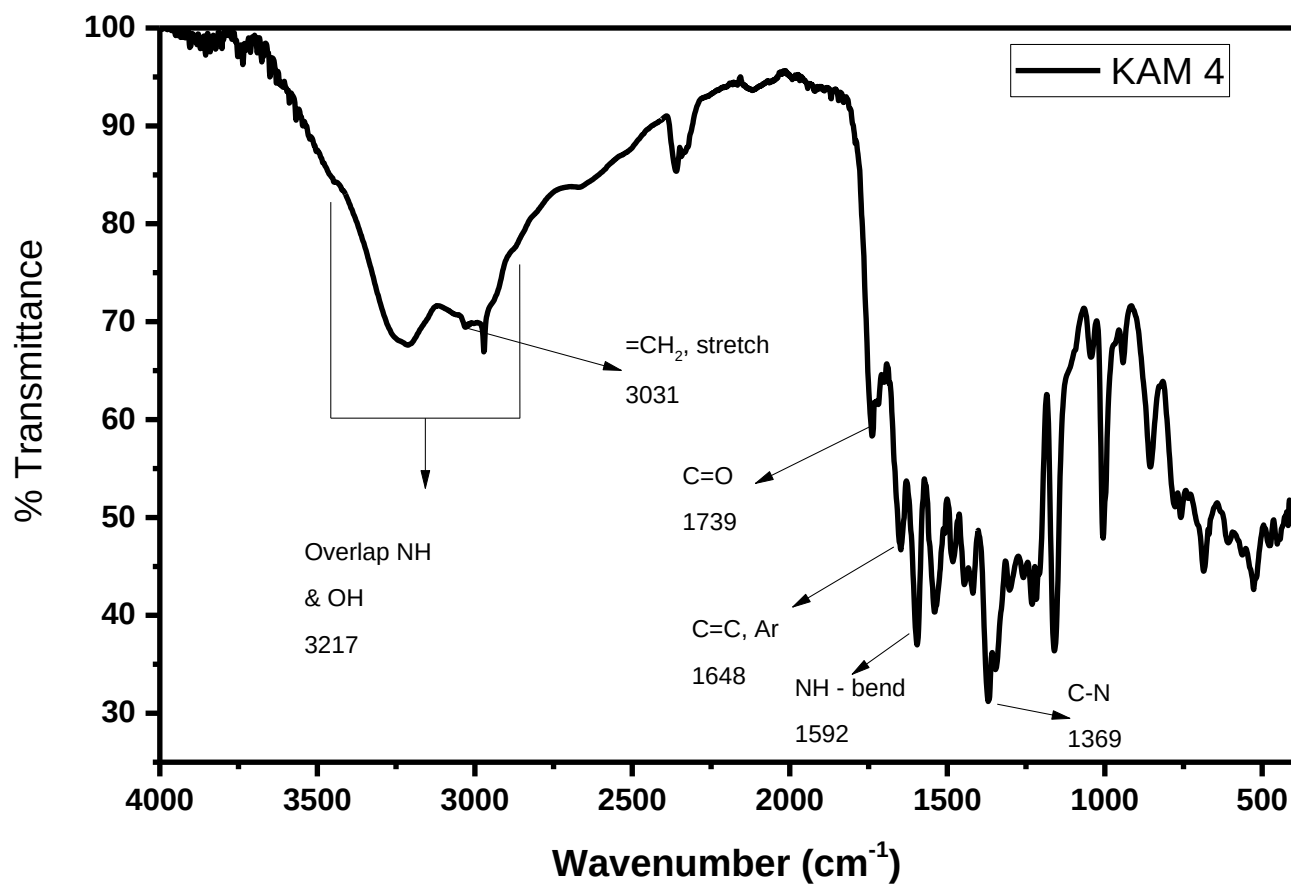
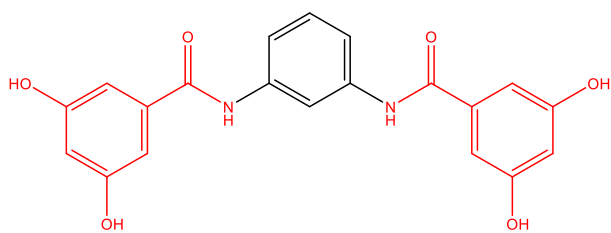


FTIR spectrum of *N,N'*-(4-hydroxy-1,3-phenylene)bis(3,5-dihydroxybenzamide)

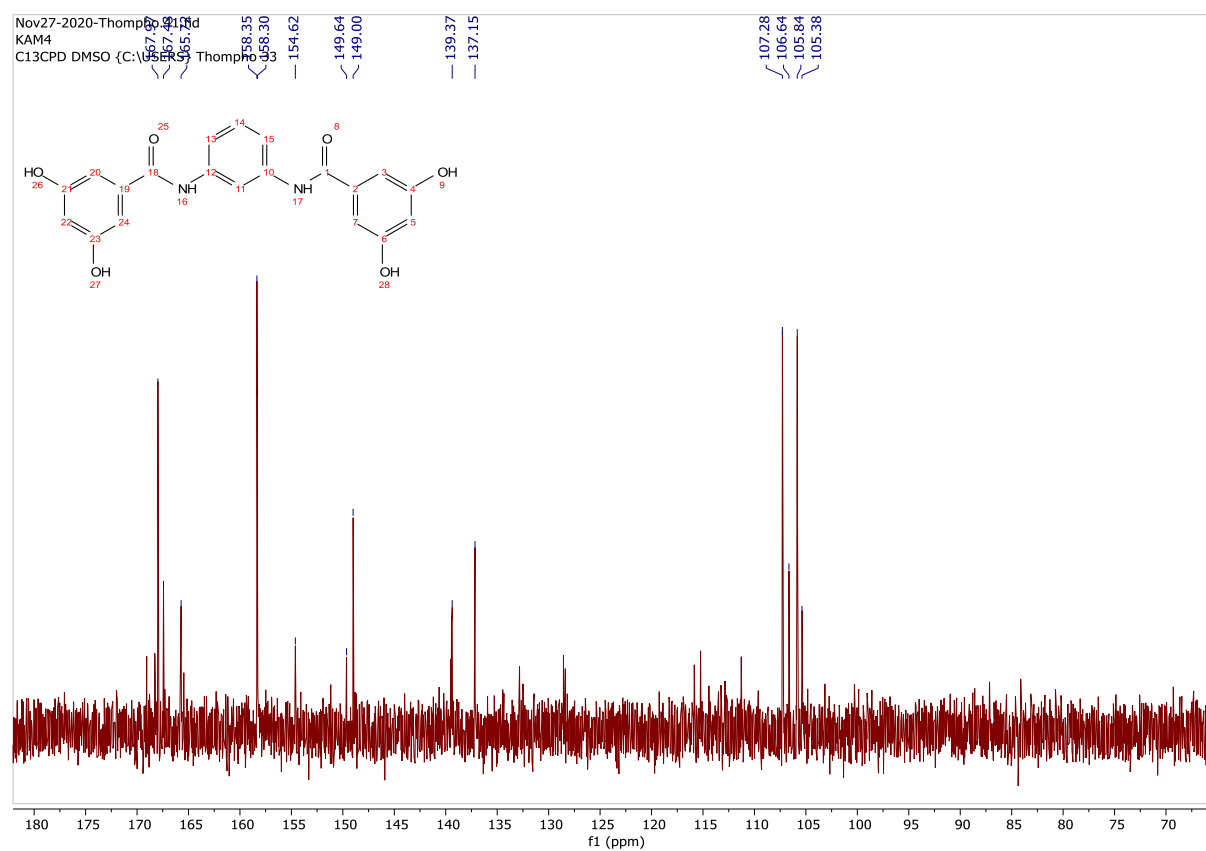
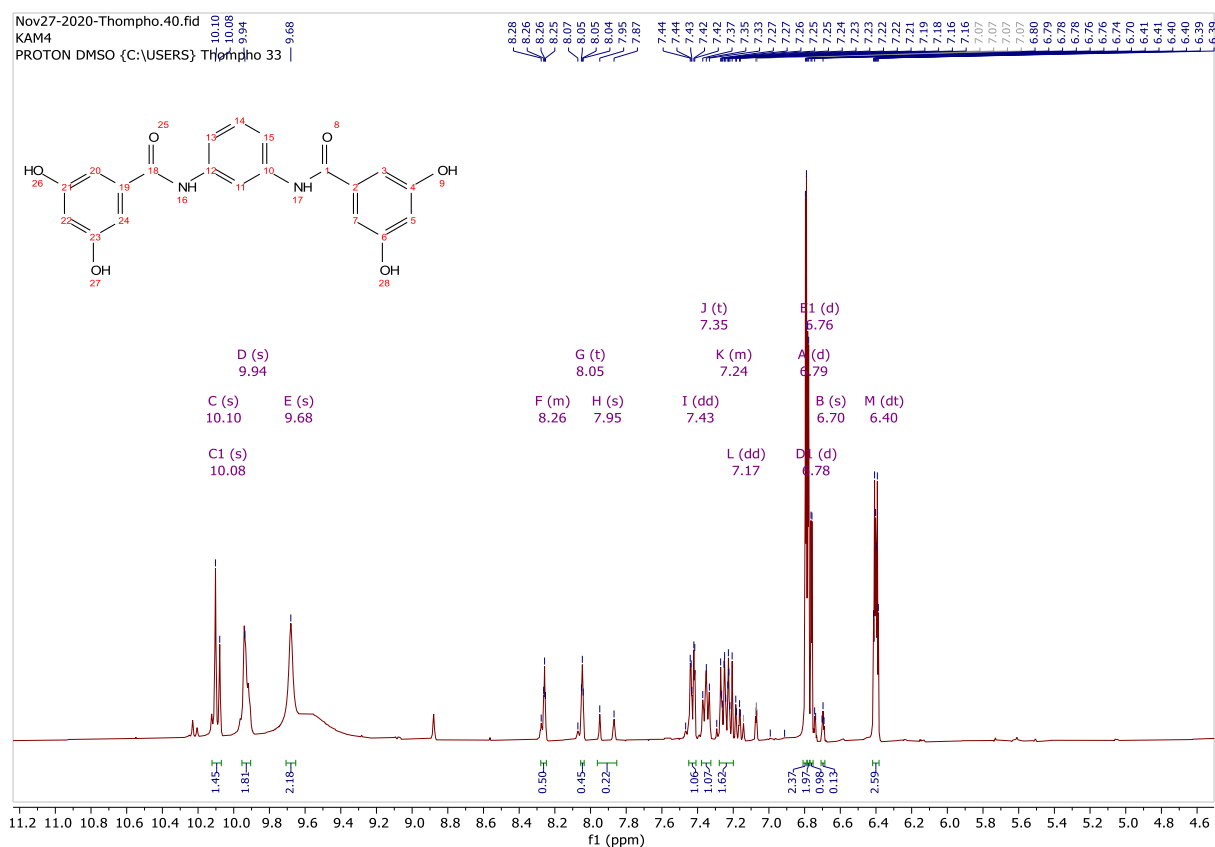


**<sup>1</sup>H and <sup>13</sup>C NMR spectra of N,N'-(4-hydroxy-1,3-phenylene)bis(3,5-dihydroxybenzamide) (KAM 3)**

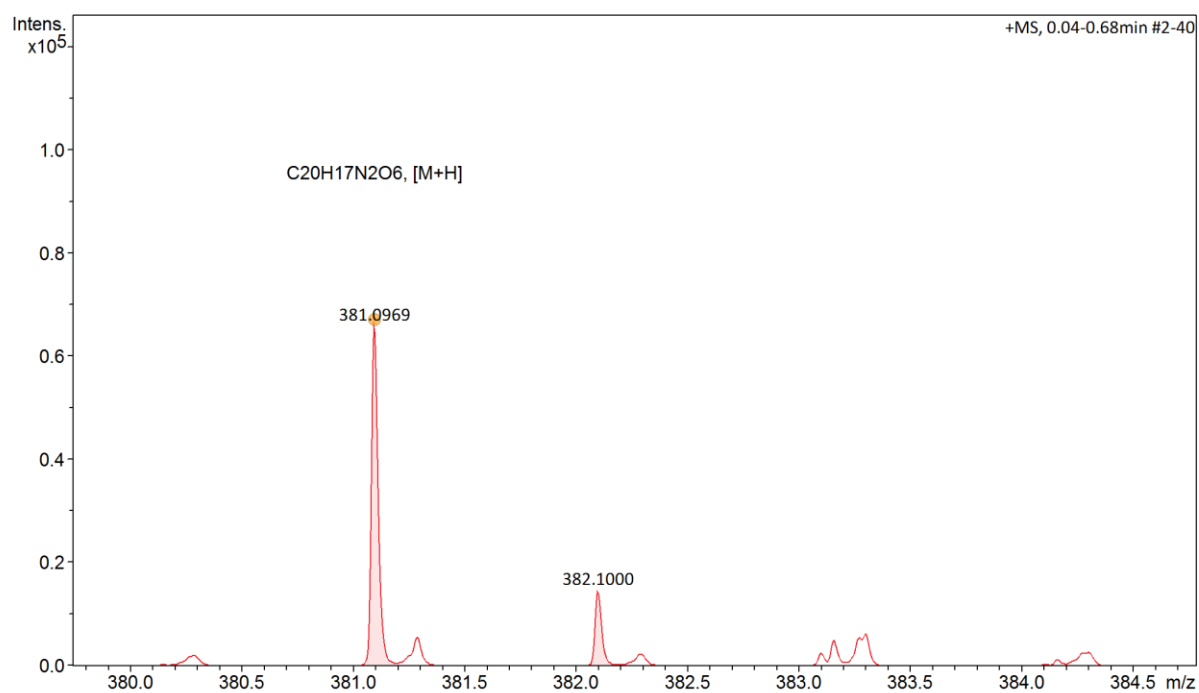
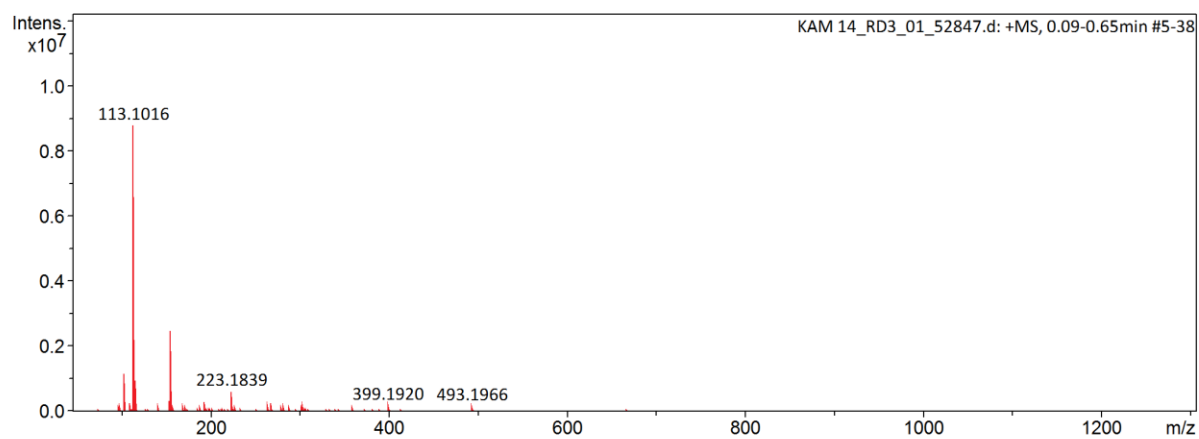




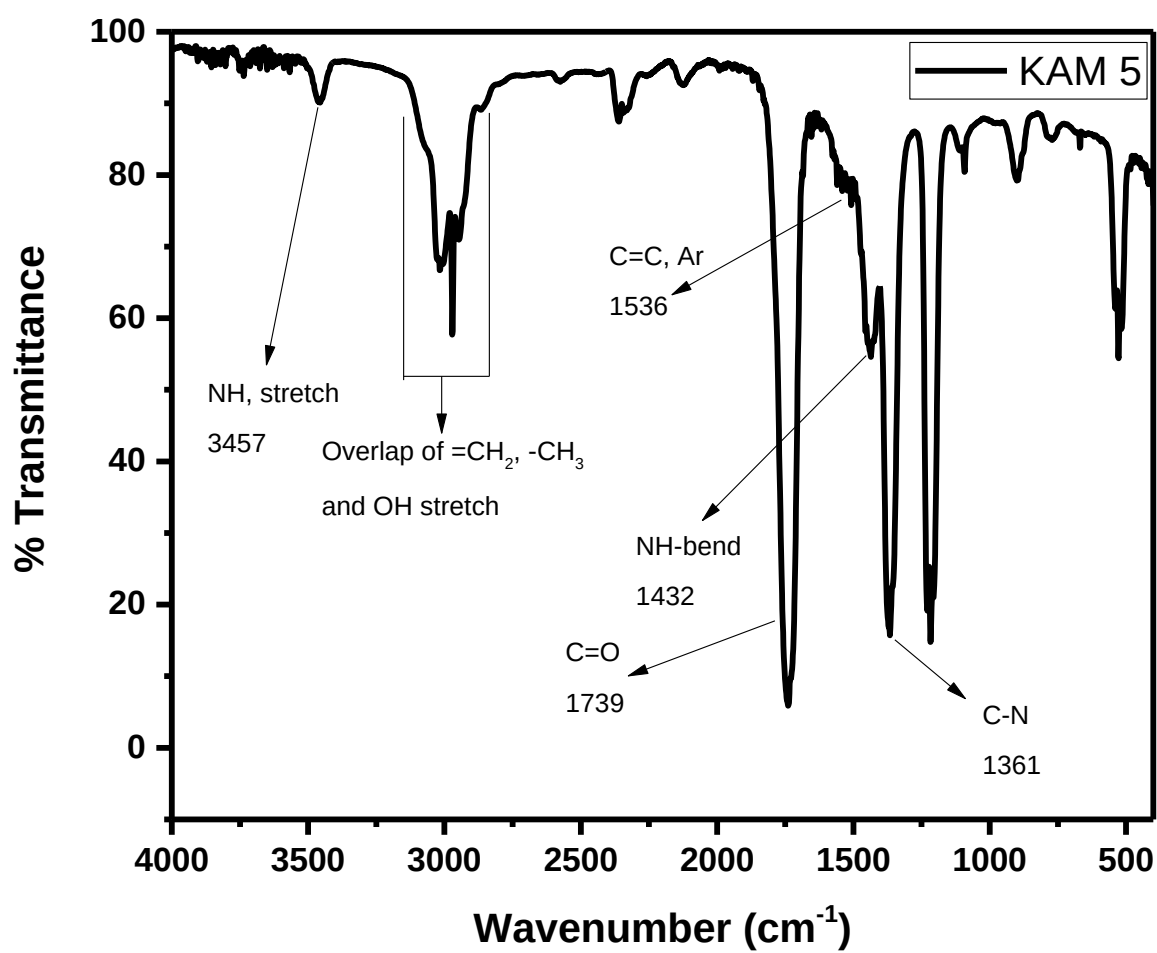
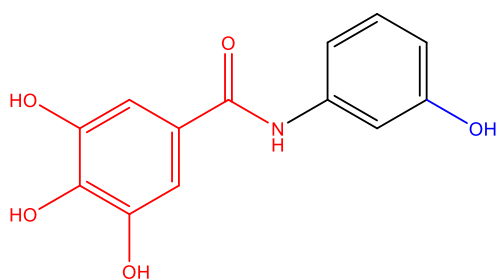
FTIR spectrum of *N,N'*-(1,3-phenylene)bis(3,5-dihydroxybenzamide) (KAM 4)



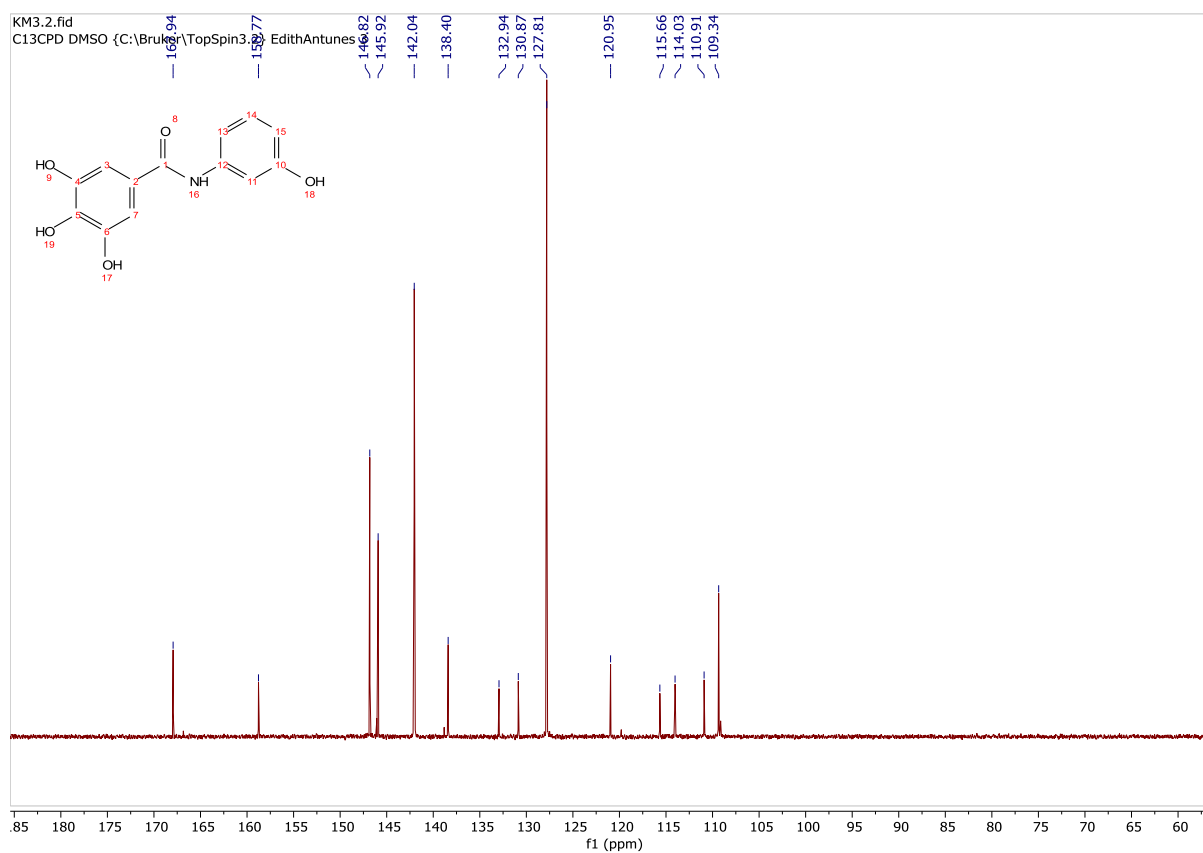
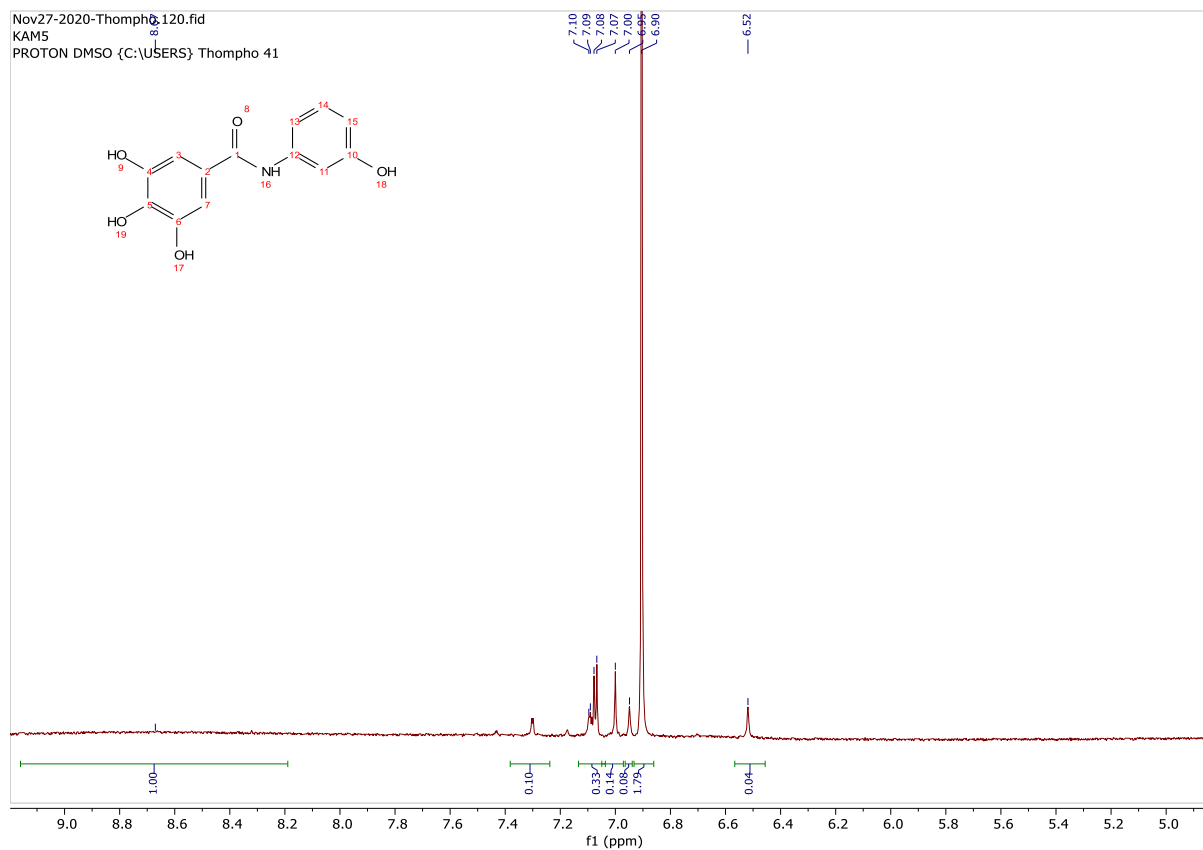
**$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of *N,N'*-(1,3-phenylene)bis(3,5-dihydroxybenzamide) (KAM 4)**



**HRMS of *N,N'*-(1,3-phenylene)bis(3,5-dihydroxybenzamide) (KAM 4)**



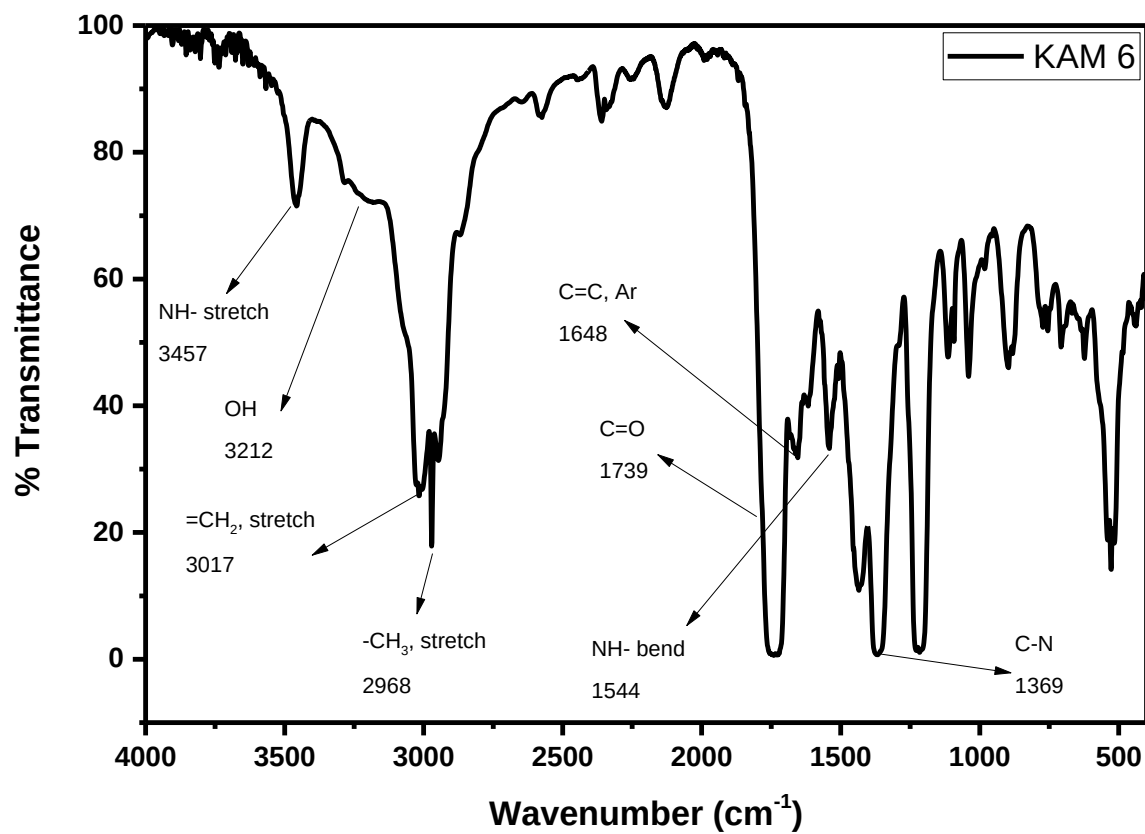
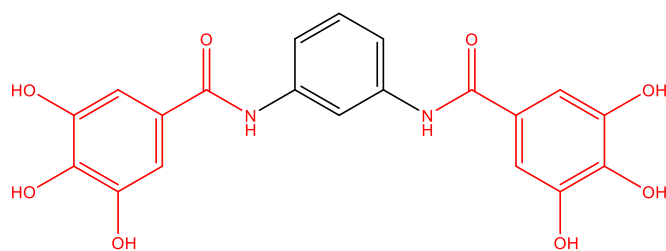
FTIR spectrum of 3,4,5-trihydroxy-N-(3-hydroxyphenyl)benzamide (KAM 5)



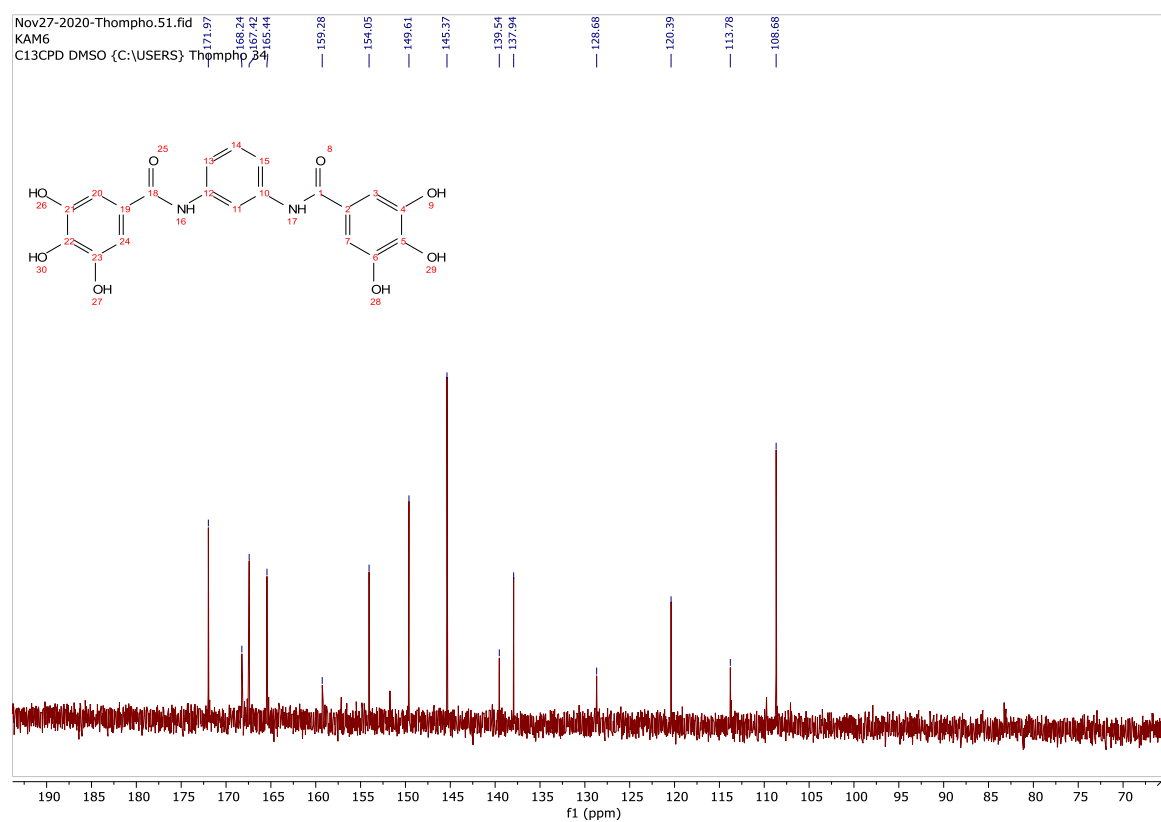
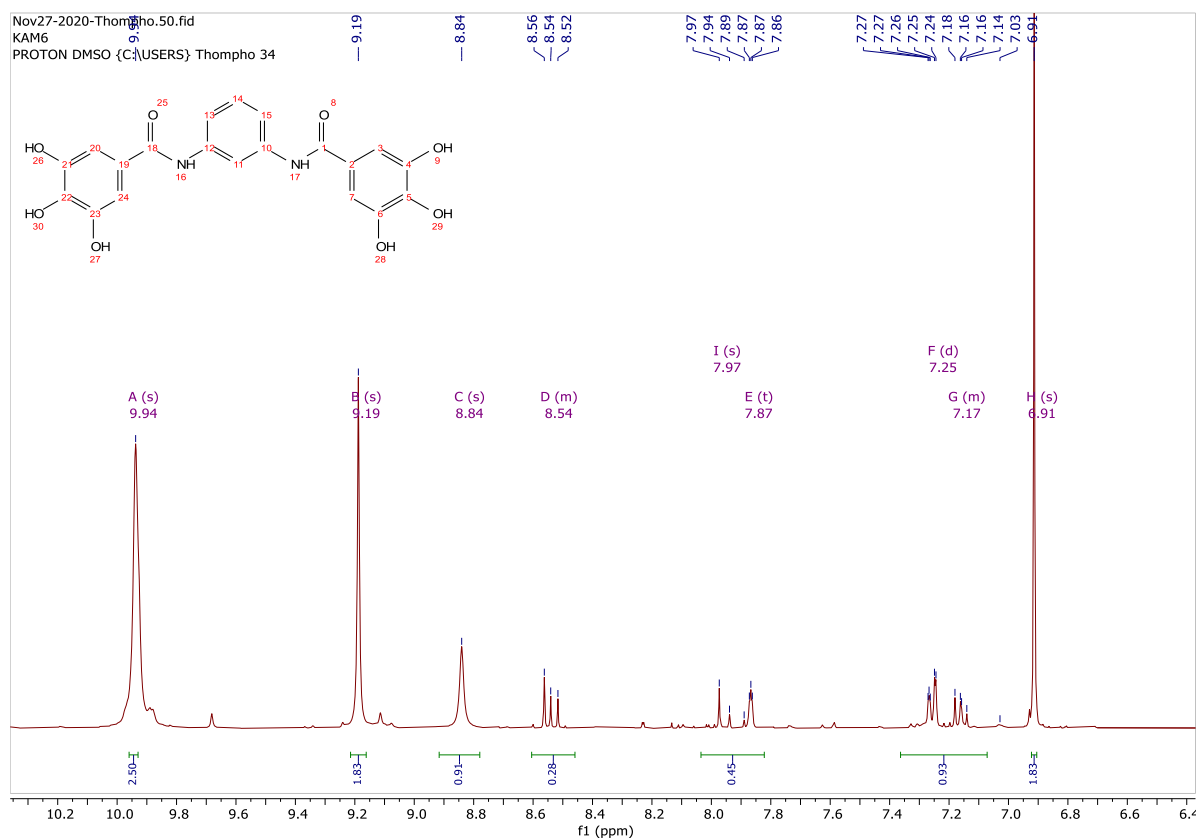
**<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3,4,5-trihydroxy-N-(3-hydroxyphenyl)benzamide (KAM 5)**



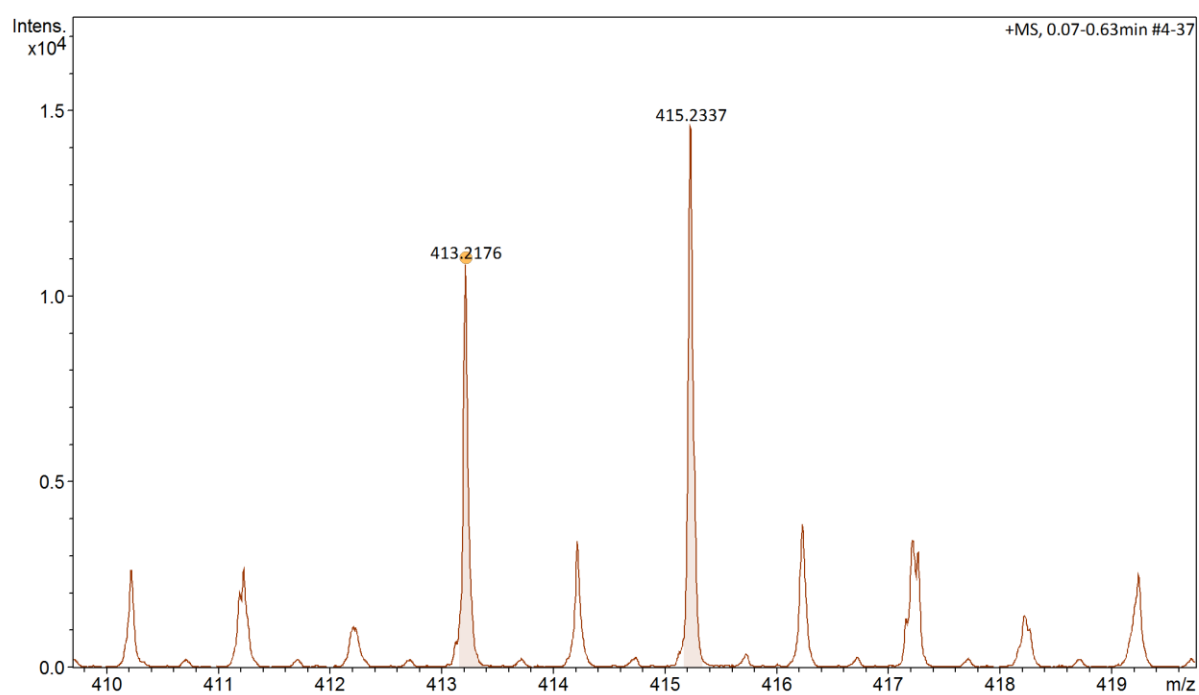
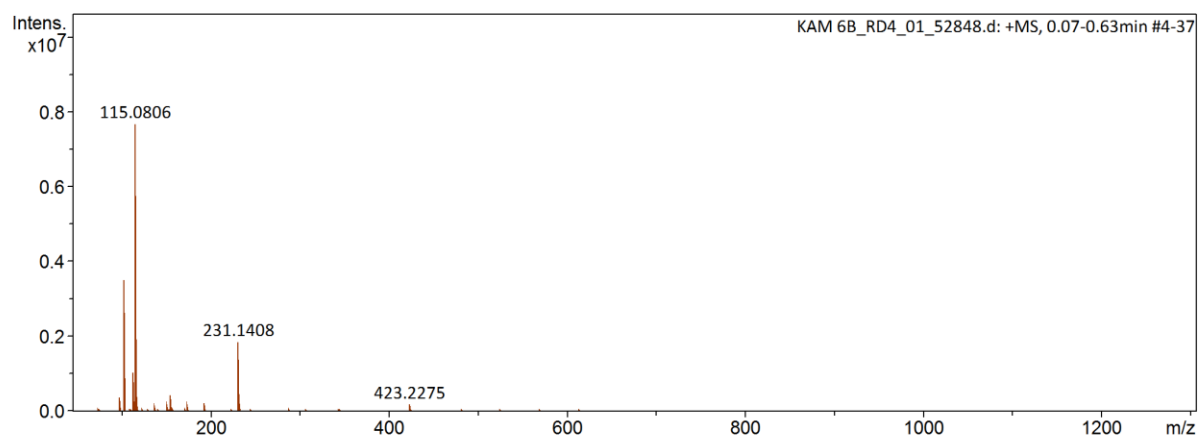




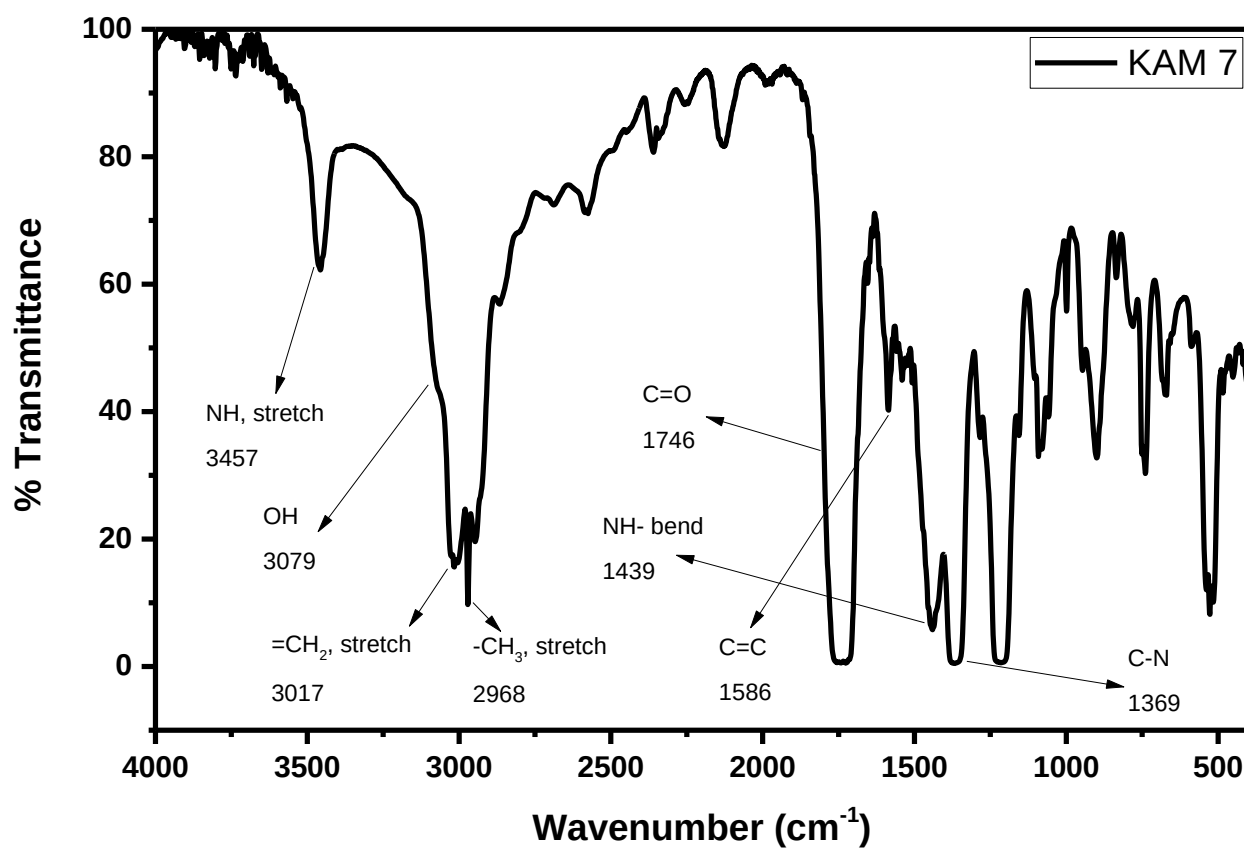
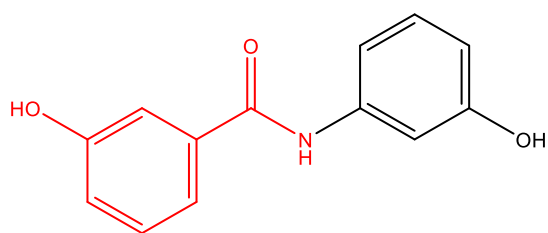
FTIR spectrum of *N,N'*-(1,3-phenylene)bis(3,4,5-trihydroxybenzamide) (KAM 6)



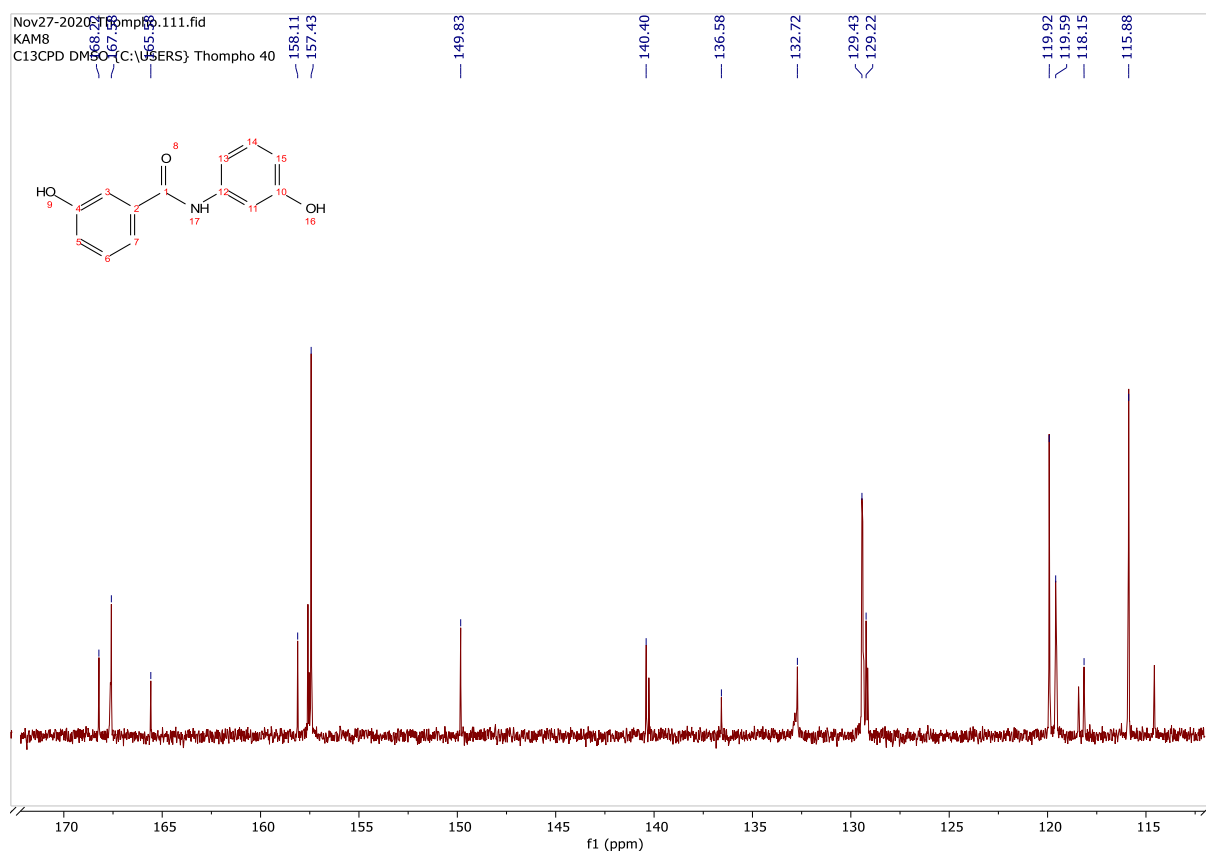
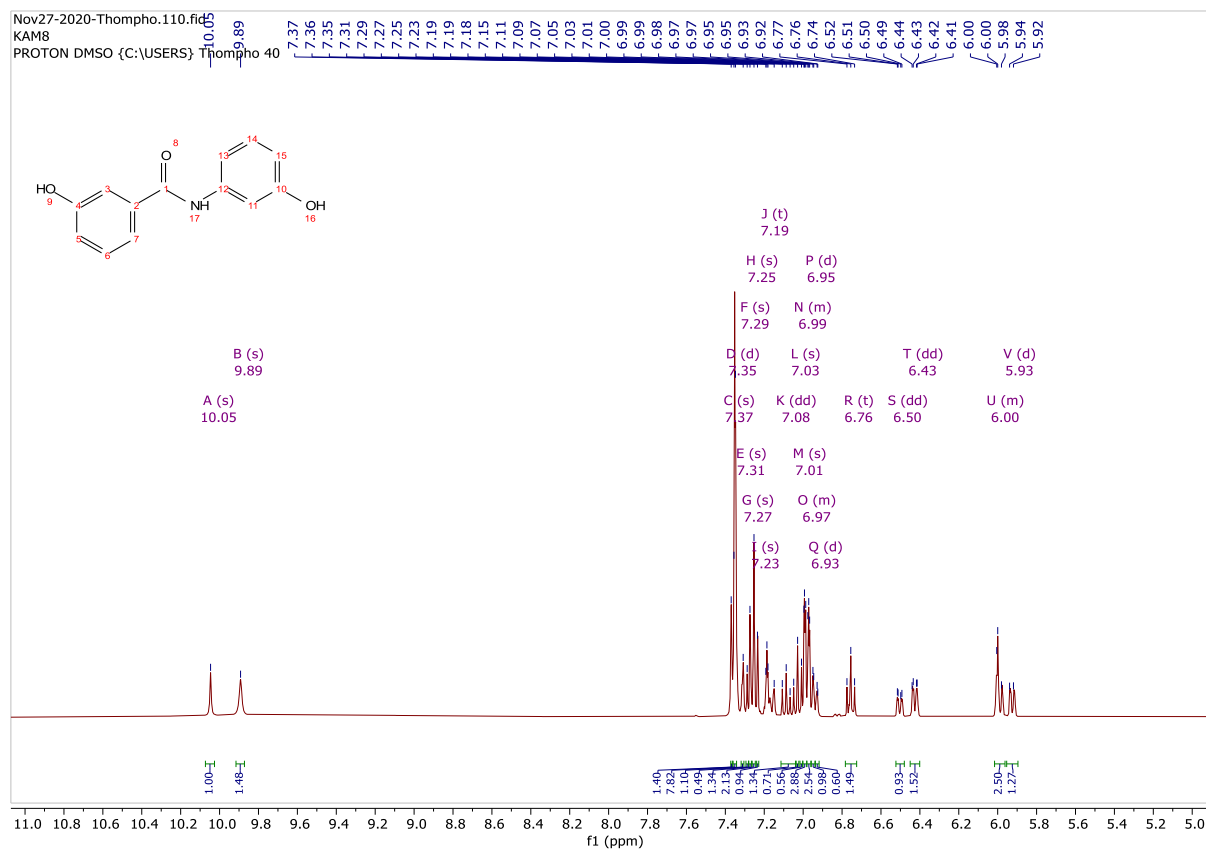
**$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of *N,N'*-(1,3-phenylene)bis(3,4,5-trihydroxybenzamide) (KAM6)**



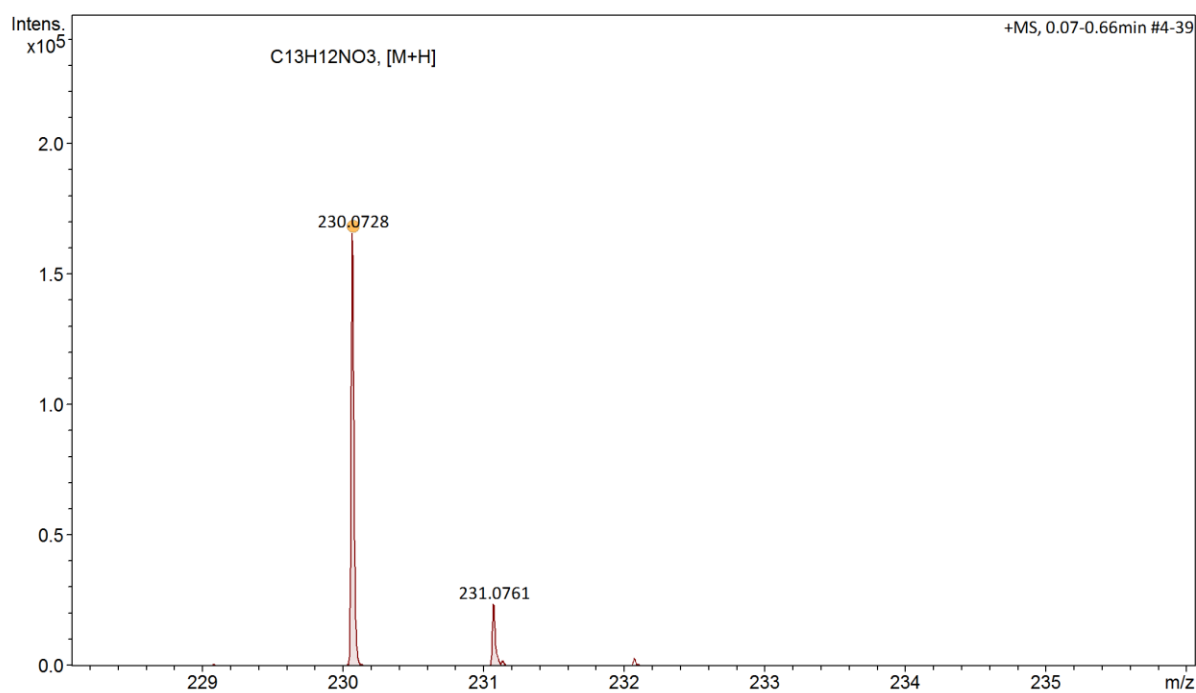
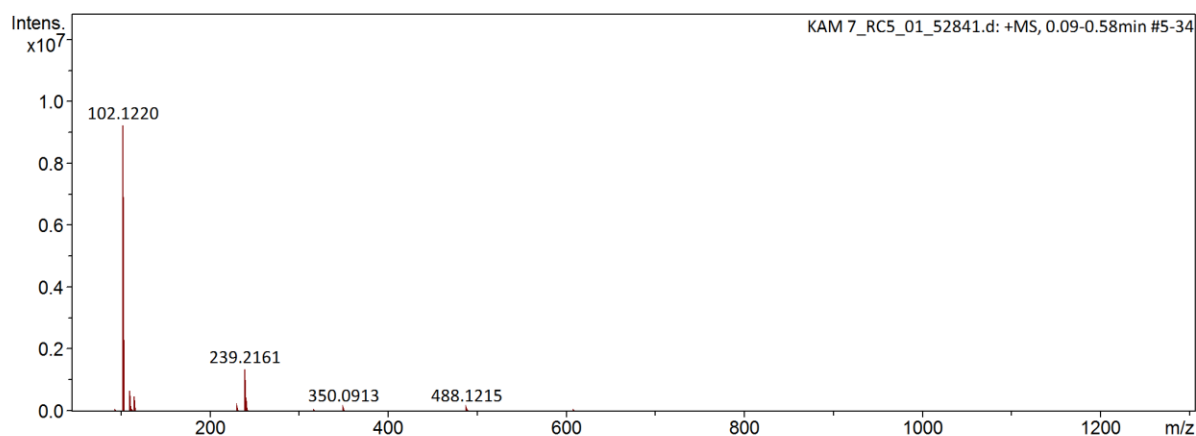
**HRMS of *N,N'*-(1,3-phenylene)bis(3,4,5-trihydroxybenzamide) (KAM 6)**



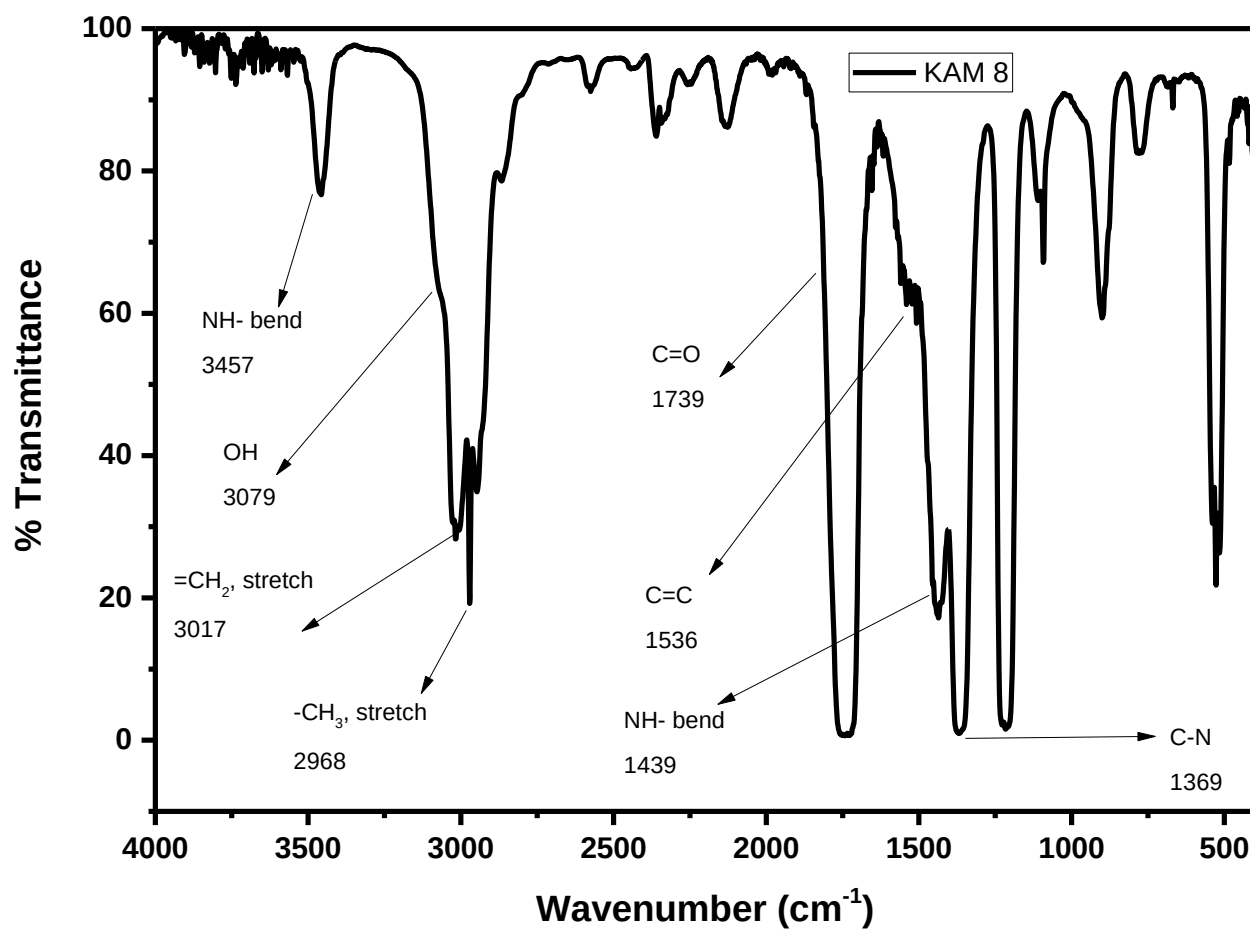
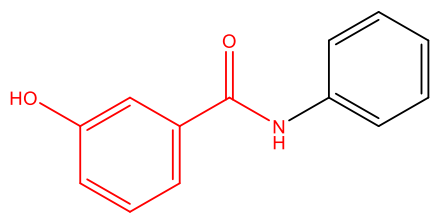
FTIR spectrum of 3-hydroxy-N-(3-hydroxyphenyl)benzamide (KAM 7)



**<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3-hydroxy-N-(3-hydroxyphenyl)benzamide (KAM 7)**

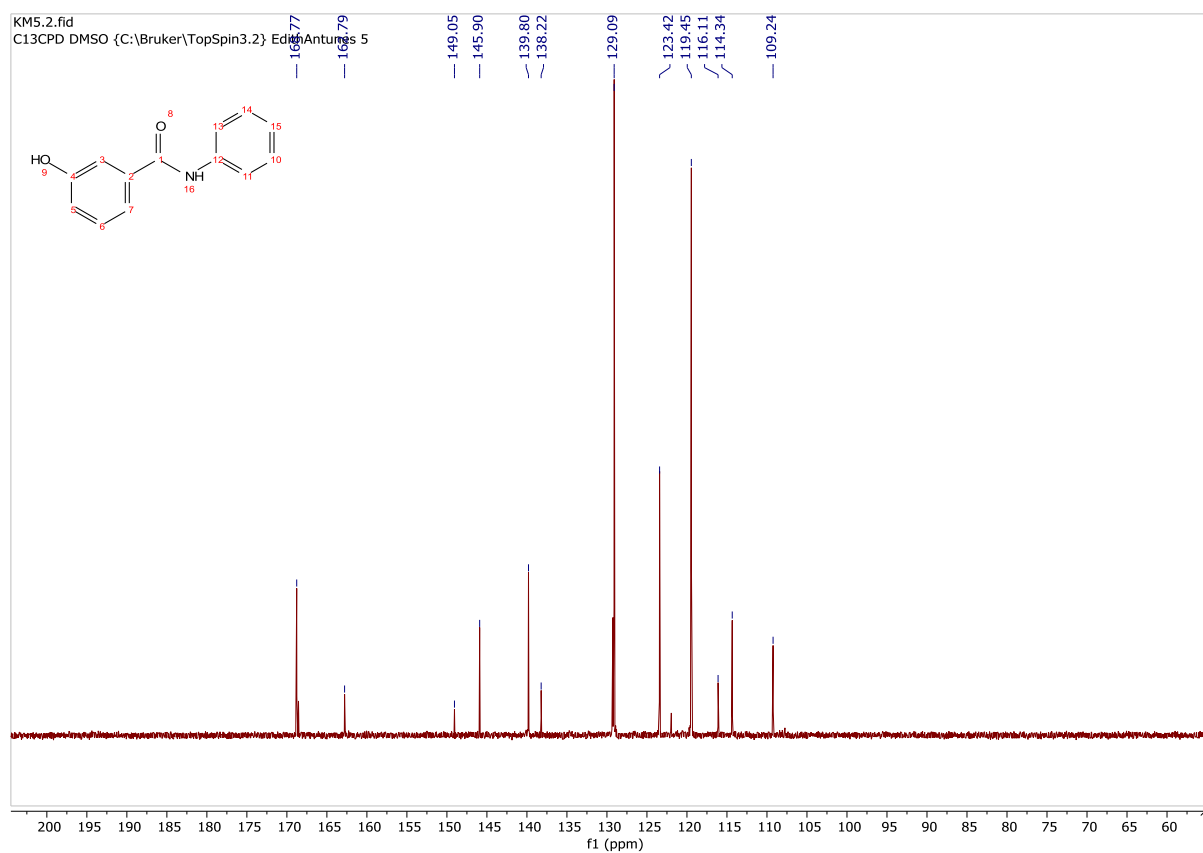
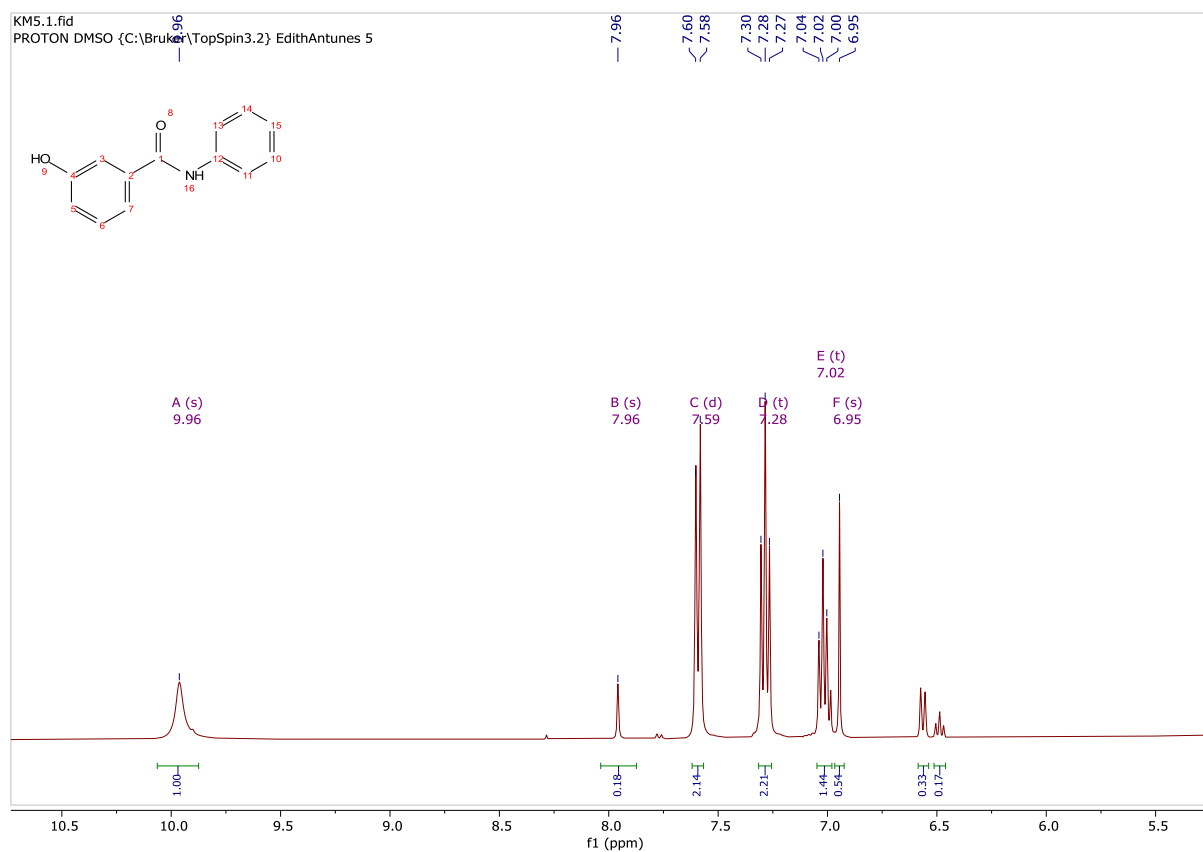


**HRMS of 3-hydroxy-*N*-(3-hydroxyphenyl)benzamide (KAM 7)**

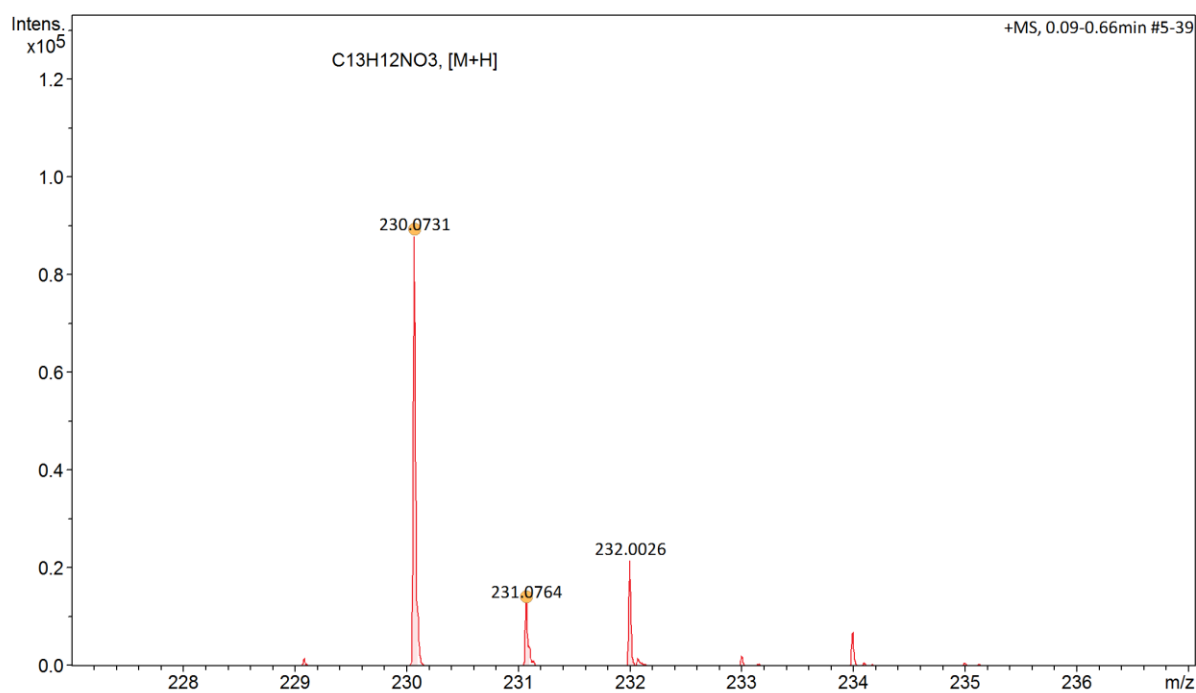
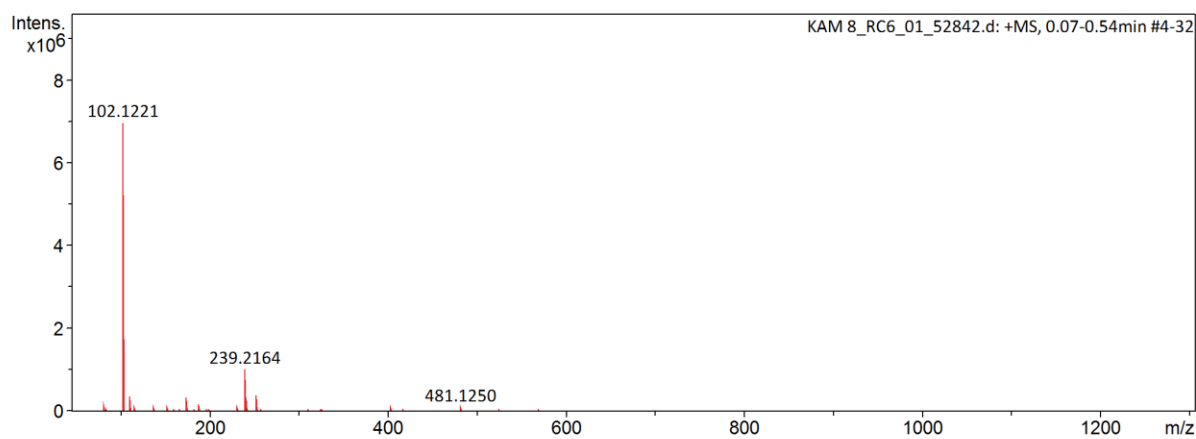


FTIR spectrum of 3-hydroxy-N-phenylbenzamide (KAM 8)

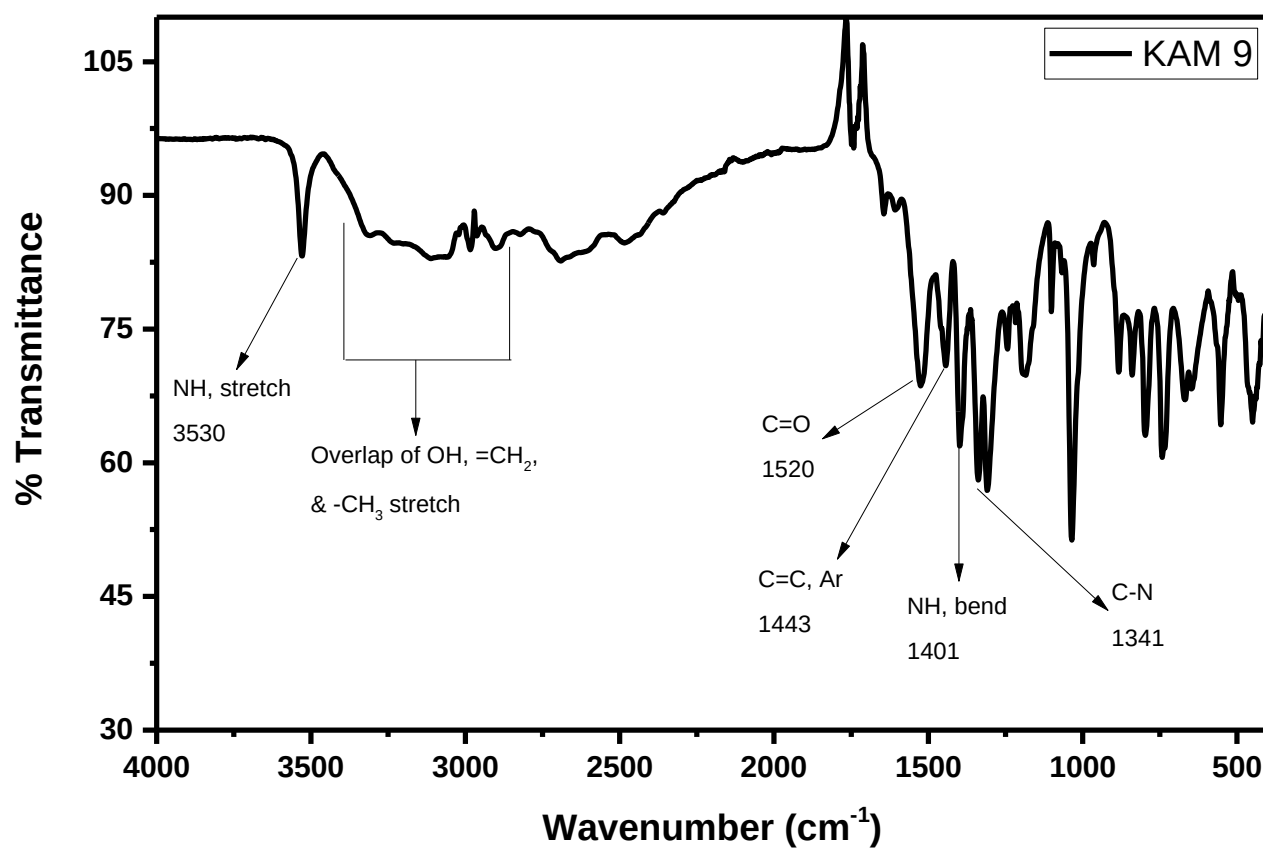
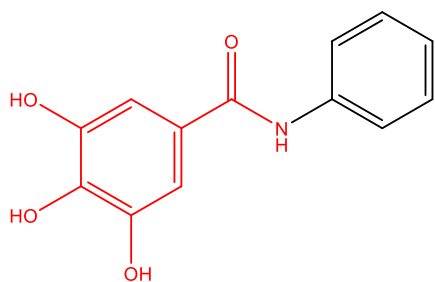




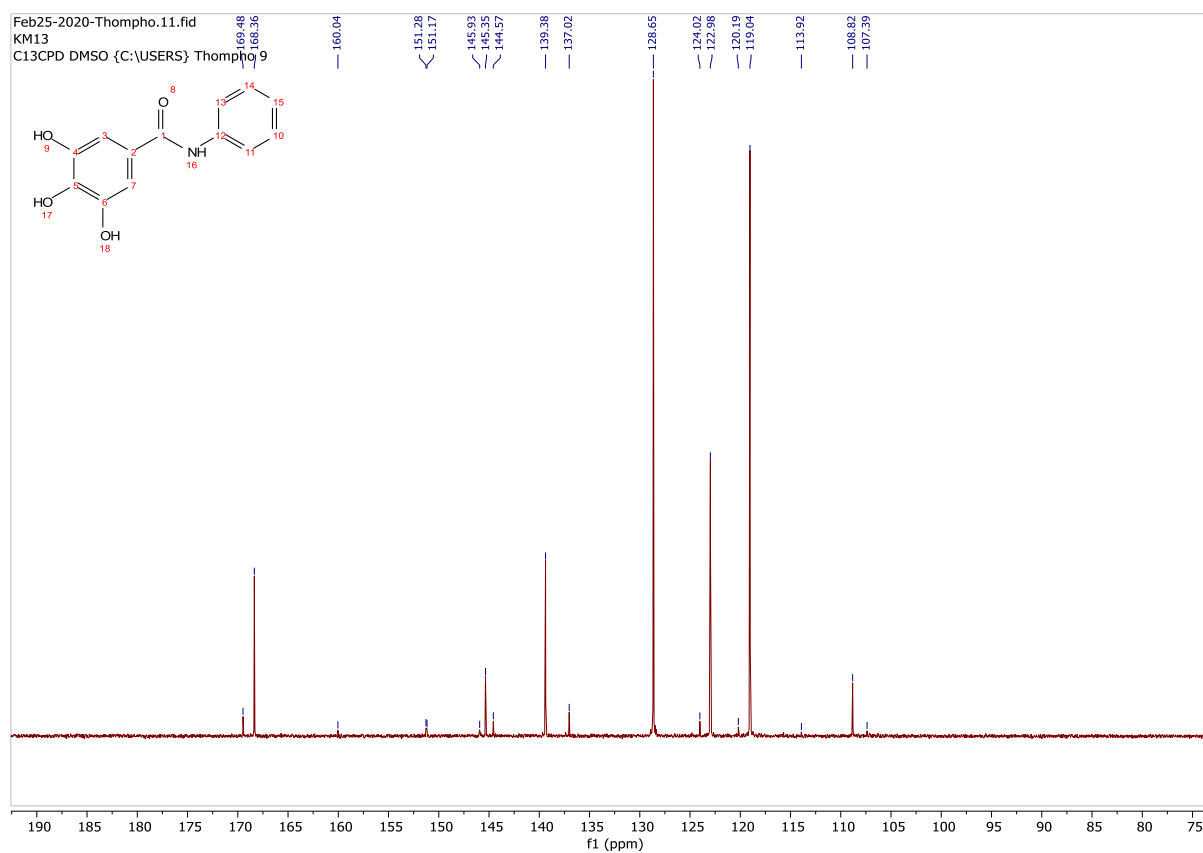
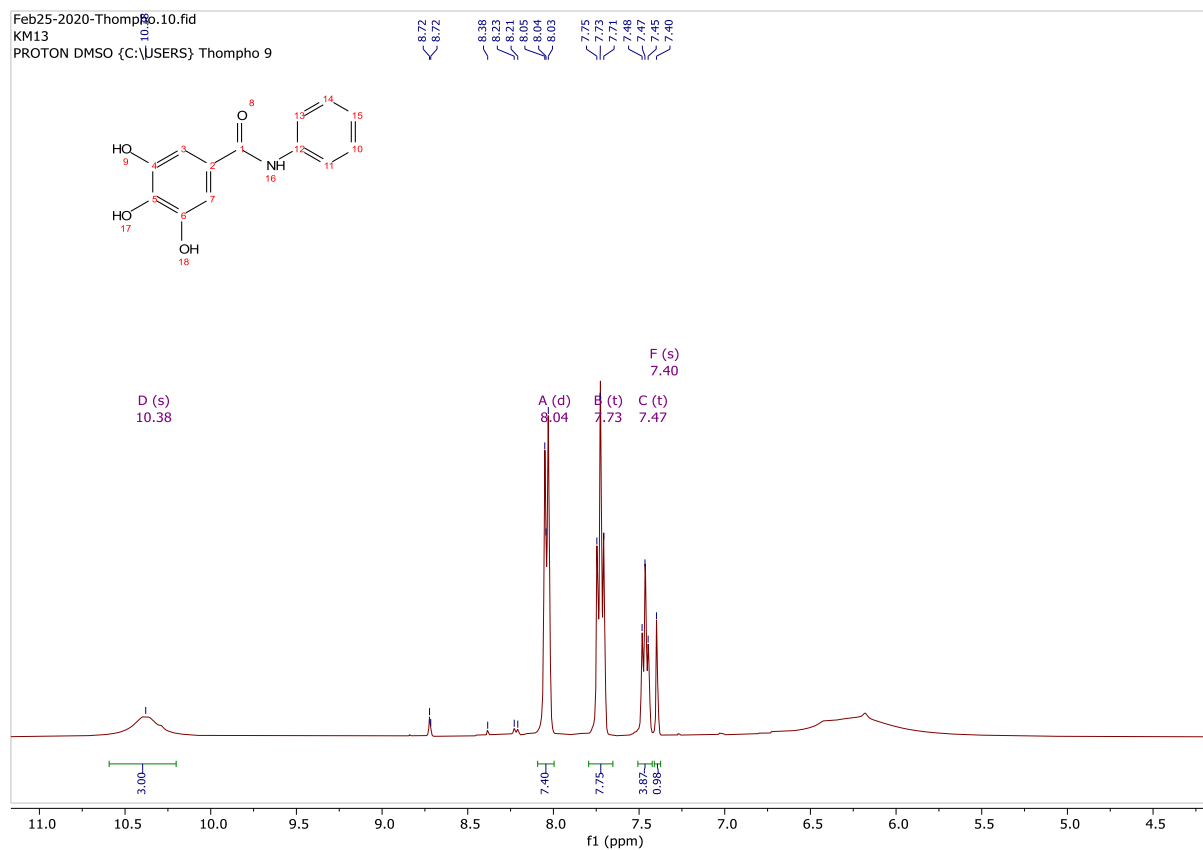
**$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-hydroxy-N-phenylbenzamide (KAM 8)**



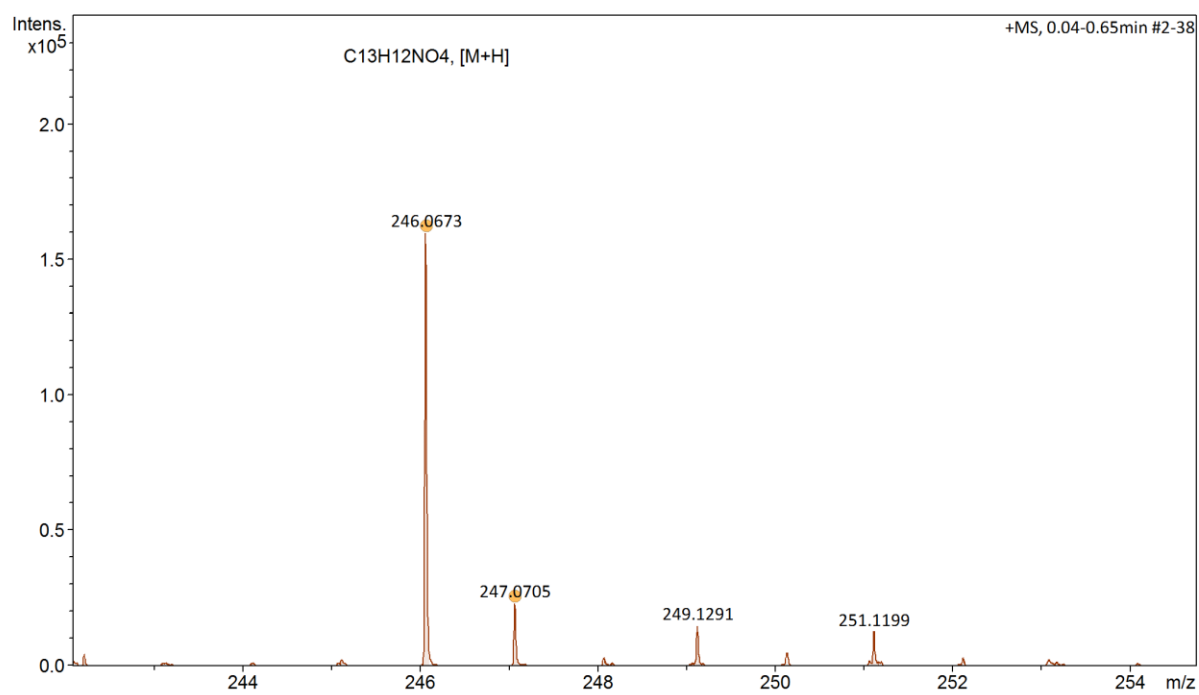
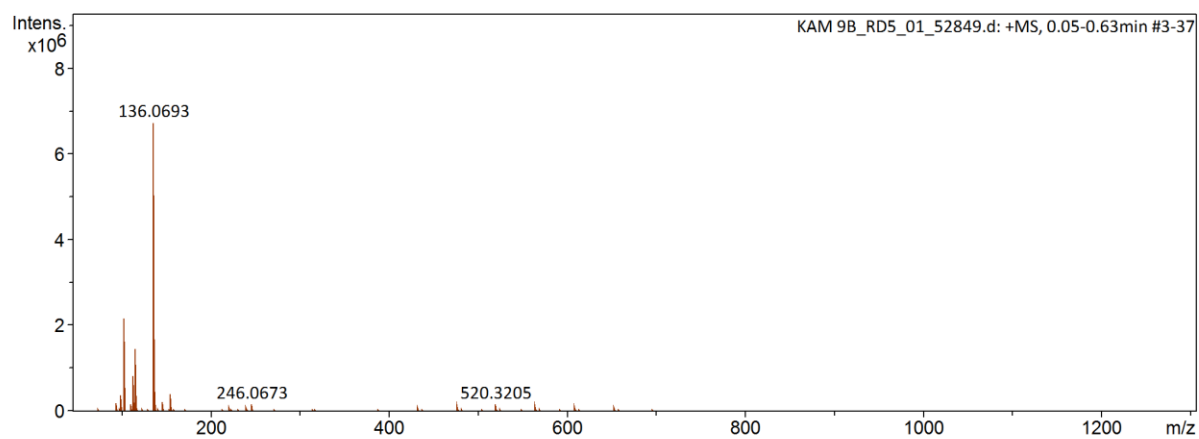
**HRMS of 3-hydroxy-*N*-phenylbenzamide (KAM 8)**



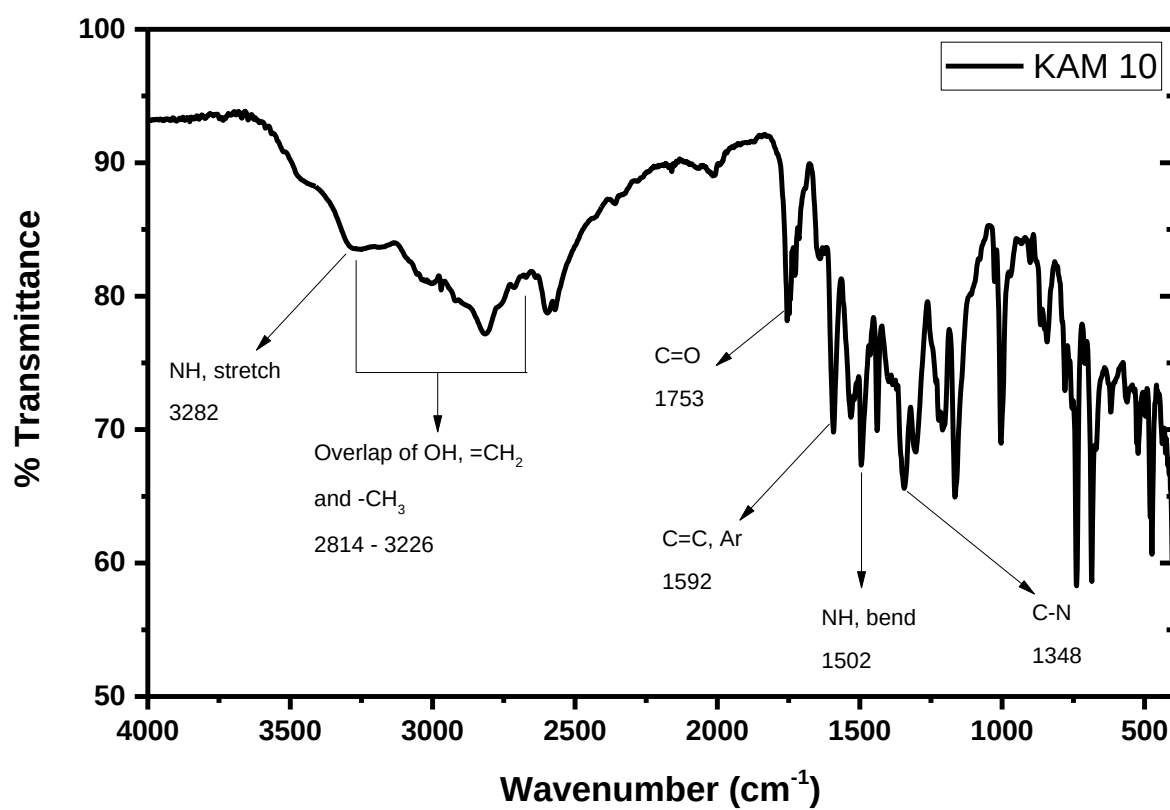
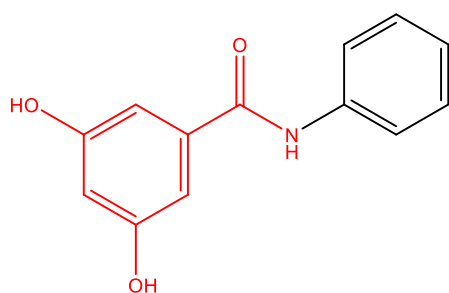
FTIR spectrum of 3,4,5-trihydroxy-N-phenylbenzamide (KAM 9)



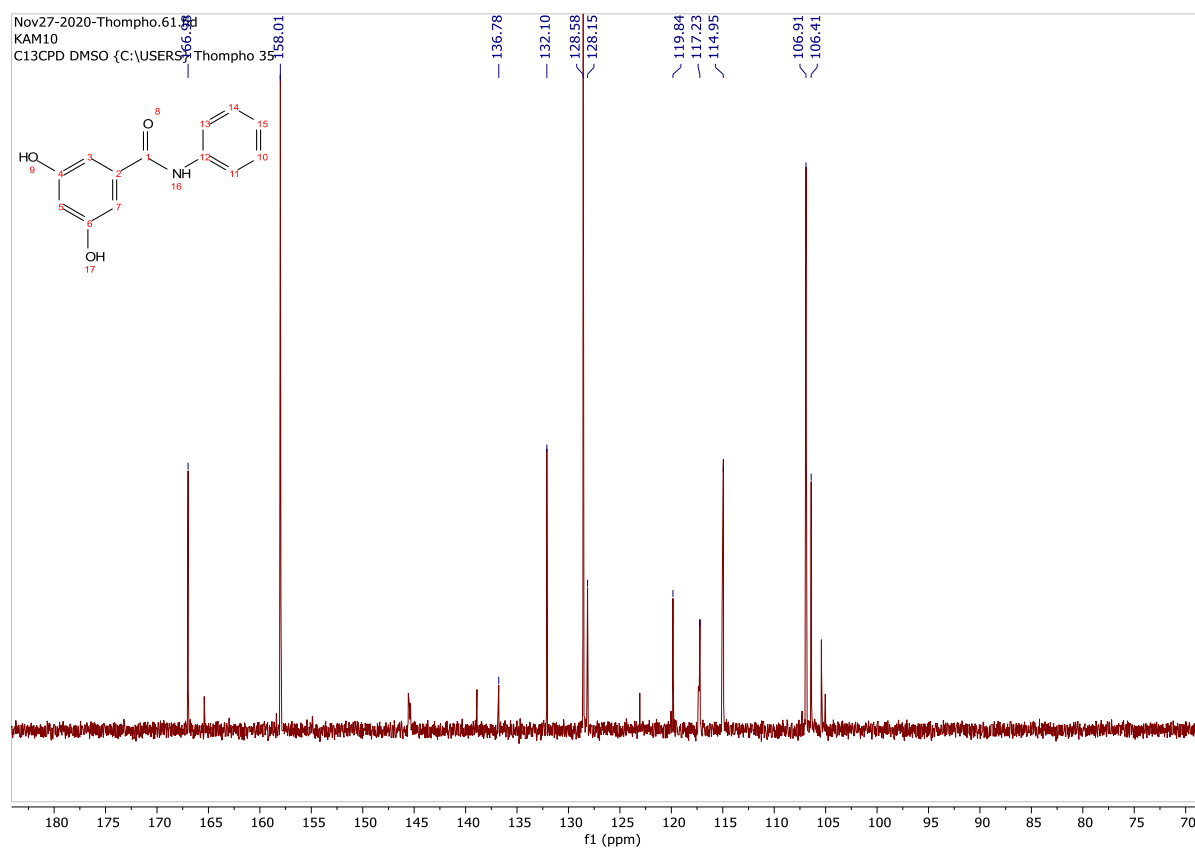
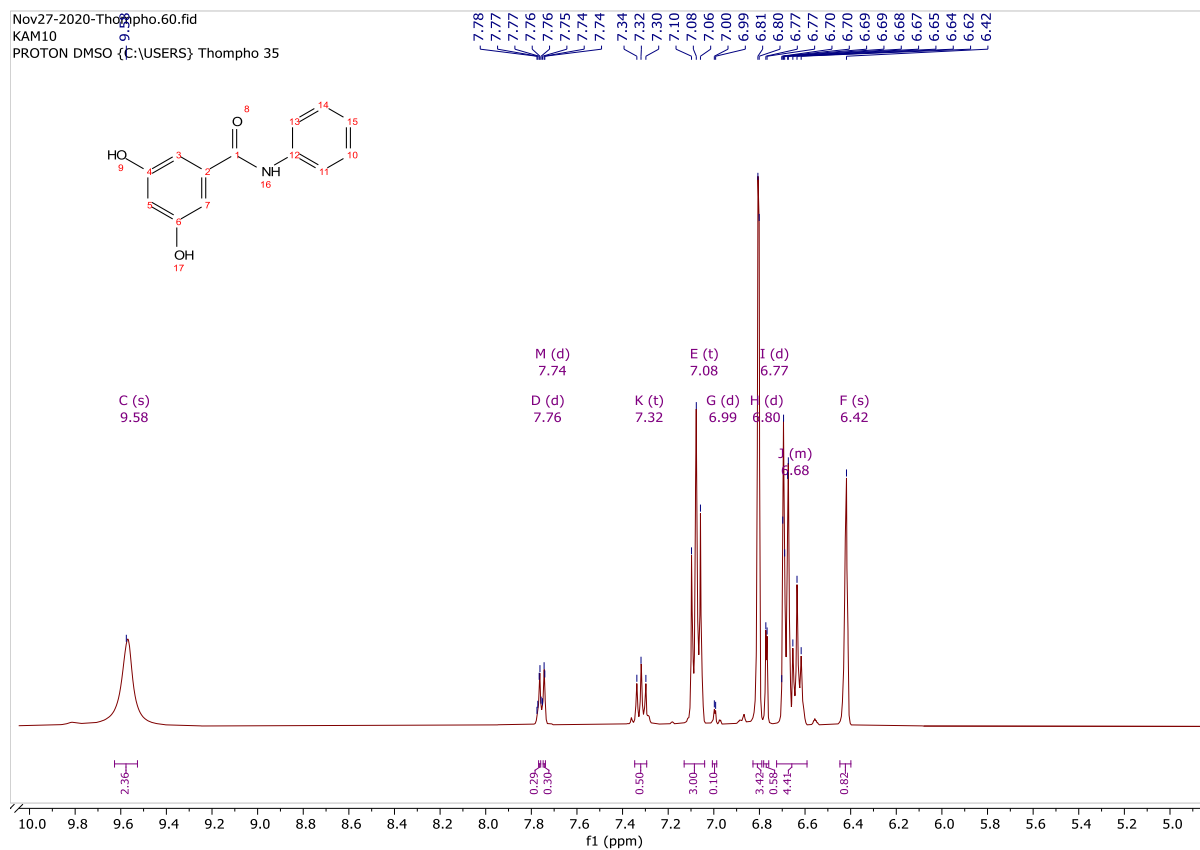
**<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3,4,5-trihydroxy-N-phenylbenzamide (KAM 9)**



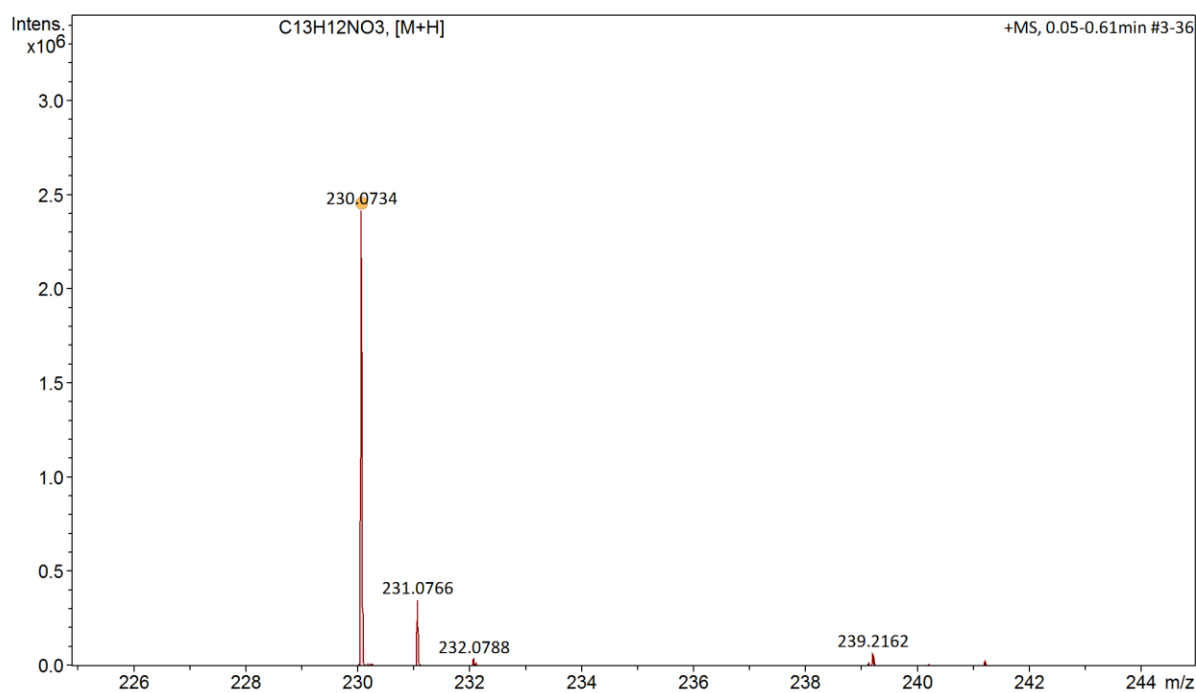
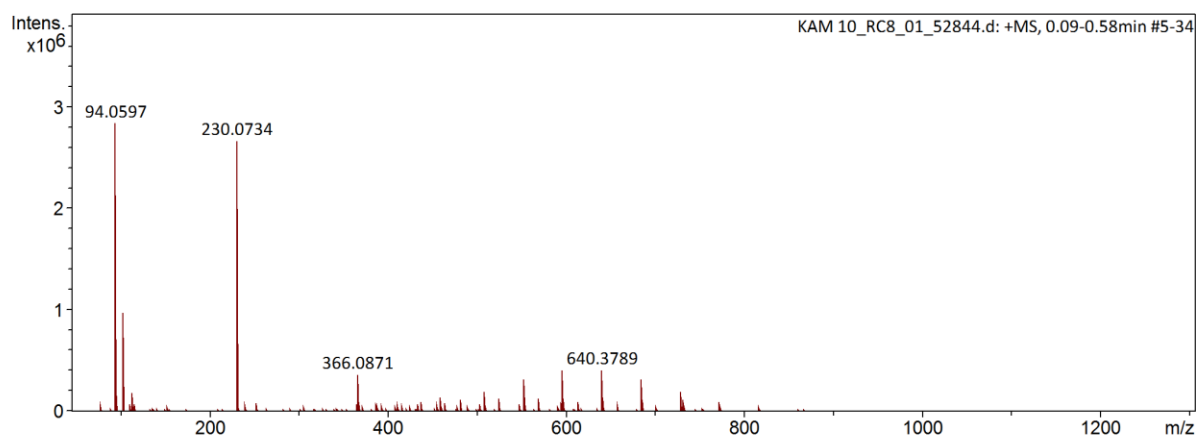
**HRMS of 3,4,5-trihydroxy-*N*-phenylbenzamide (KAM 9)**



FTIR spectrum of 3,5-dihydroxy-N-phenylbenzamide (KAM 10)

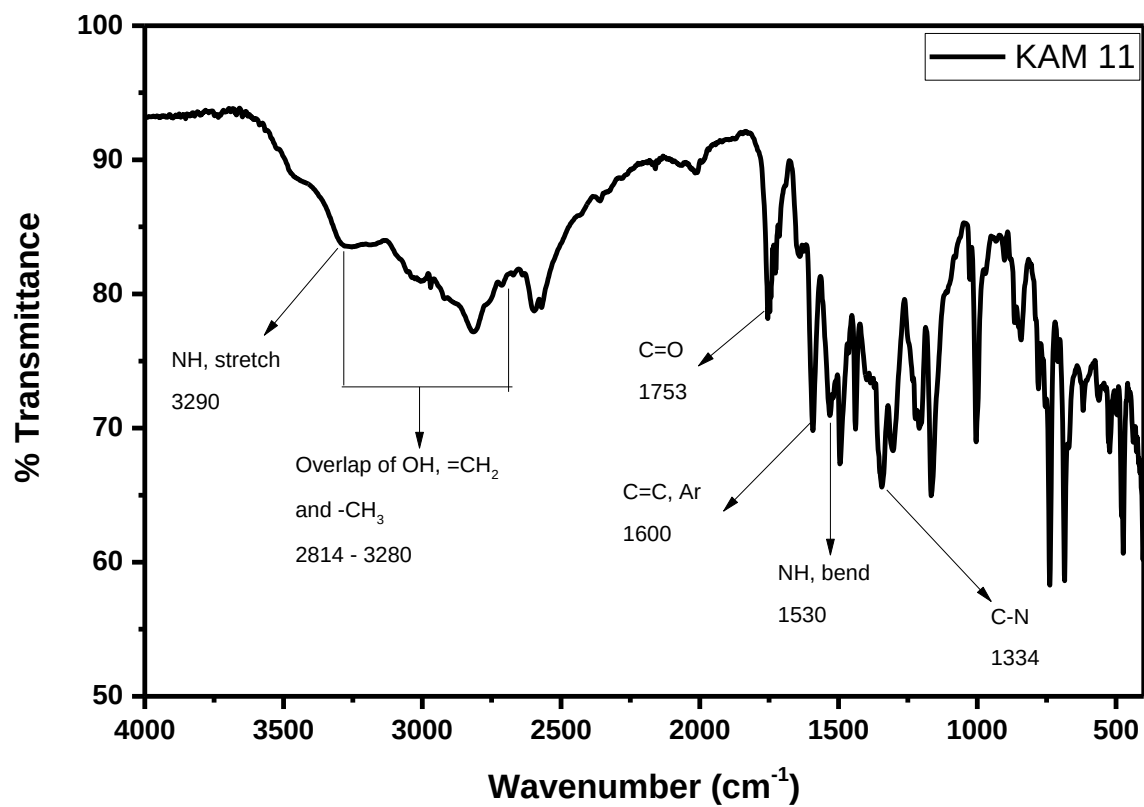
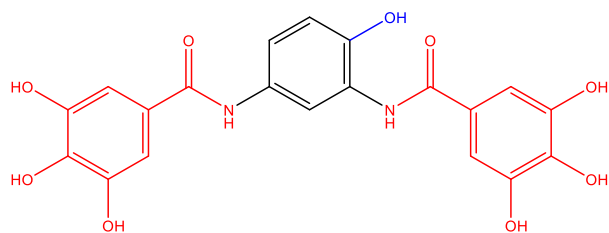


**$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3,5-dihydroxy-*N*-phenylbenzamide (KAM 10)**

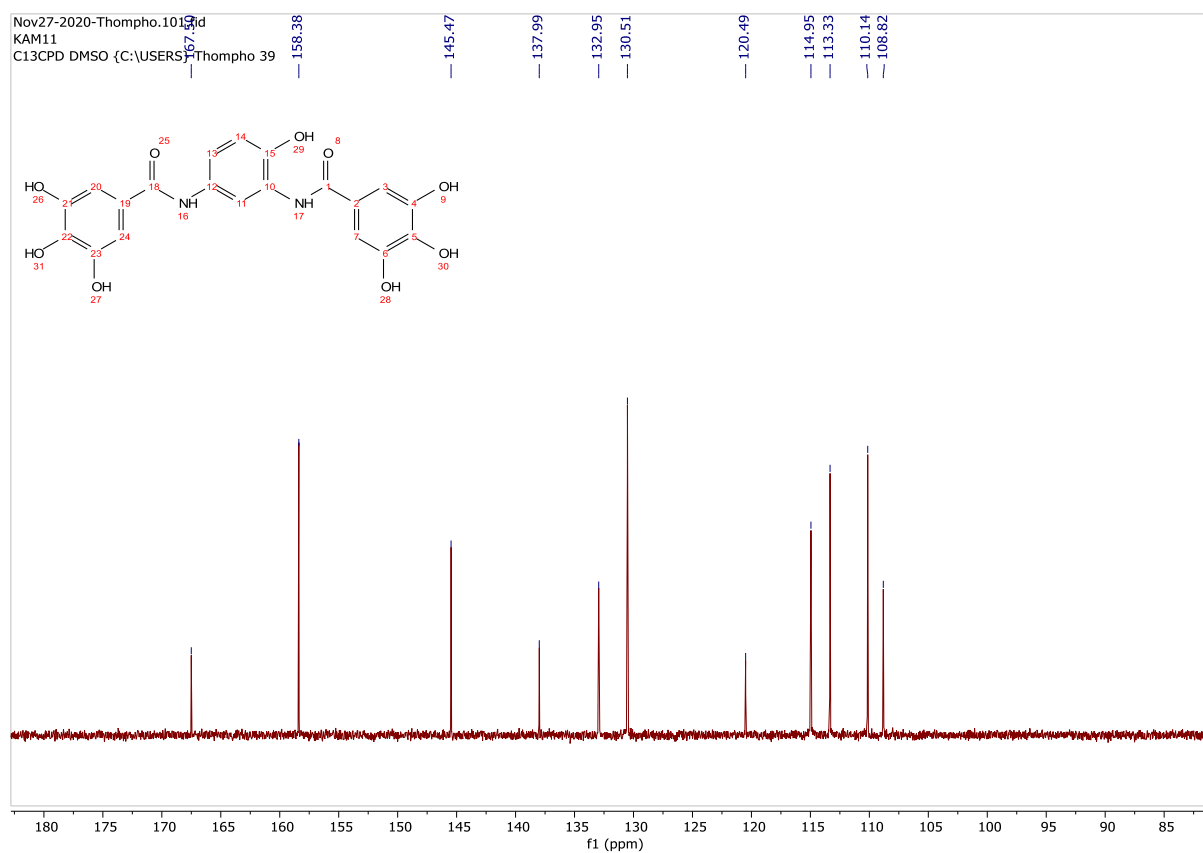
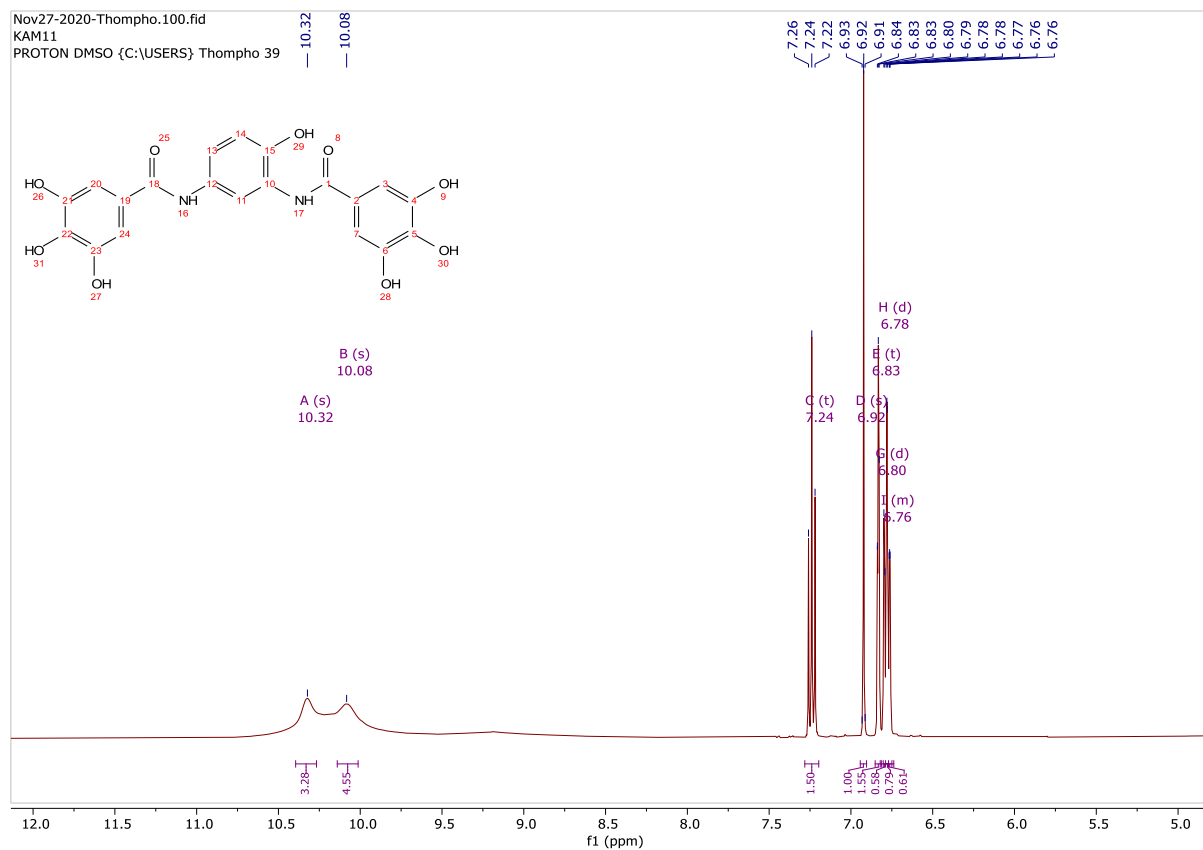


**HRMS of 3,5-dihydroxy-*N*-phenylbenzamide (KAM 10)**

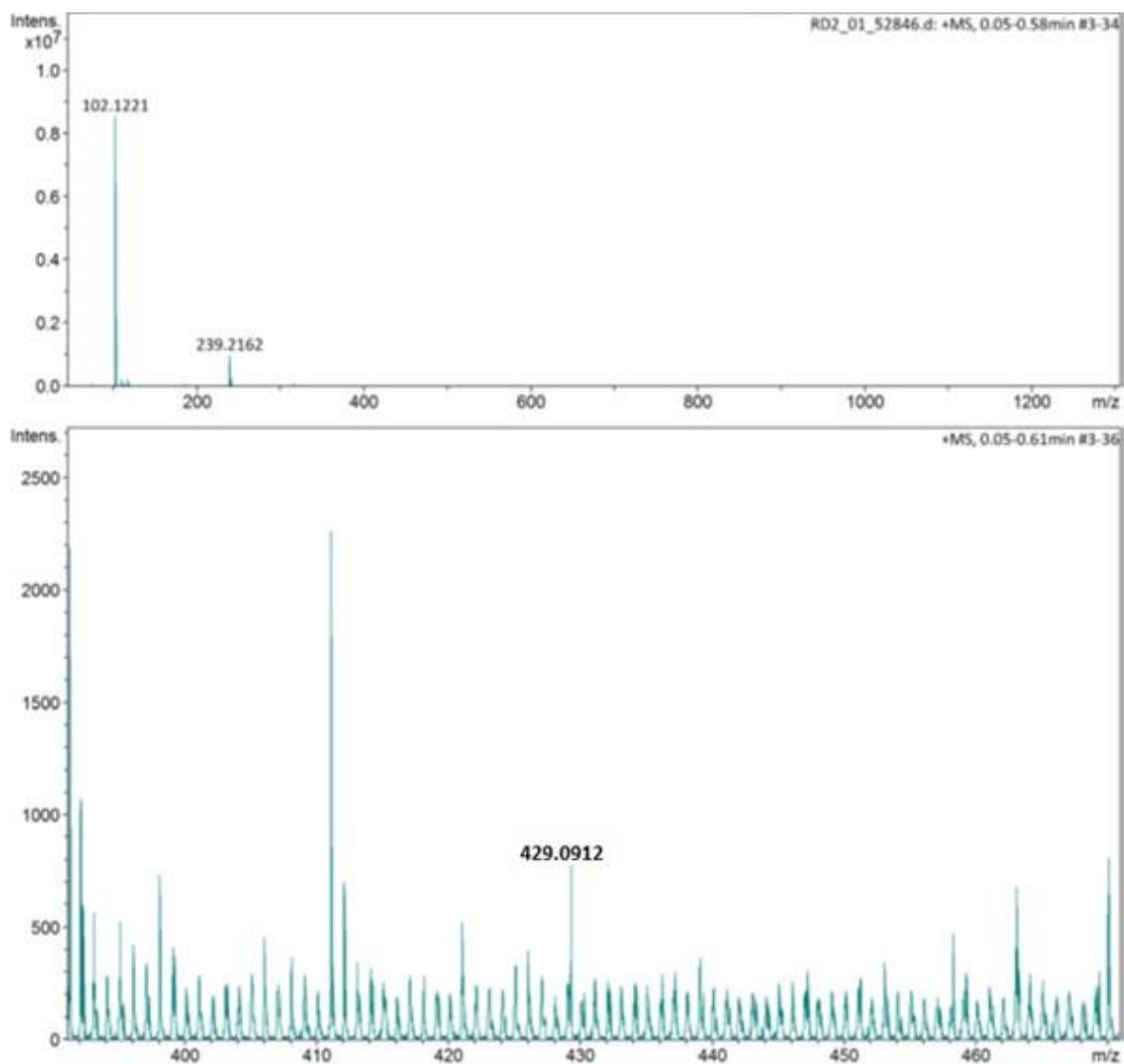




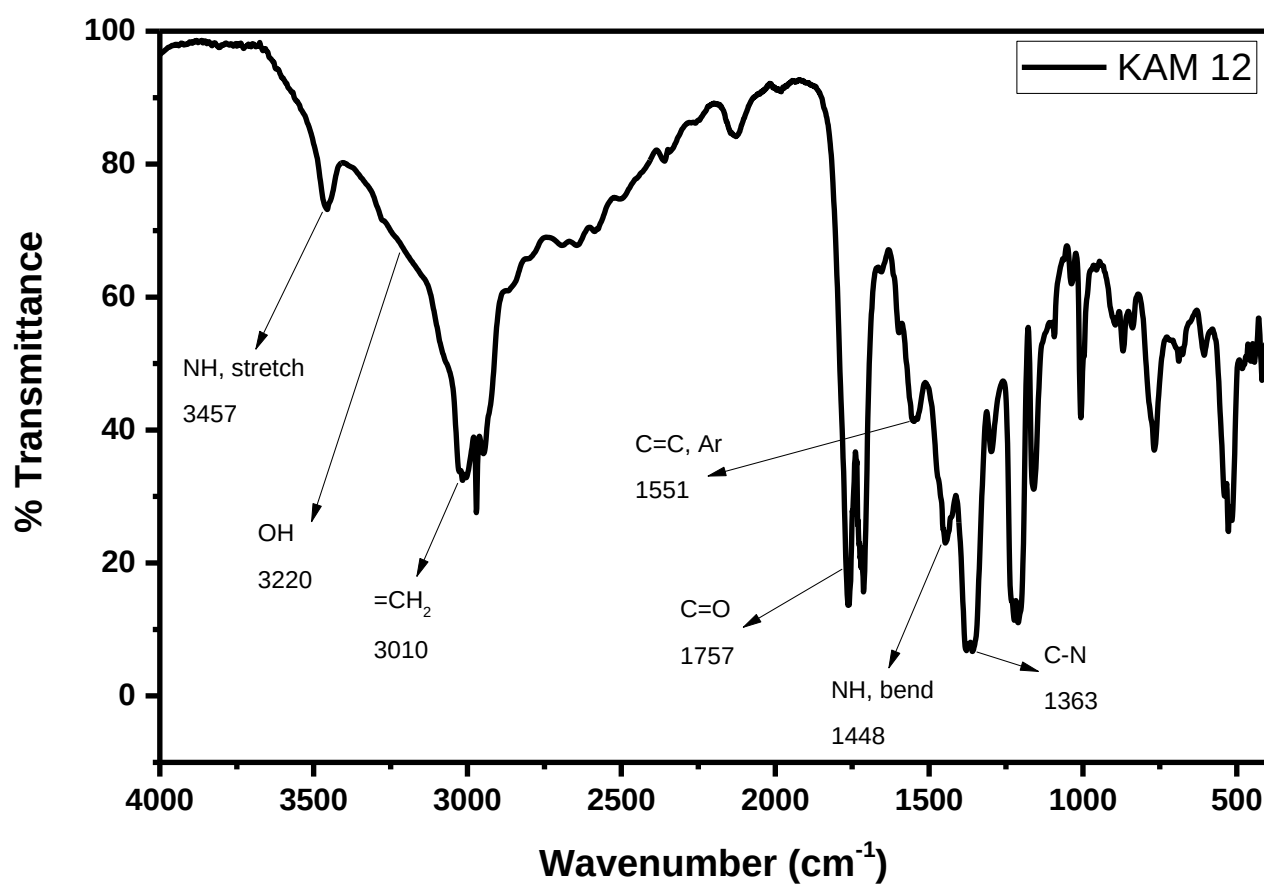
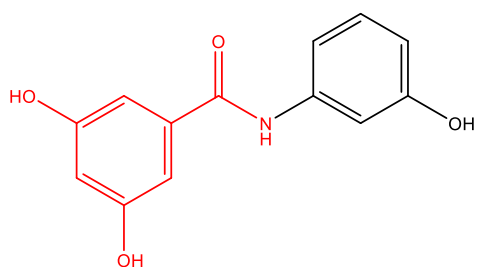
FTIR spectrum of *N,N'*-(4-hydroxy-1,3-phenylene)bis(3,4,5-trihydroxybenzamide)



**<sup>1</sup>H and <sup>13</sup>C NMR spectra of *N,N'*-(4-hydroxy-1,3-phenylene)bis(3,4,5-trihydroxybenzamide) (KAM 11)**



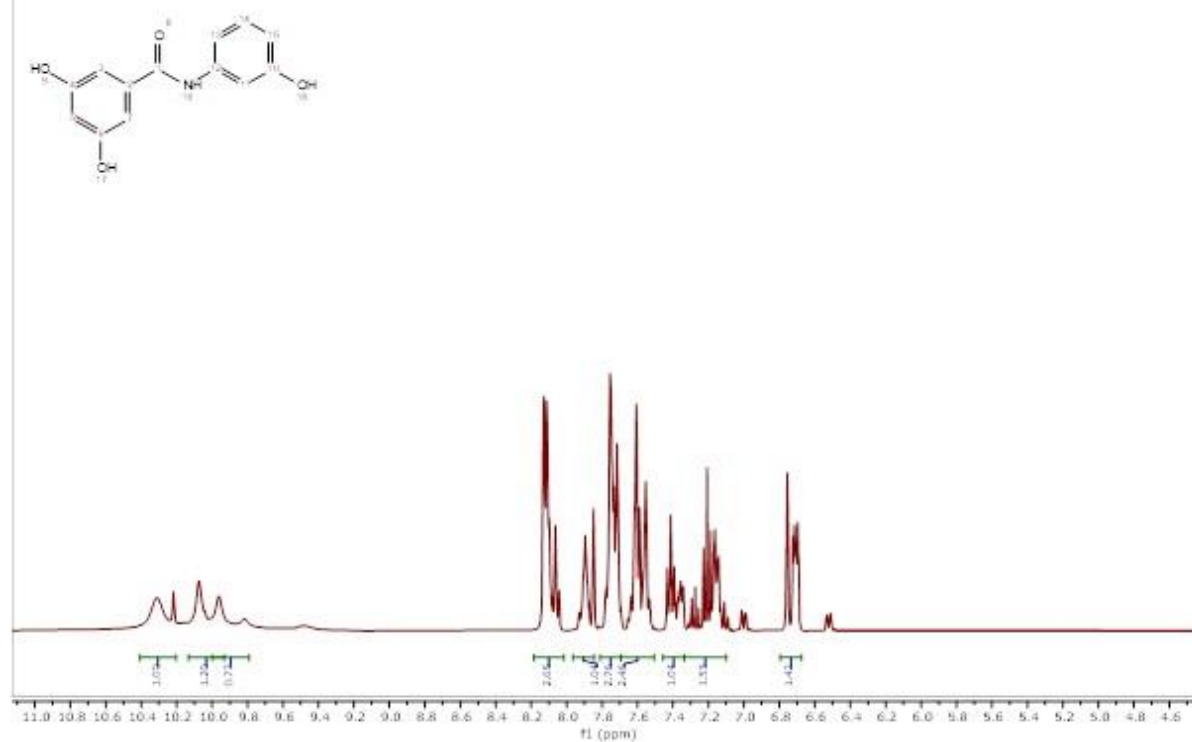
**HRMS of *N,N'*-(4-hydroxy-1,3-phenylene)bis(3,4,5-trihydroxybenzamide) (KAM 11)**



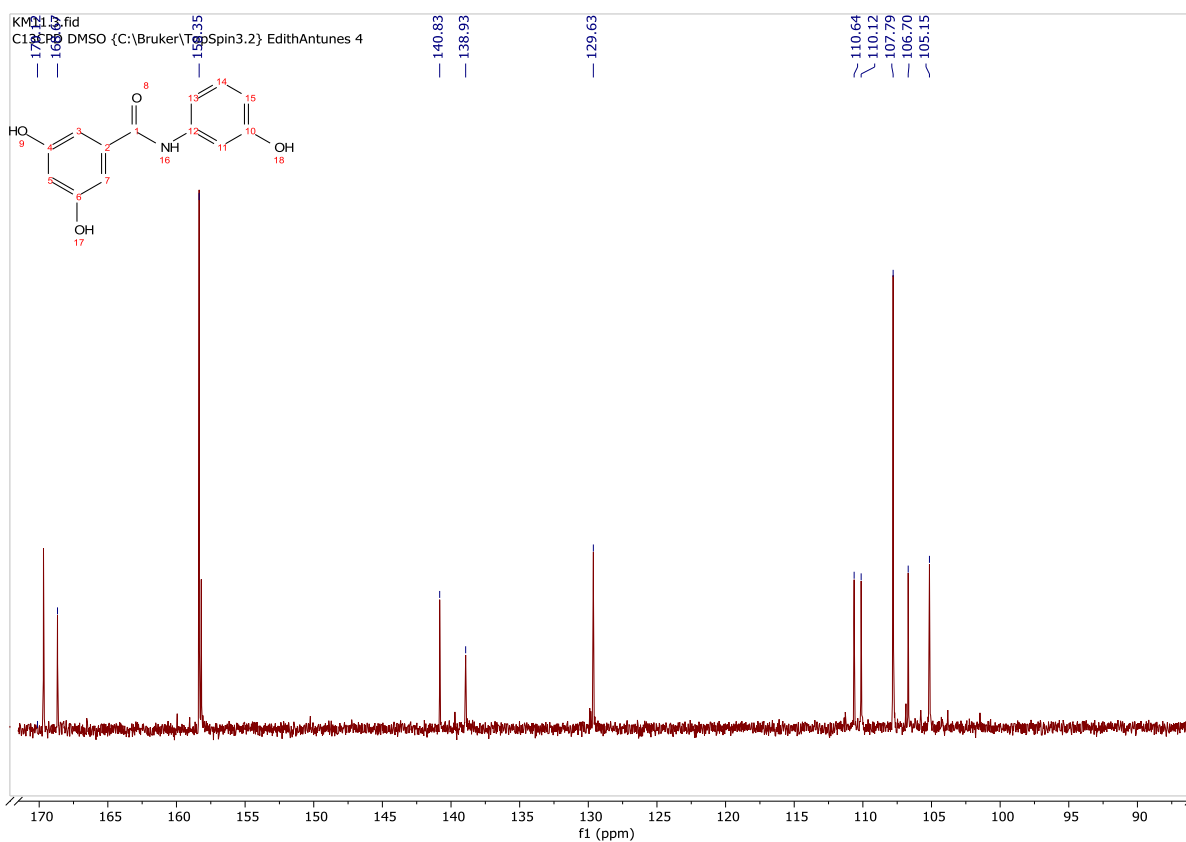
FTIR spectrum of 3,5-dihydroxy-*N*-(3-hydroxyphenyl)benzamide (KAM 12)

Nov27-2020-Thompho.90.fid

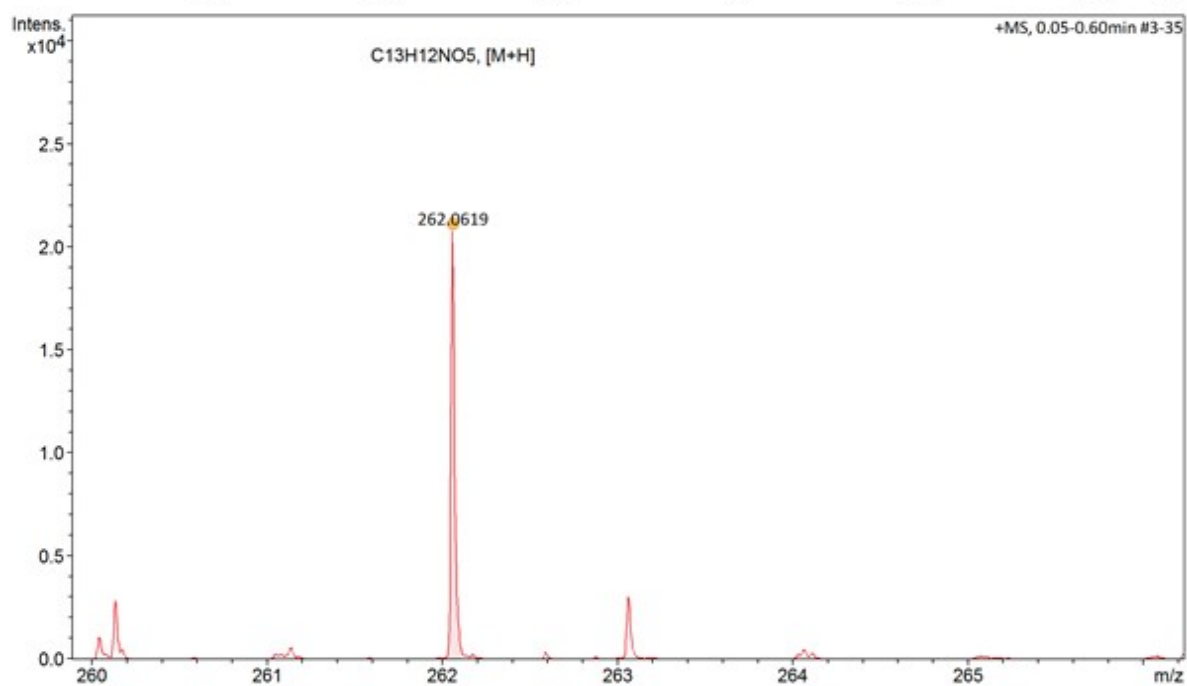
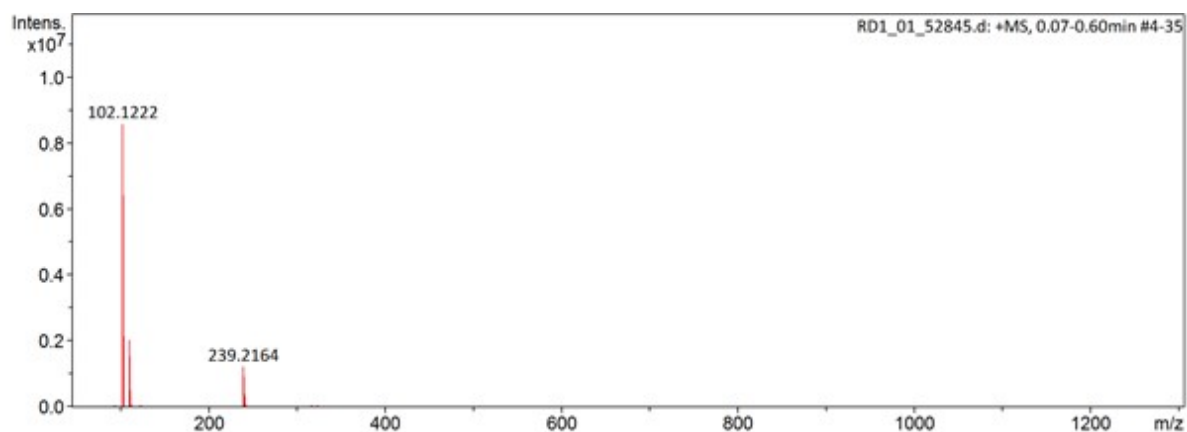
PROTON DMSO (C:\USERS\Thompho.38



KM12.fid  
C13CPD DMSO (C:\Bruker\TopSpin3.2) EdithAntunes 4

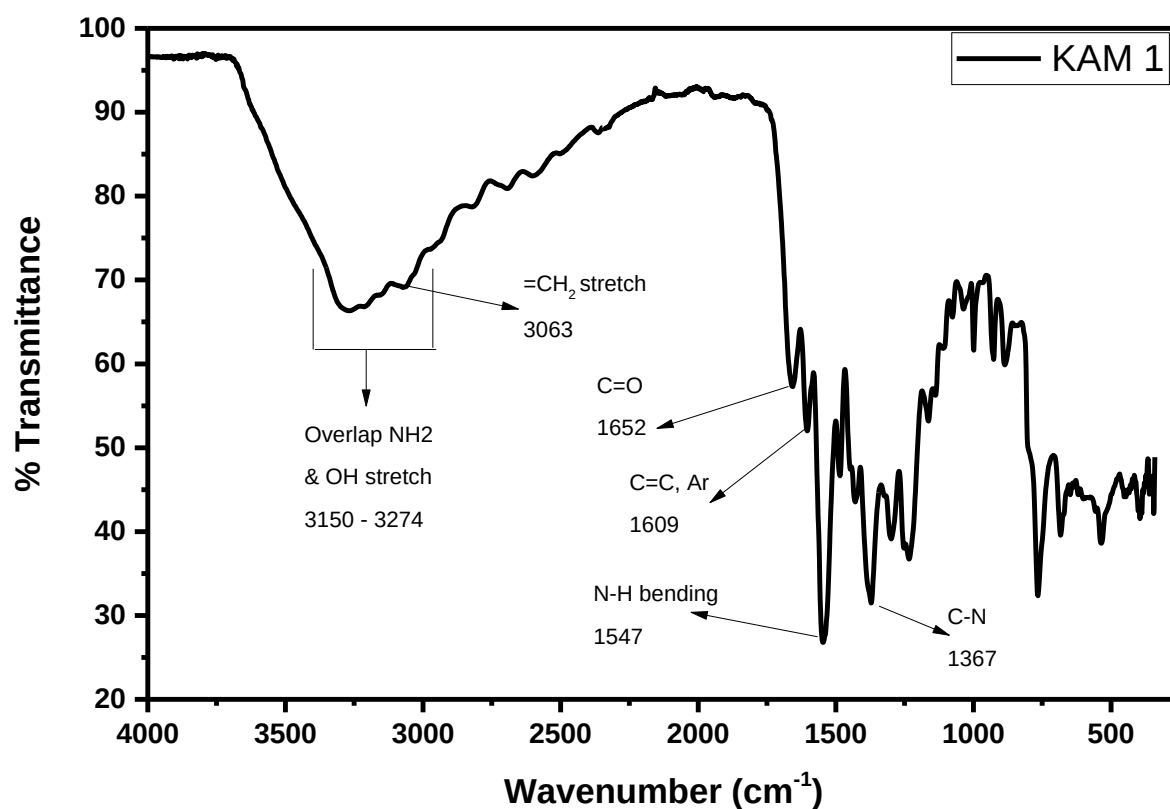
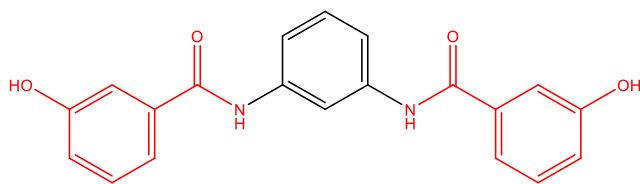


**<sup>1</sup>H and <sup>13</sup>C NMR of 3,5-dihydroxy-N-(3-hydroxyphenyl)benzamide (KAM 12)**

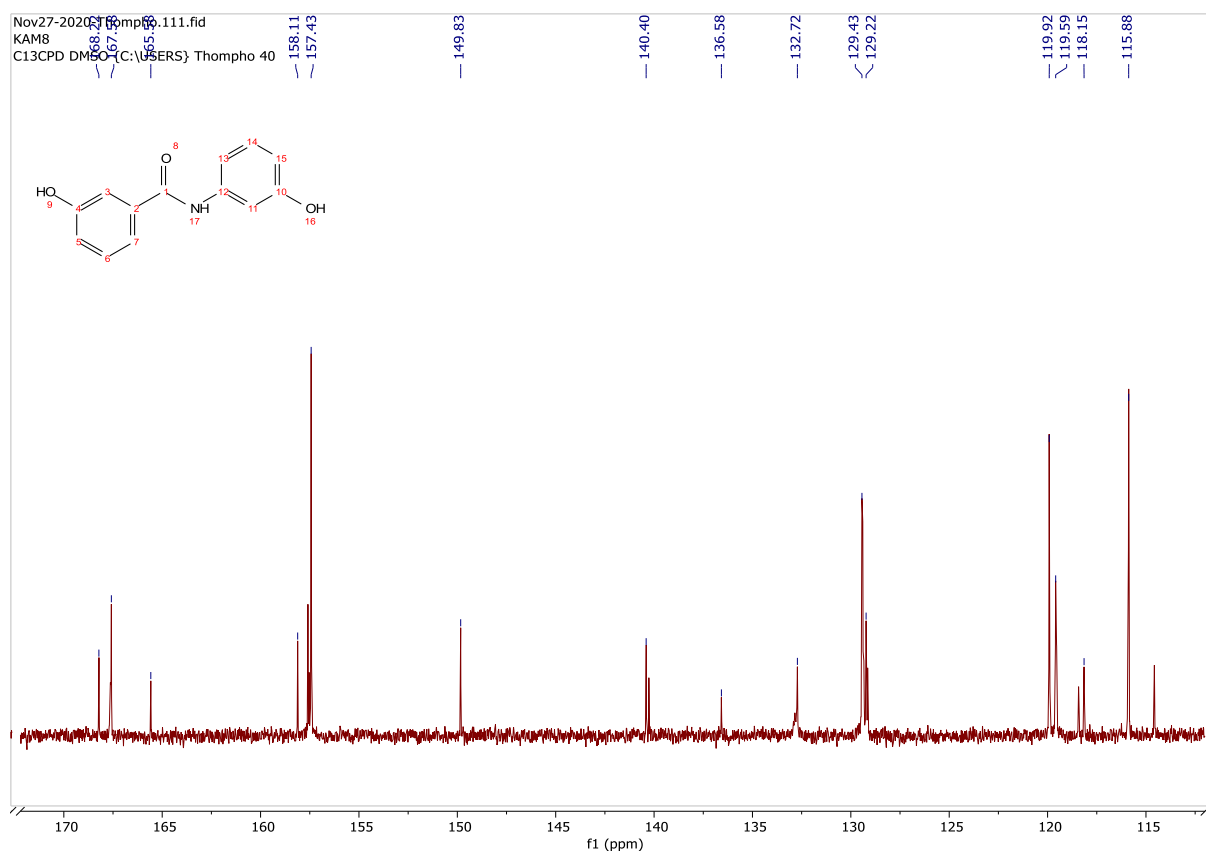
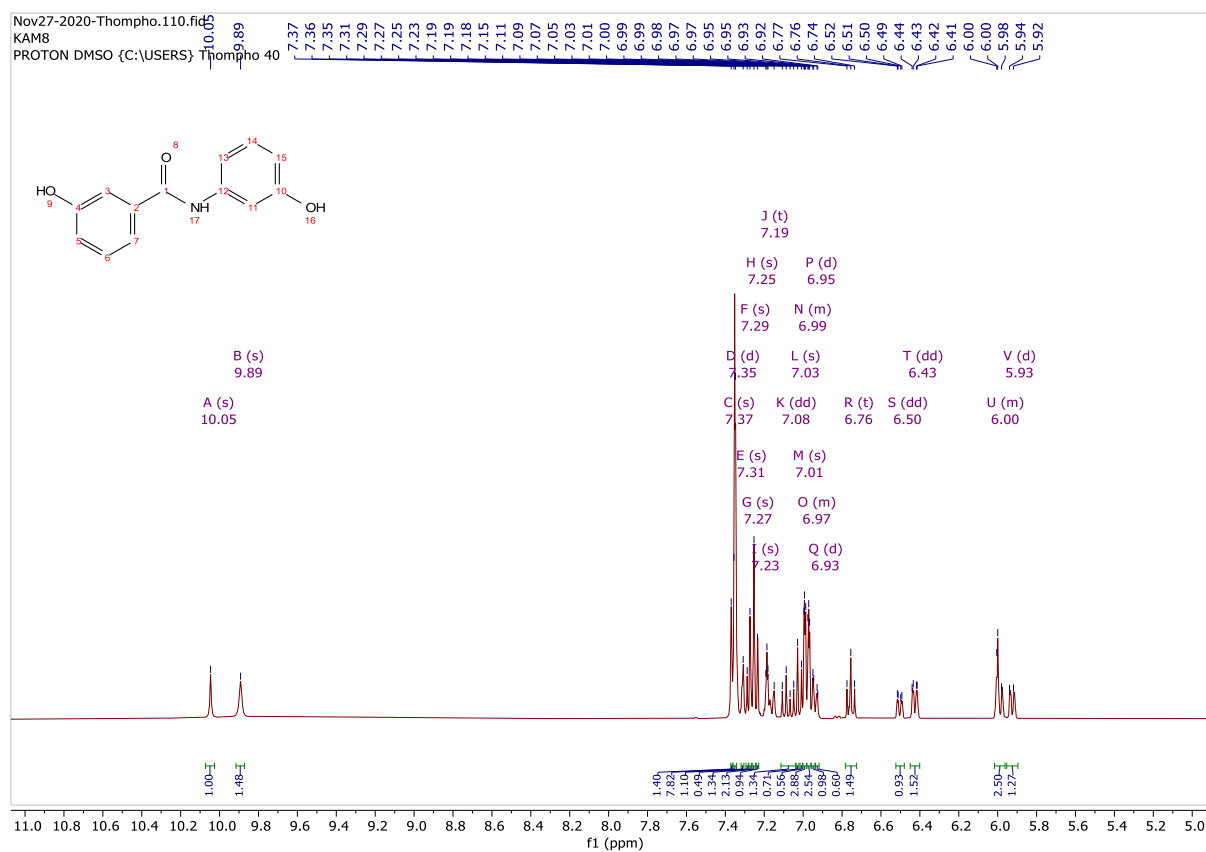


**HRMS of 3,5-dihydroxy-*N*-(3-hydroxyphenyl)benzamide (KAM 12)**

## Appendix C: Characterization data for amide compounds

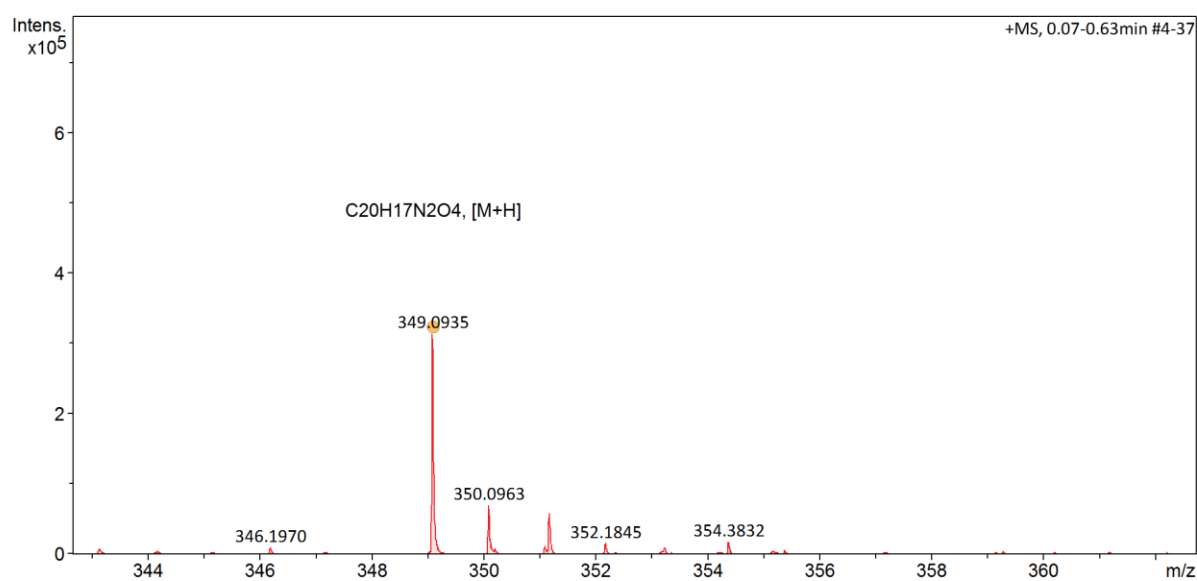
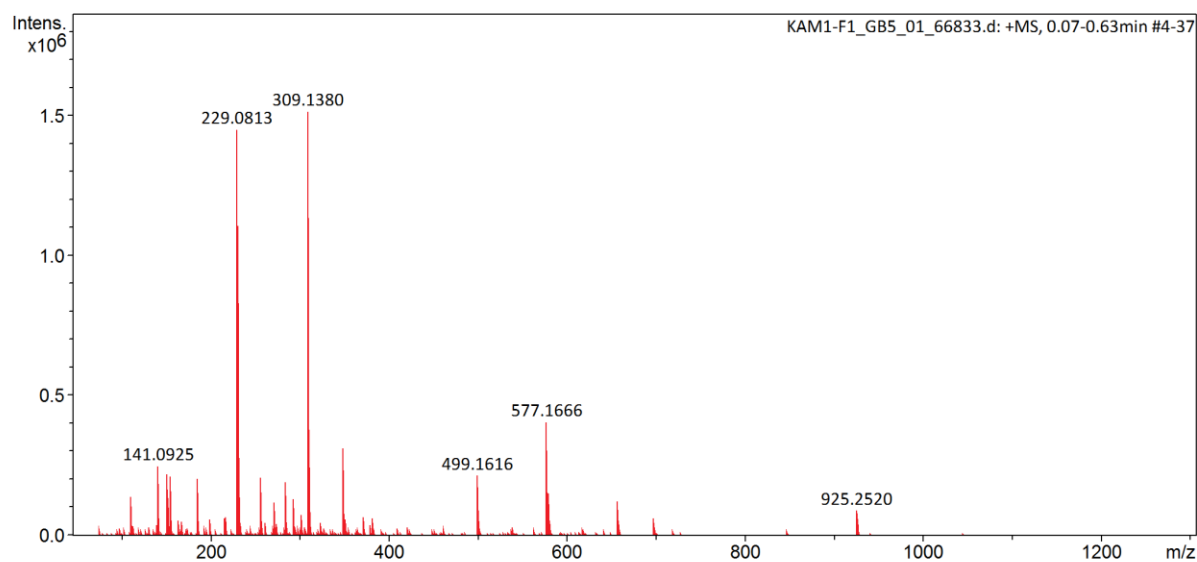


FTIR spectrum of *N,N'*-(1,3-phenylene)bis(3-hydroxybenzamide) (KAM 1)

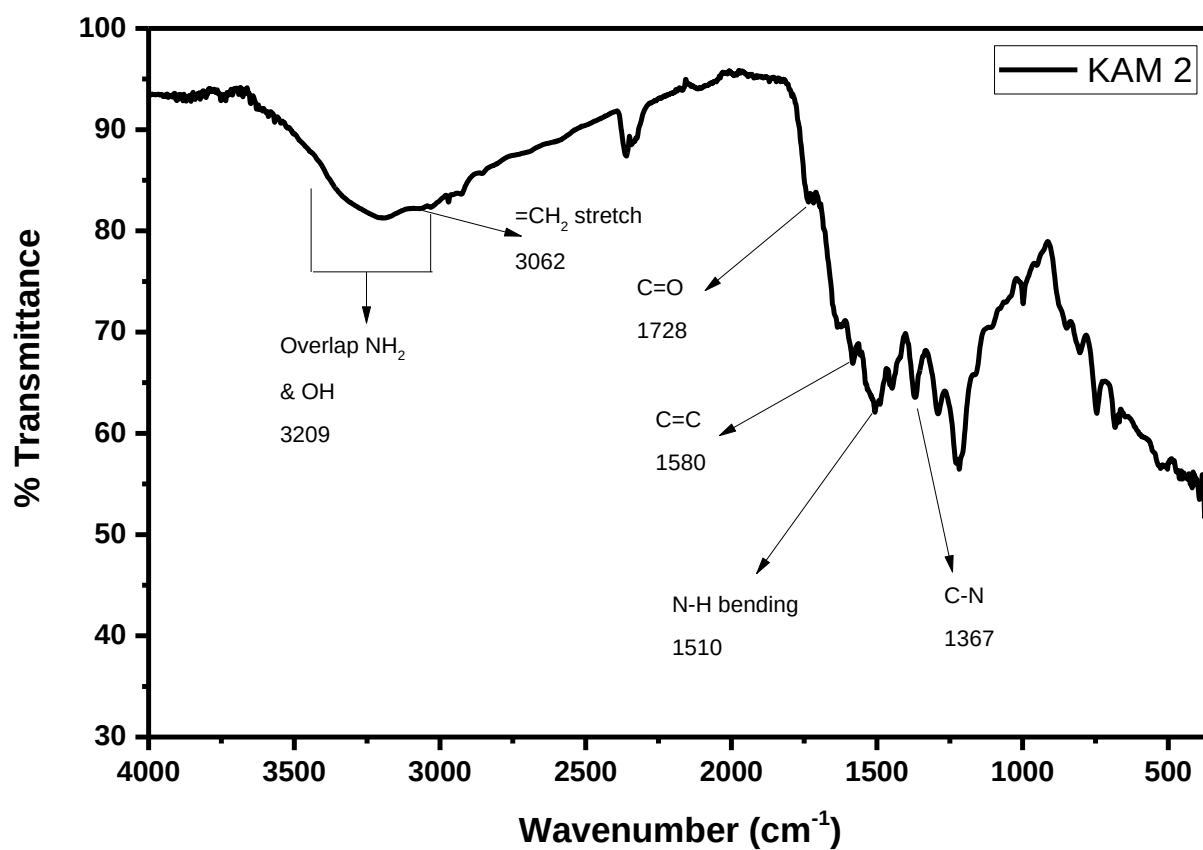
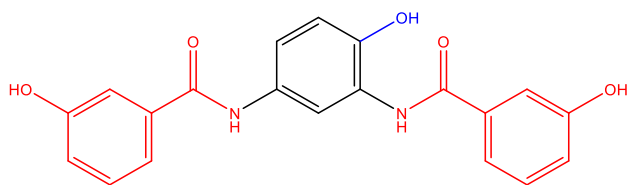


**<sup>1</sup>H and <sup>13</sup>C NMR spectra of *N,N'*-(1,3-phenylene)bis(3-hydroxybenzamide) (KAM 1)**

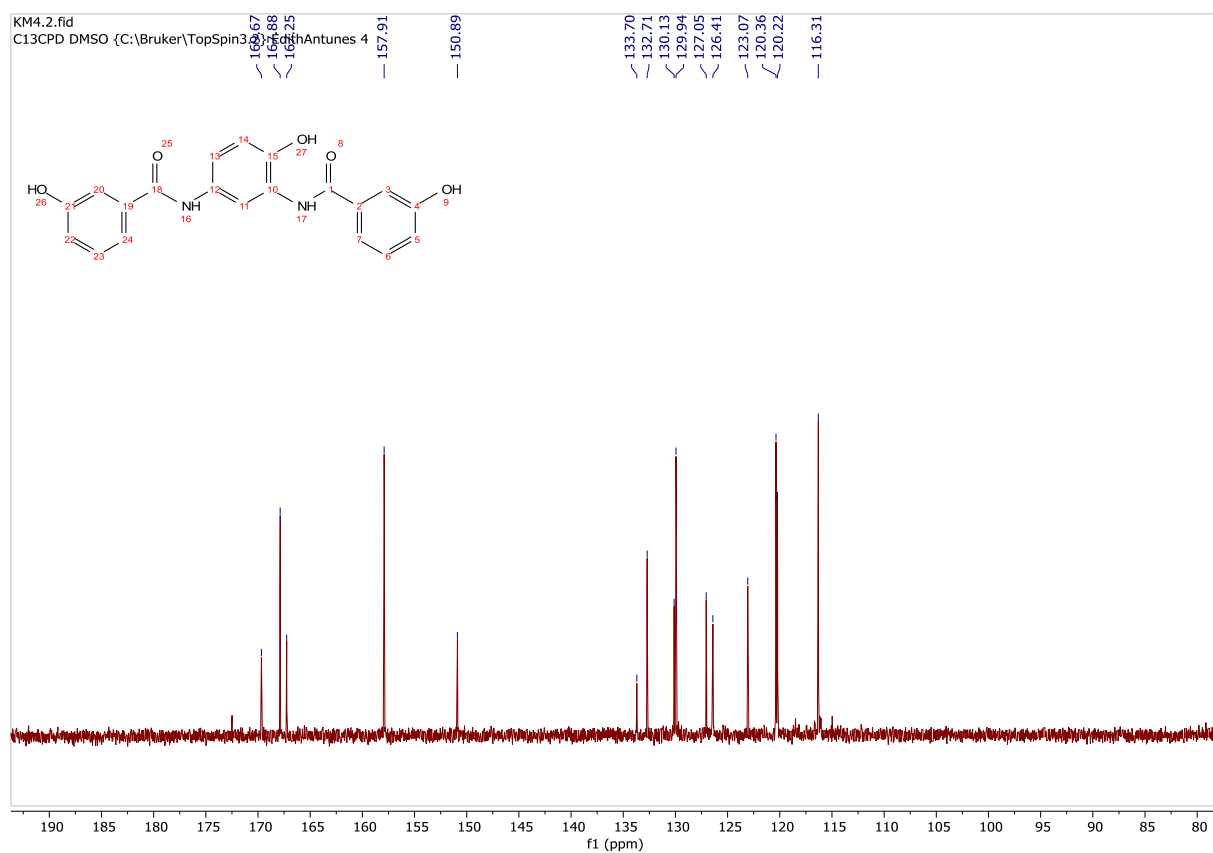
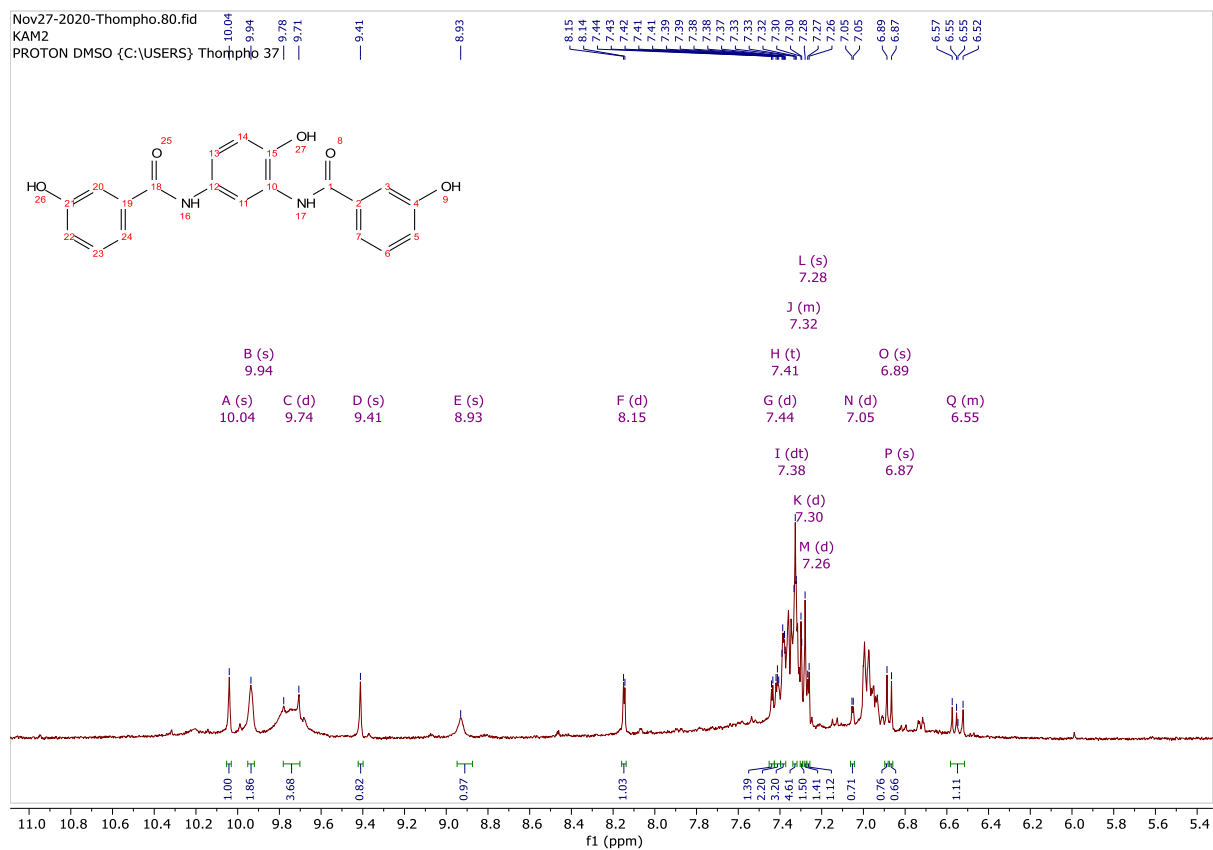




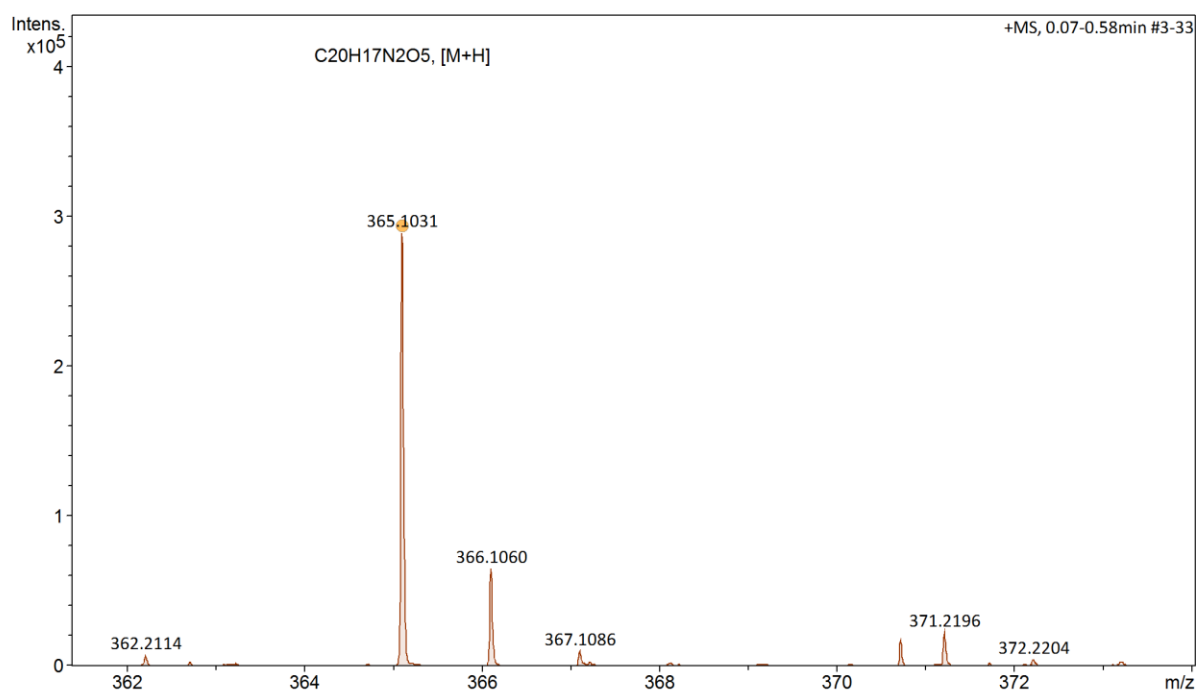
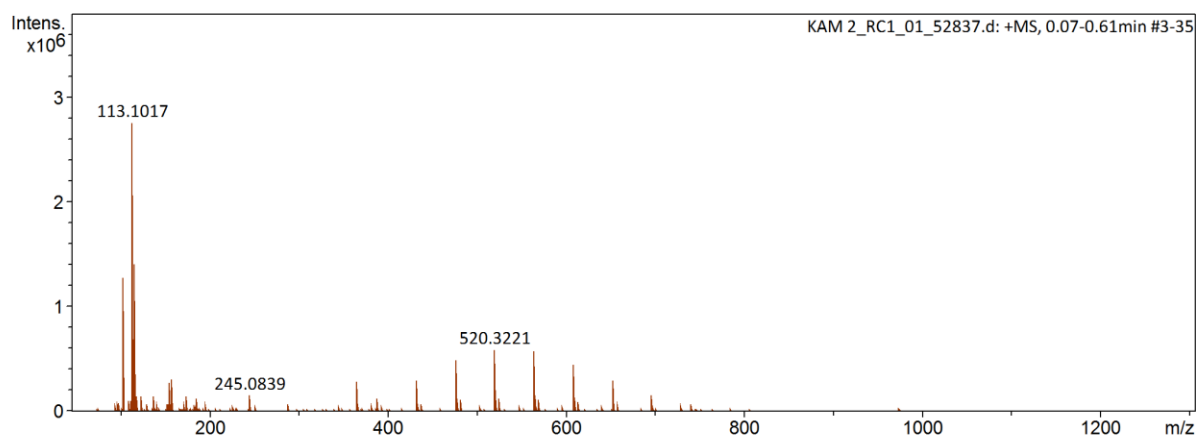
**HRMS of *N,N'*-(1,3-phenylene)bis(3-hydroxybenzamide) (KAM 1)**



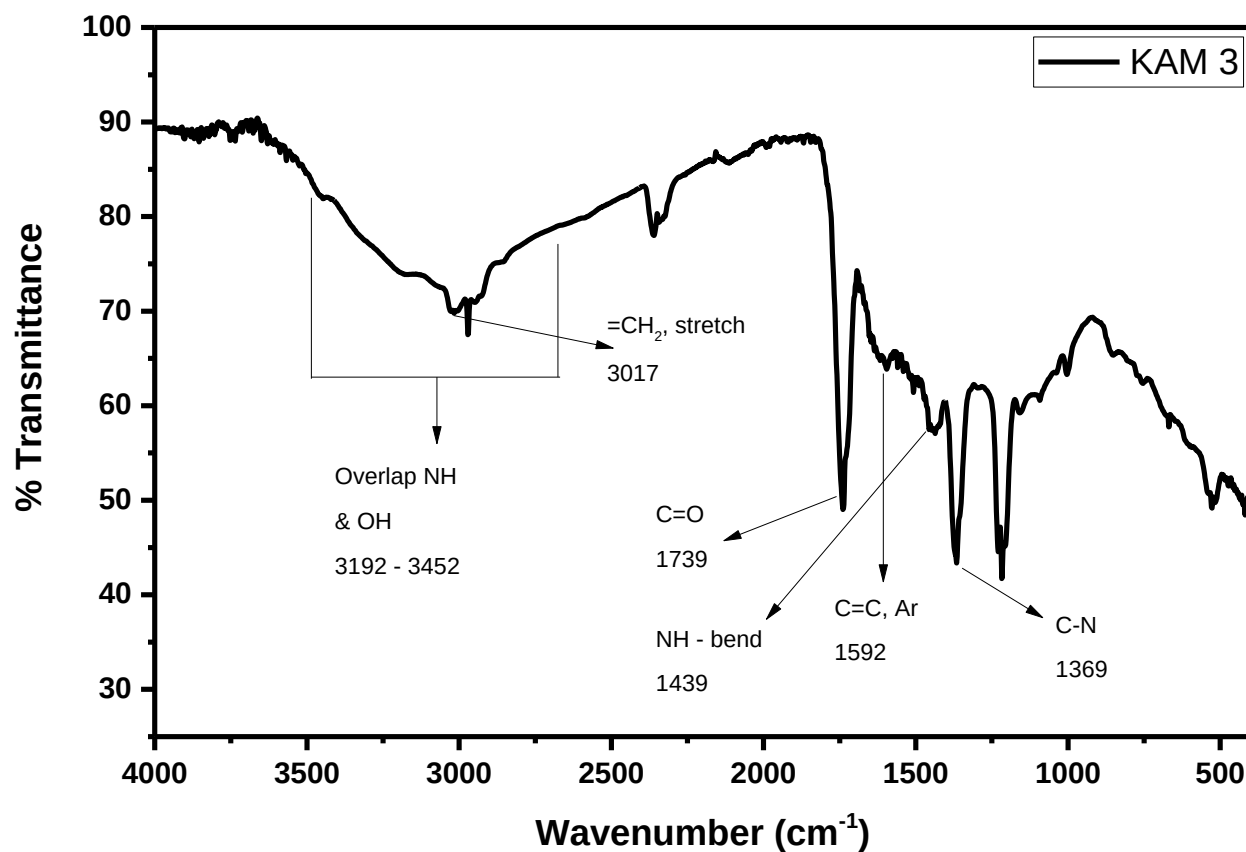
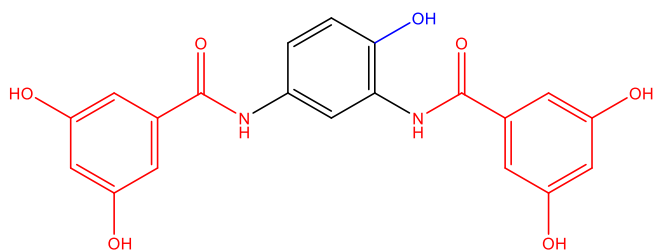
FTIR spectrum of *N,N'*-(4-hydroxy-1,3-phenylene)bis(3-hydroxybenzamide) (KAM 2)



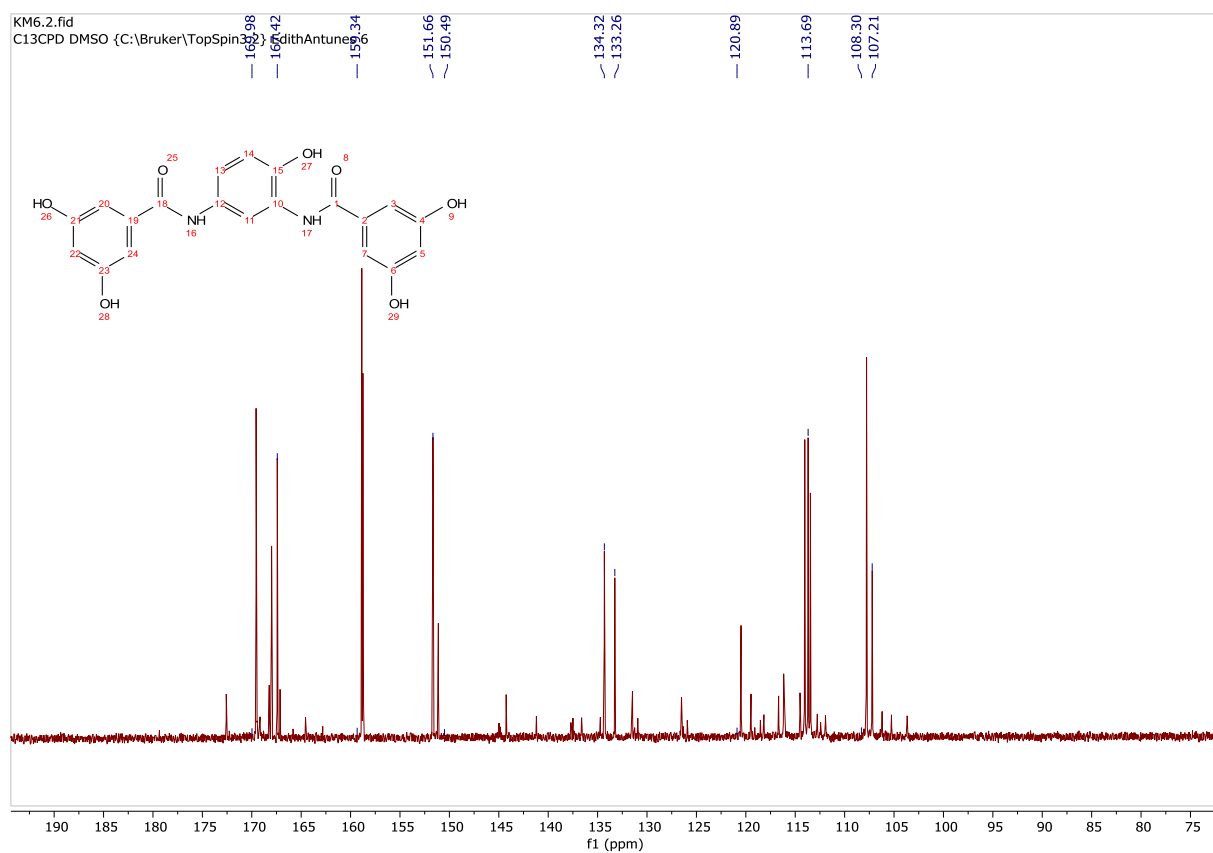
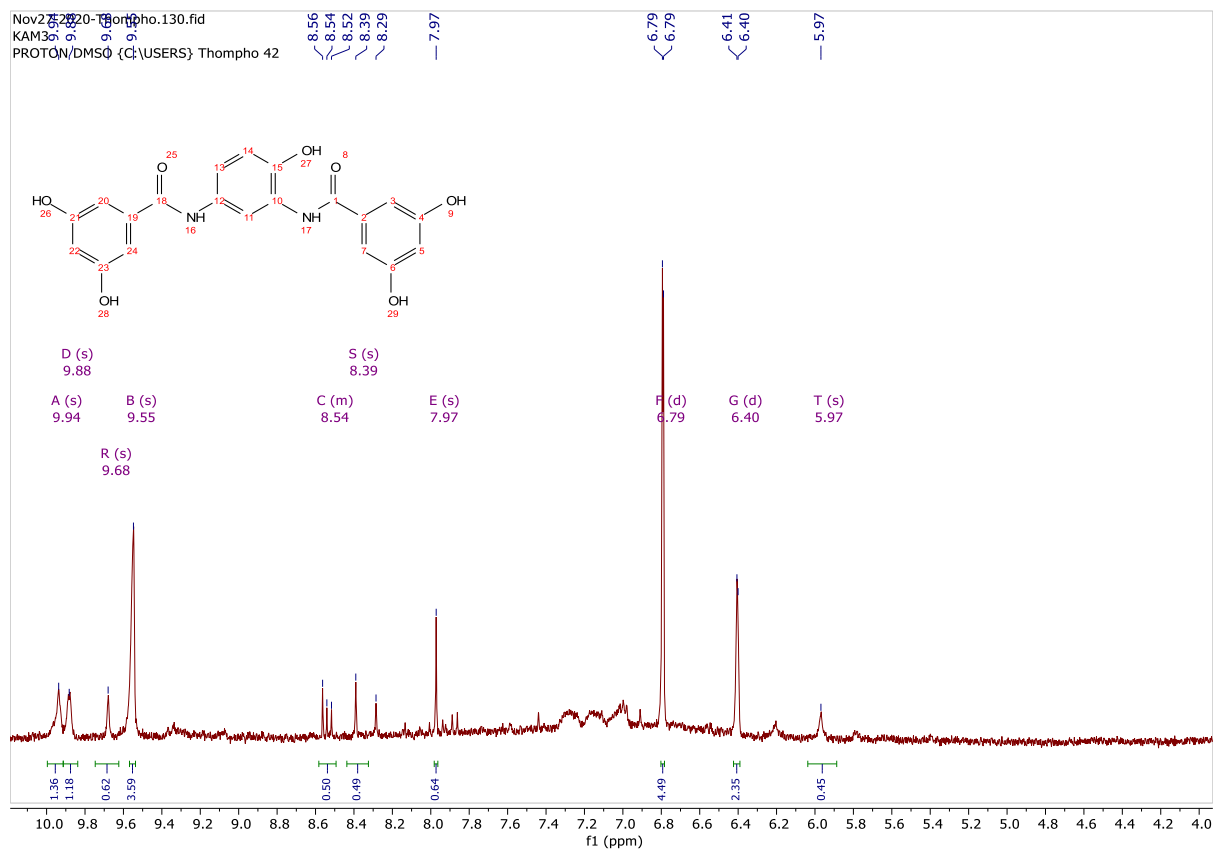
**<sup>1</sup>H and <sup>13</sup>C NMR spectra of *N,N'*-(4-hydroxy-1,3-phenylene)bis(3-hydroxybenzamide) (KAM 2)**



**HRMS of *N,N'*-(4-hydroxy-1,3-phenylene)bis(3-hydroxybenzamide) (KAM 2)**

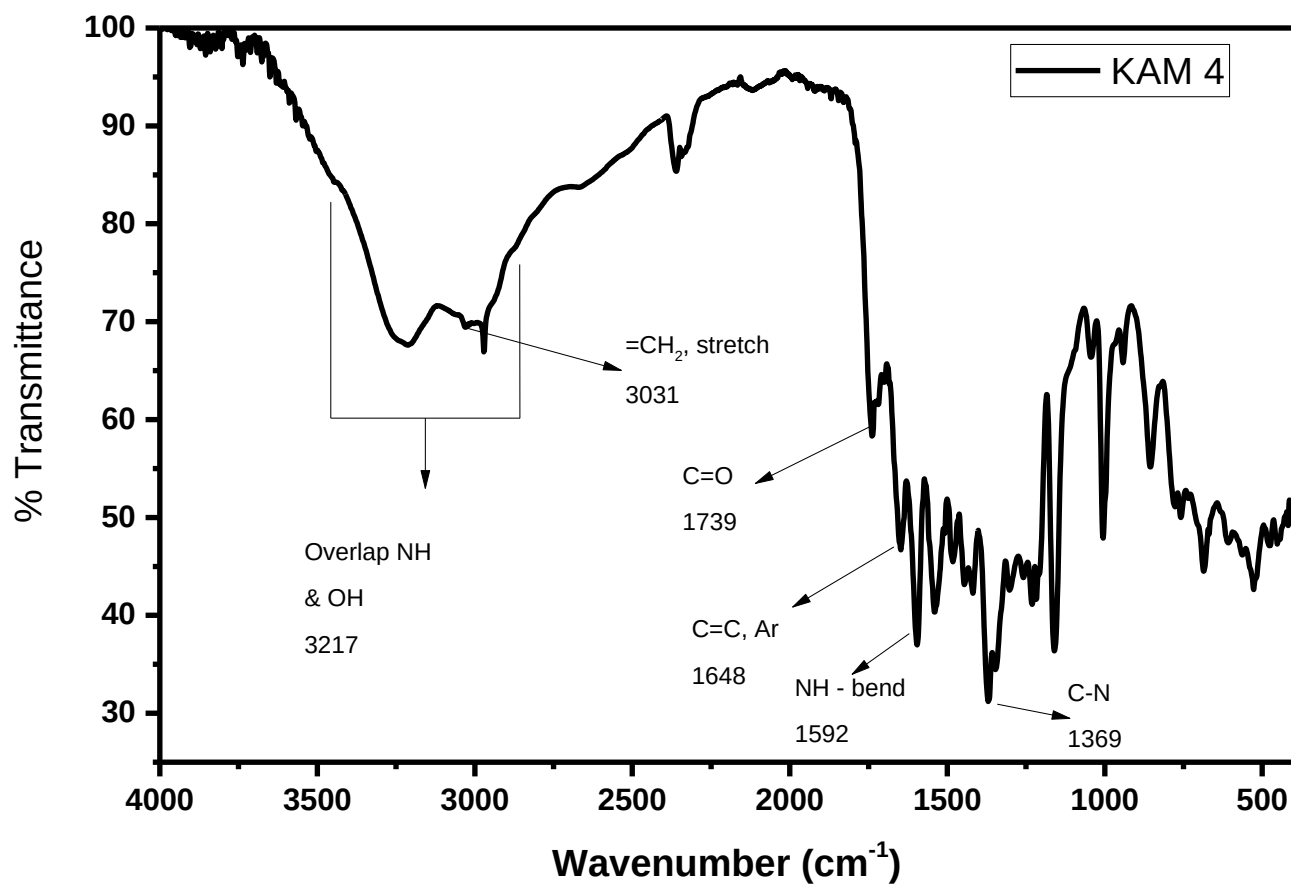
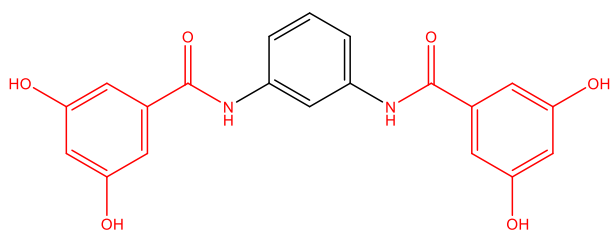


FTIR spectrum of *N,N'*-(4-hydroxy-1,3-phenylene)bis(3,5-dihydroxybenzamide)



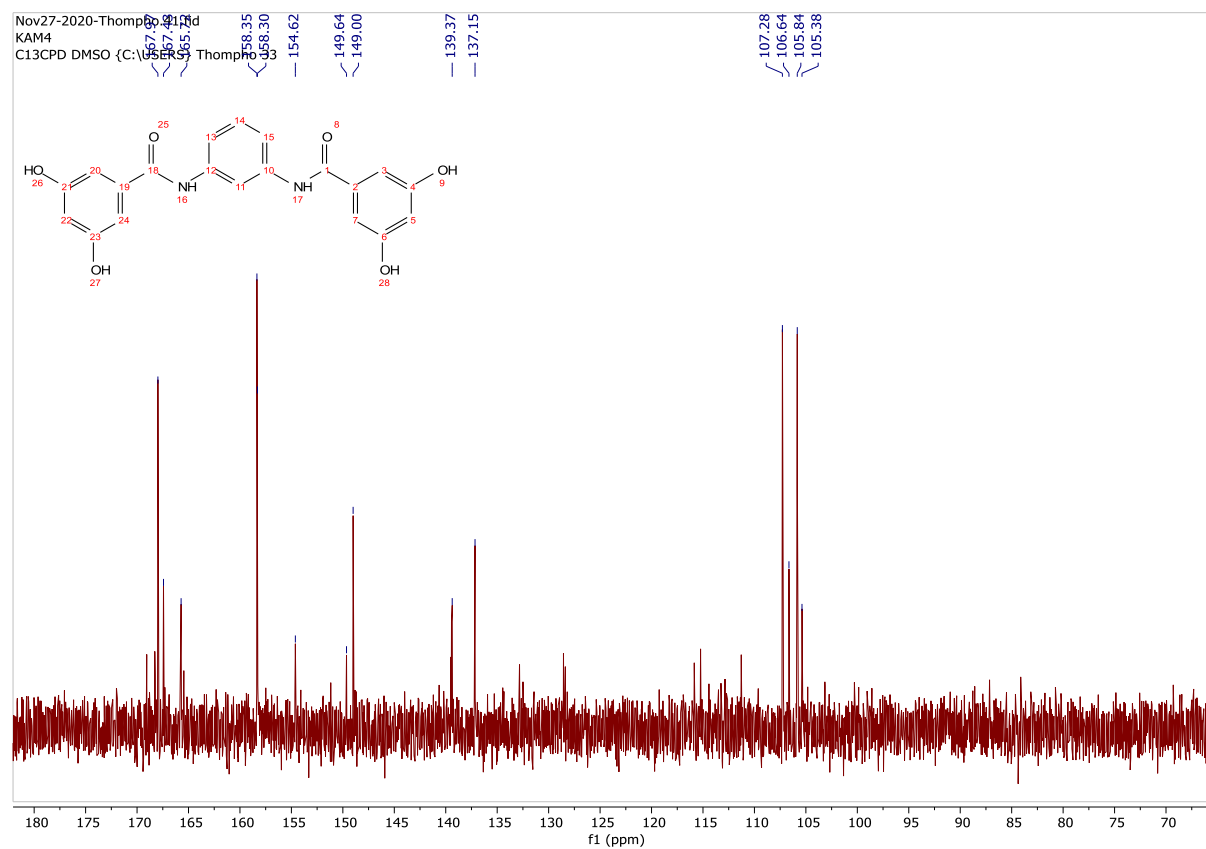
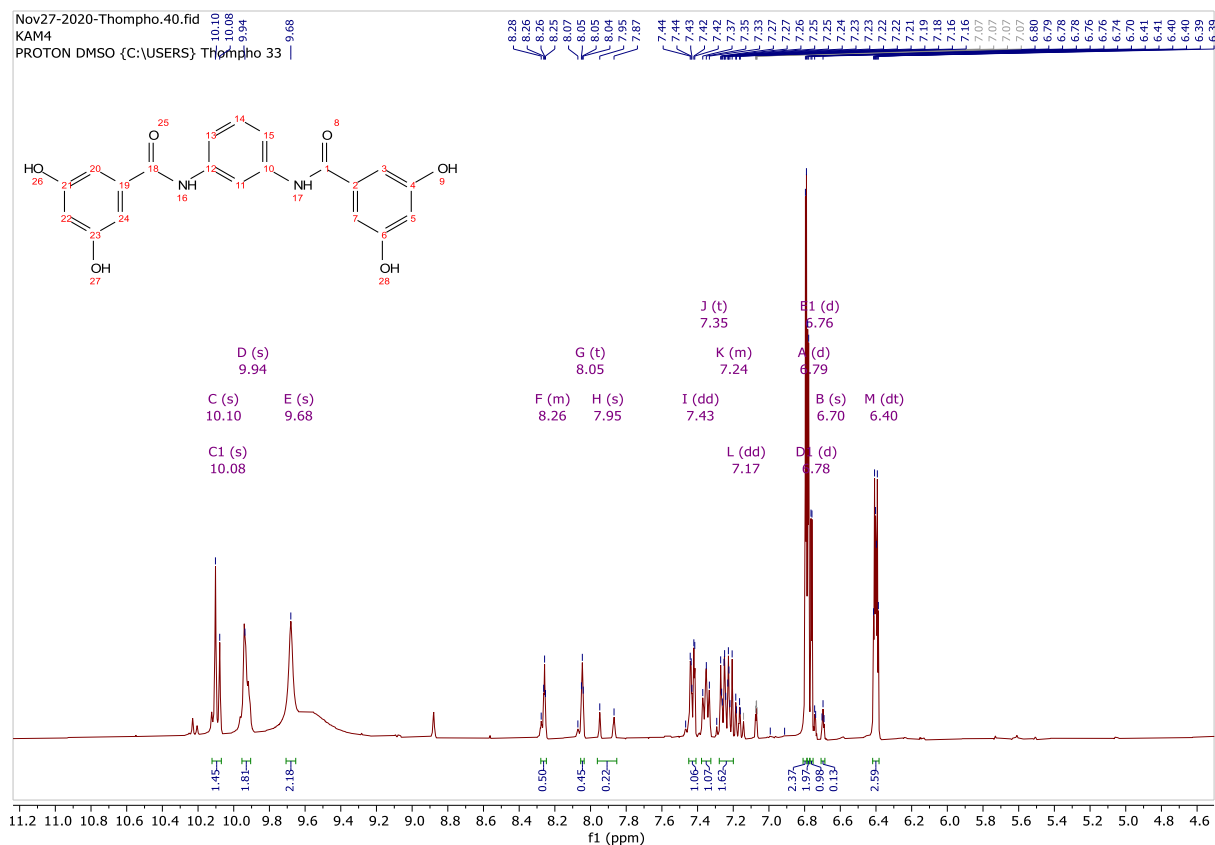
**<sup>1</sup>H and <sup>13</sup>C NMR spectra of N,N'-(4-hydroxy-1,3-phenylene)bis(3,5-dihydroxybenzamide) (KAM 3)**



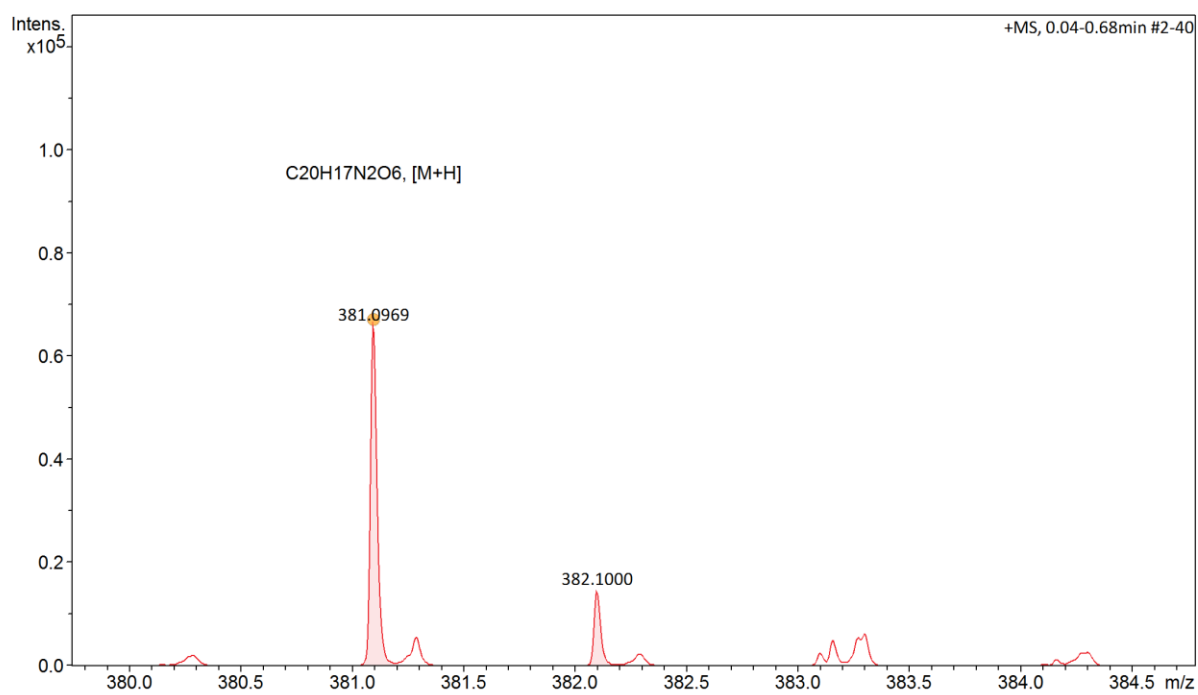
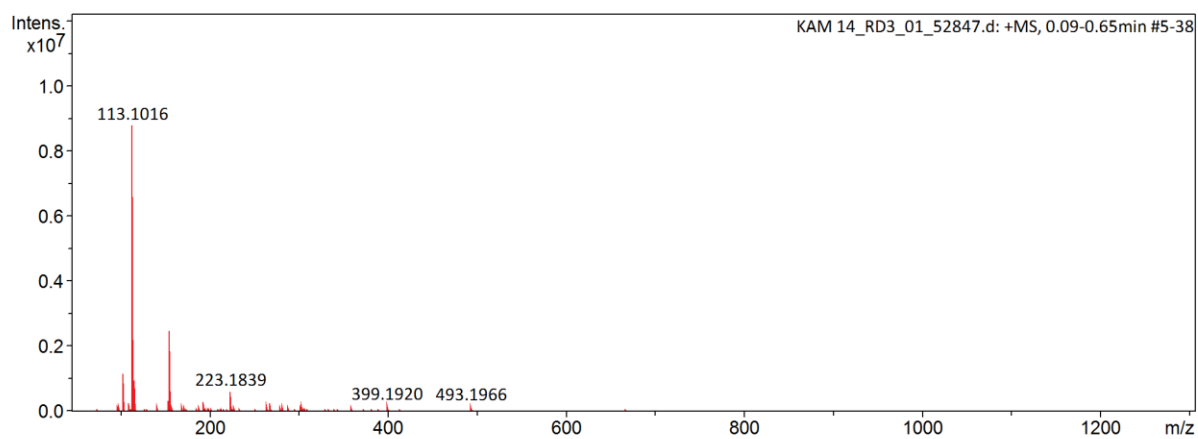


FTIR spectrum of *N,N'*-(1,3-phenylene)bis(3,5-dihydroxybenzamide) (KAM 4)

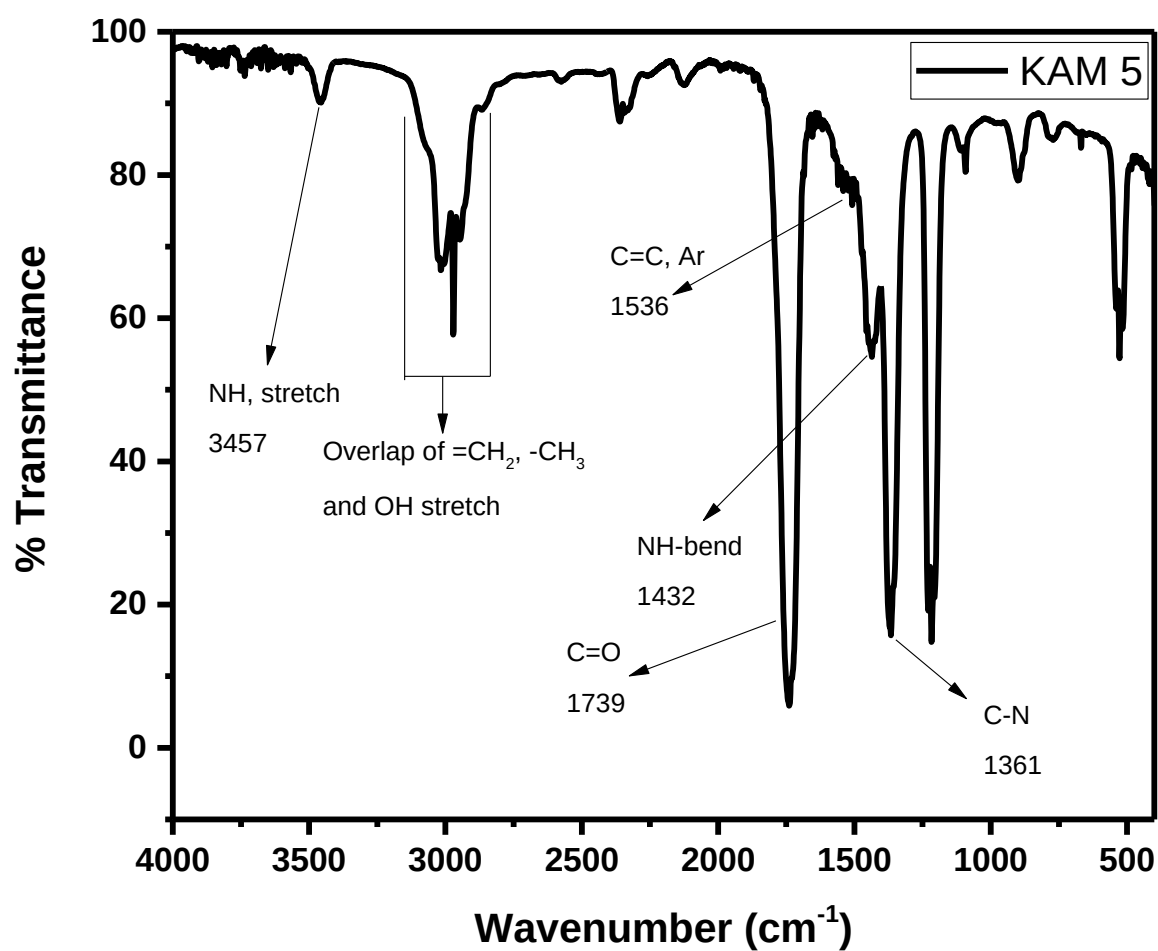
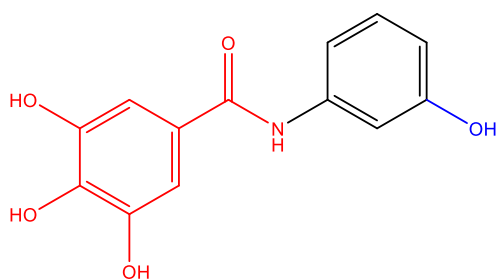




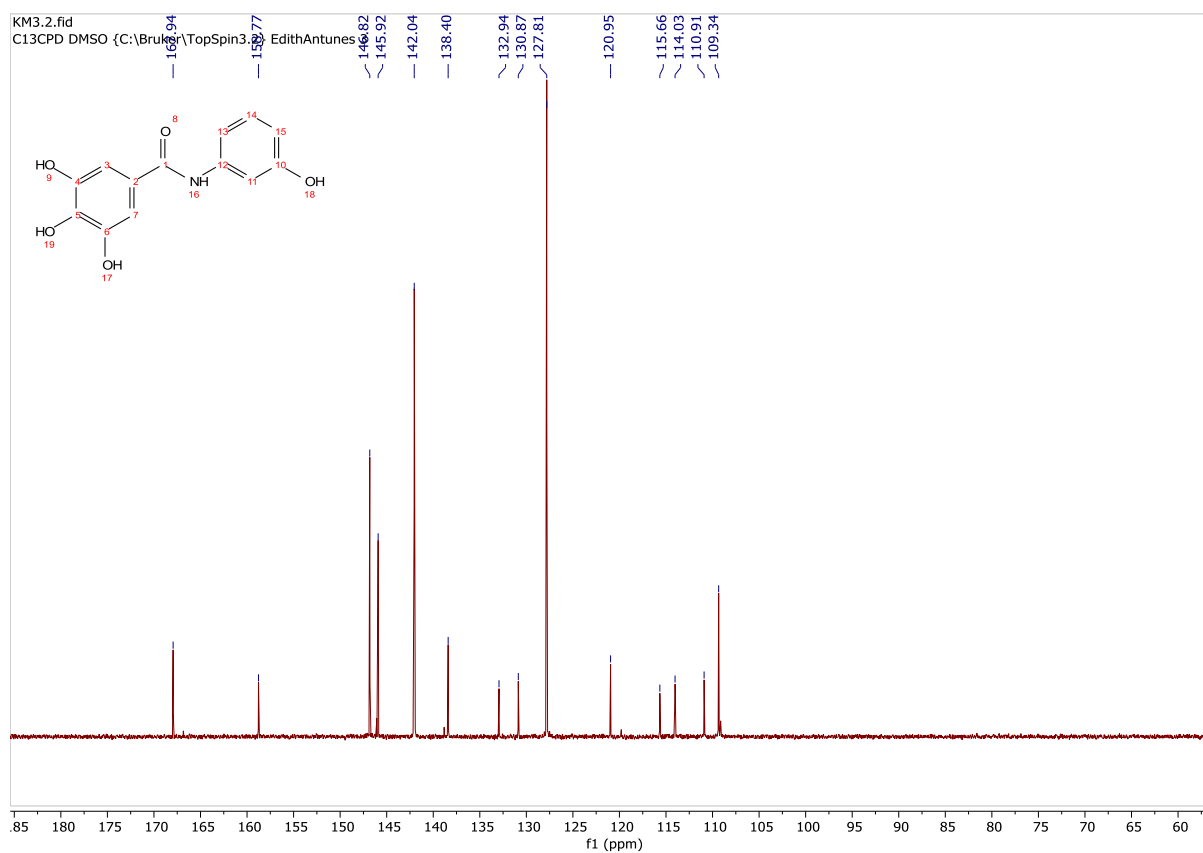
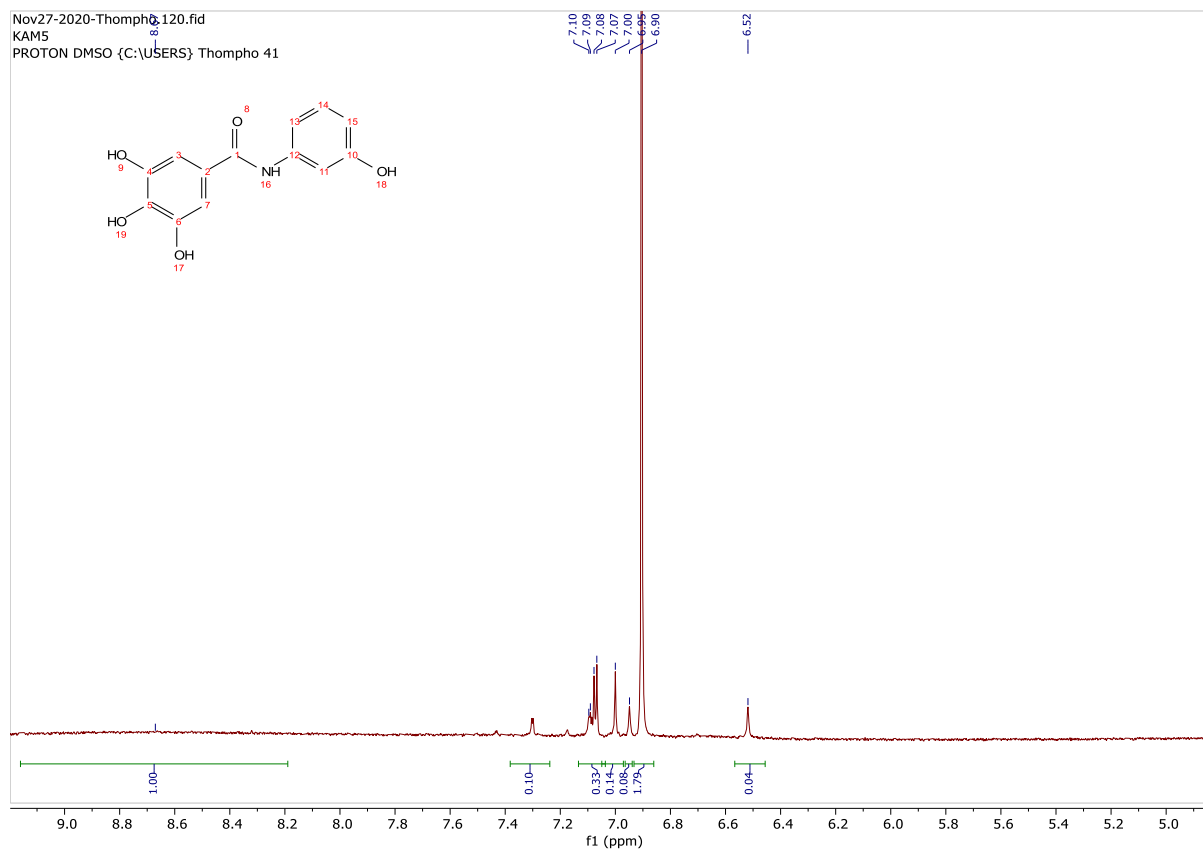
**$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of *N,N'*-(1,3-phenylene)bis(3,5-dihydroxybenzamide) (KAM 4)**



**HRMS of *N,N'*-(1,3-phenylene)bis(3,5-dihydroxybenzamide) (KAM 4)**

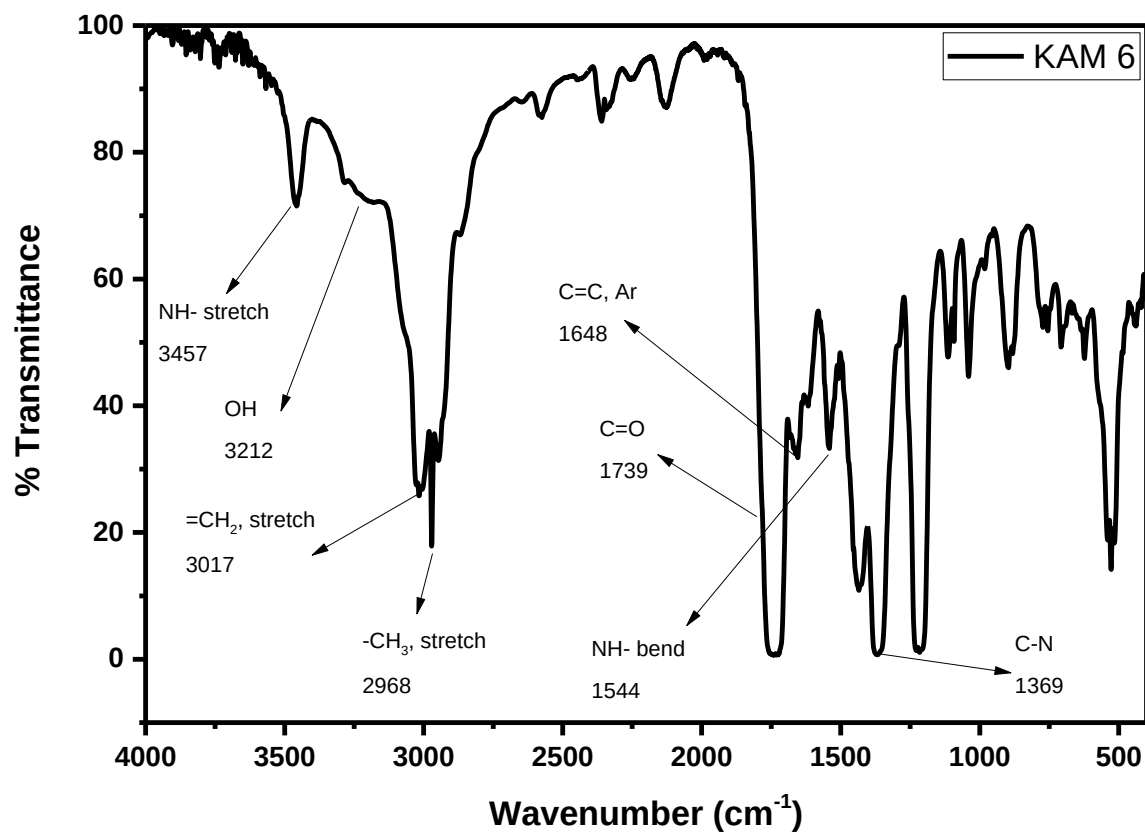
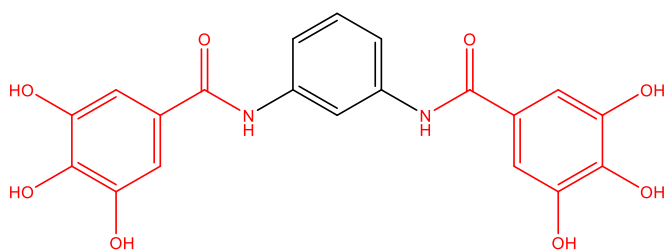


FTIR spectrum of 3,4,5-trihydroxy-N-(3-hydroxyphenyl)benzamide (KAM 5)

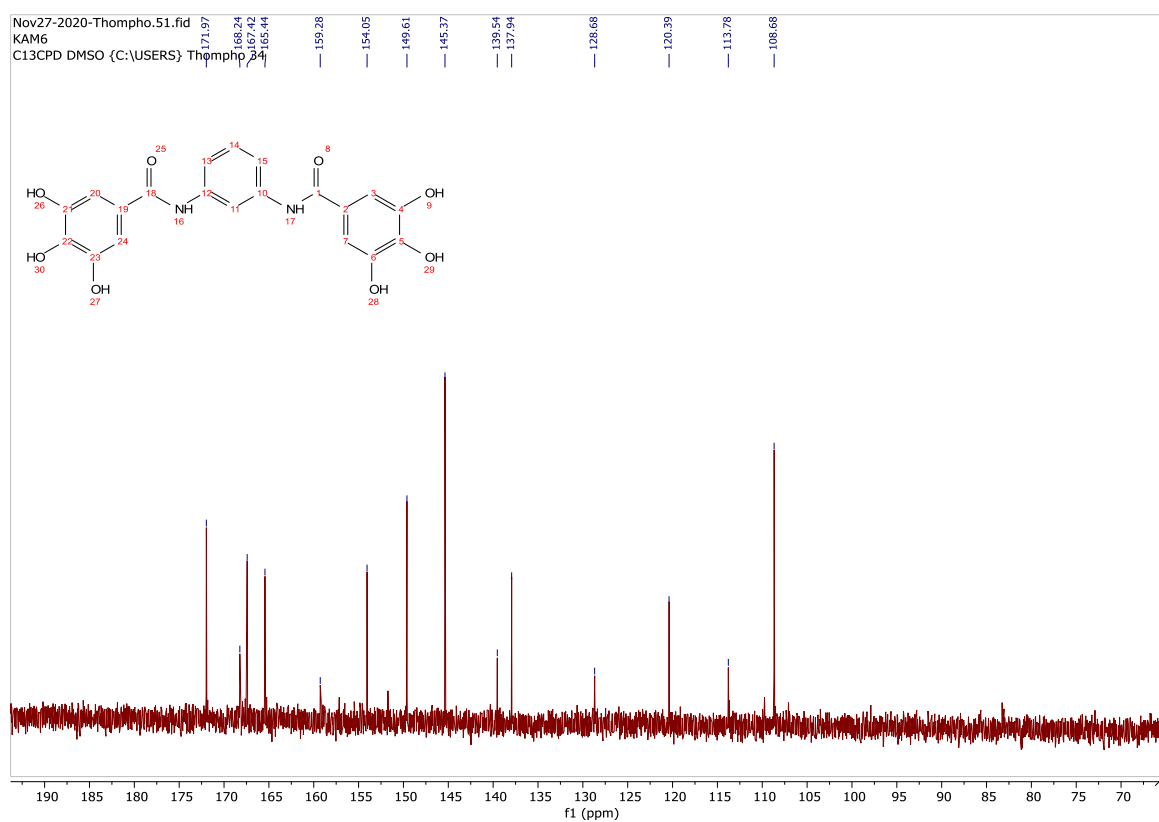
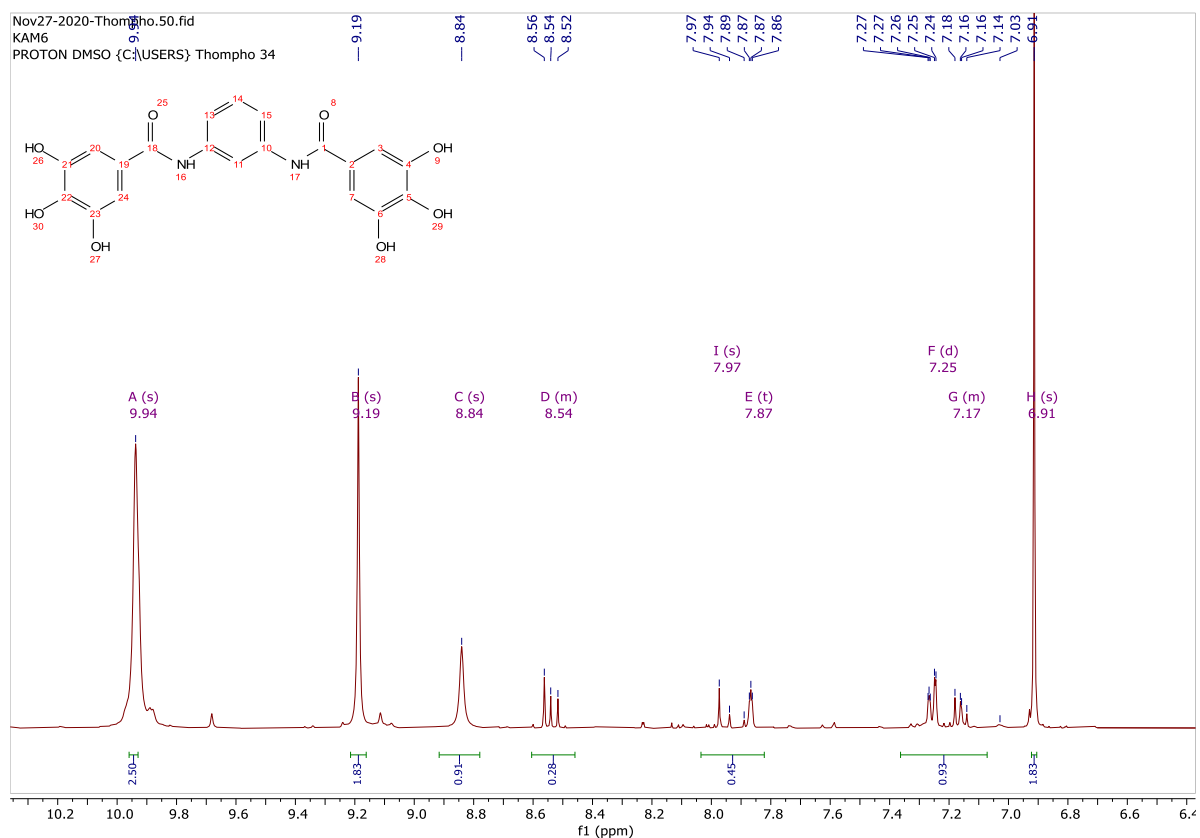


**<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3,4,5-trihydroxy-N-(3-hydroxyphenyl)benzamide (KAM 5)**

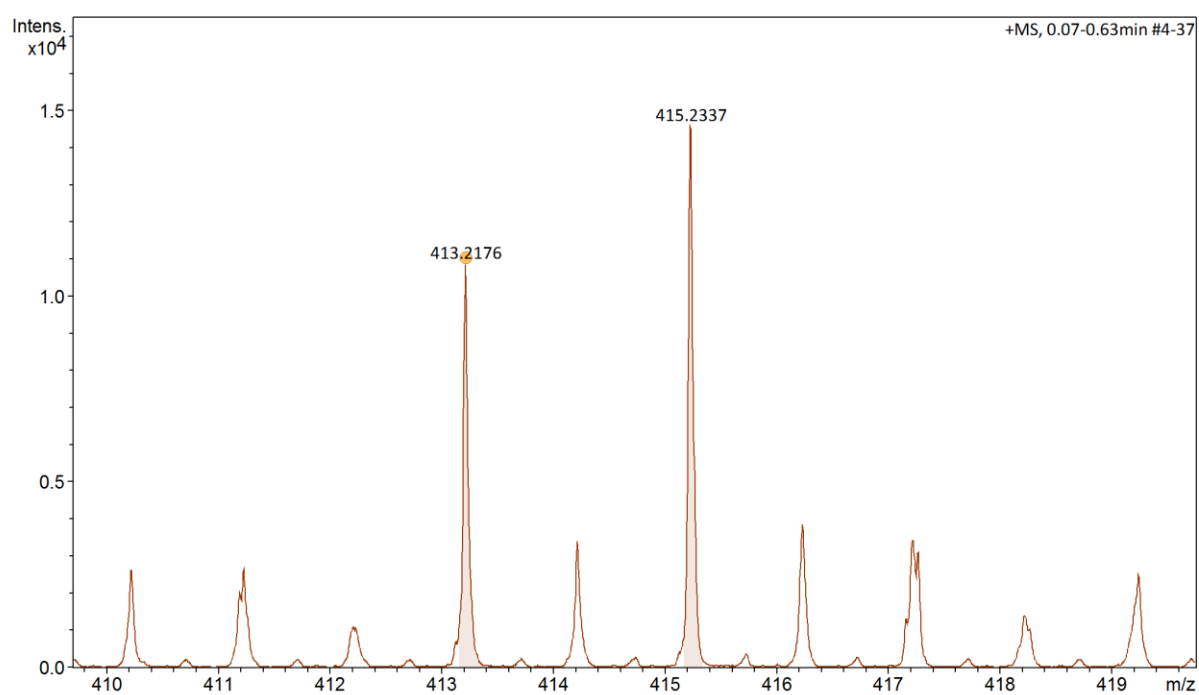
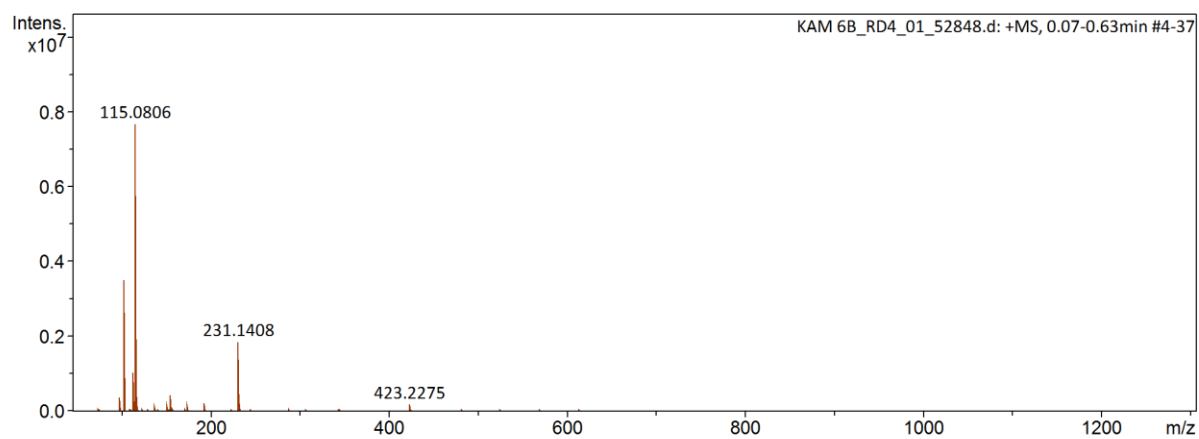




FTIR spectrum of *N,N'*-(1,3-phenylene)bis(3,4,5-trihydroxybenzamide) (KAM 6)

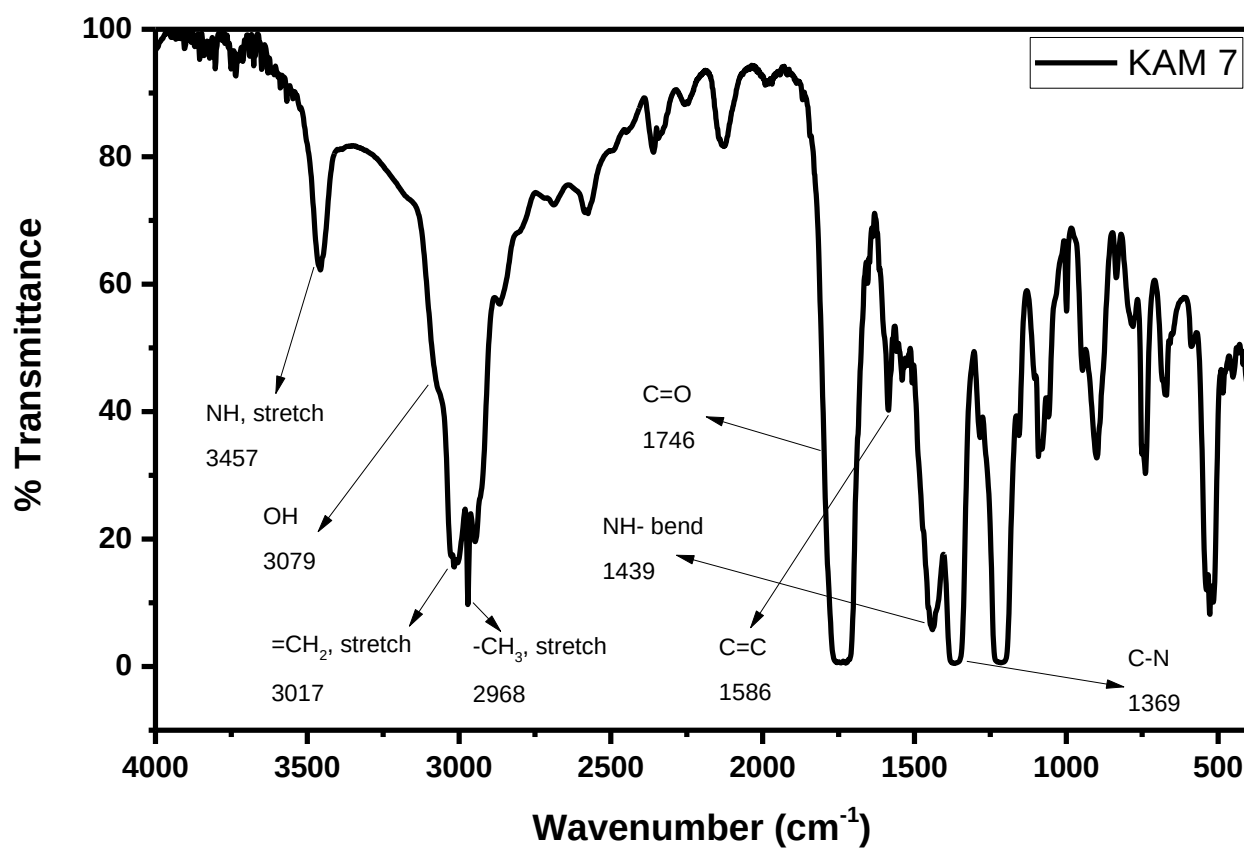
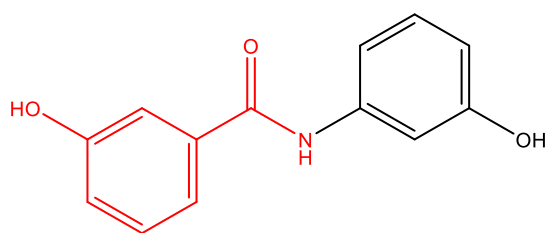


**$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of  $N,N'$ -(1,3-phenylene)bis(3,4,5-trihydroxybenzamide) (KAM6)**

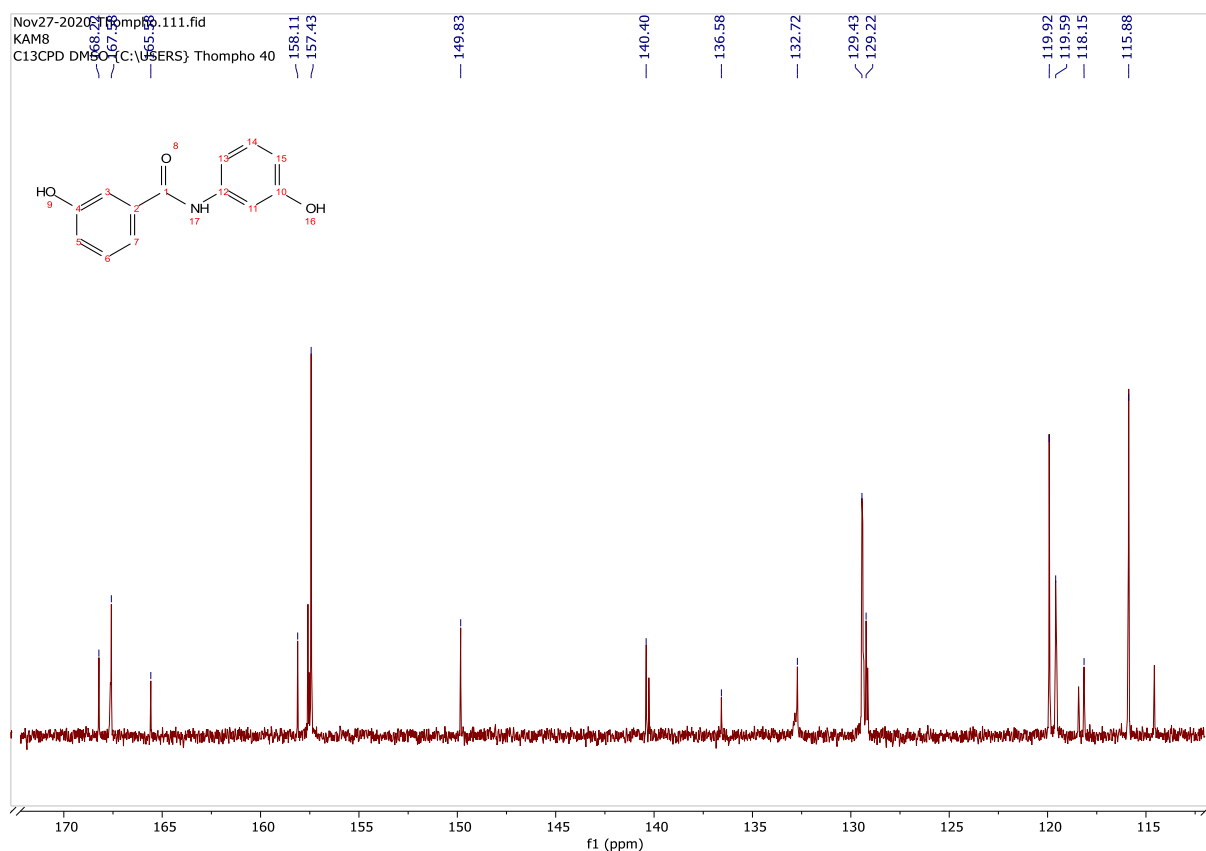
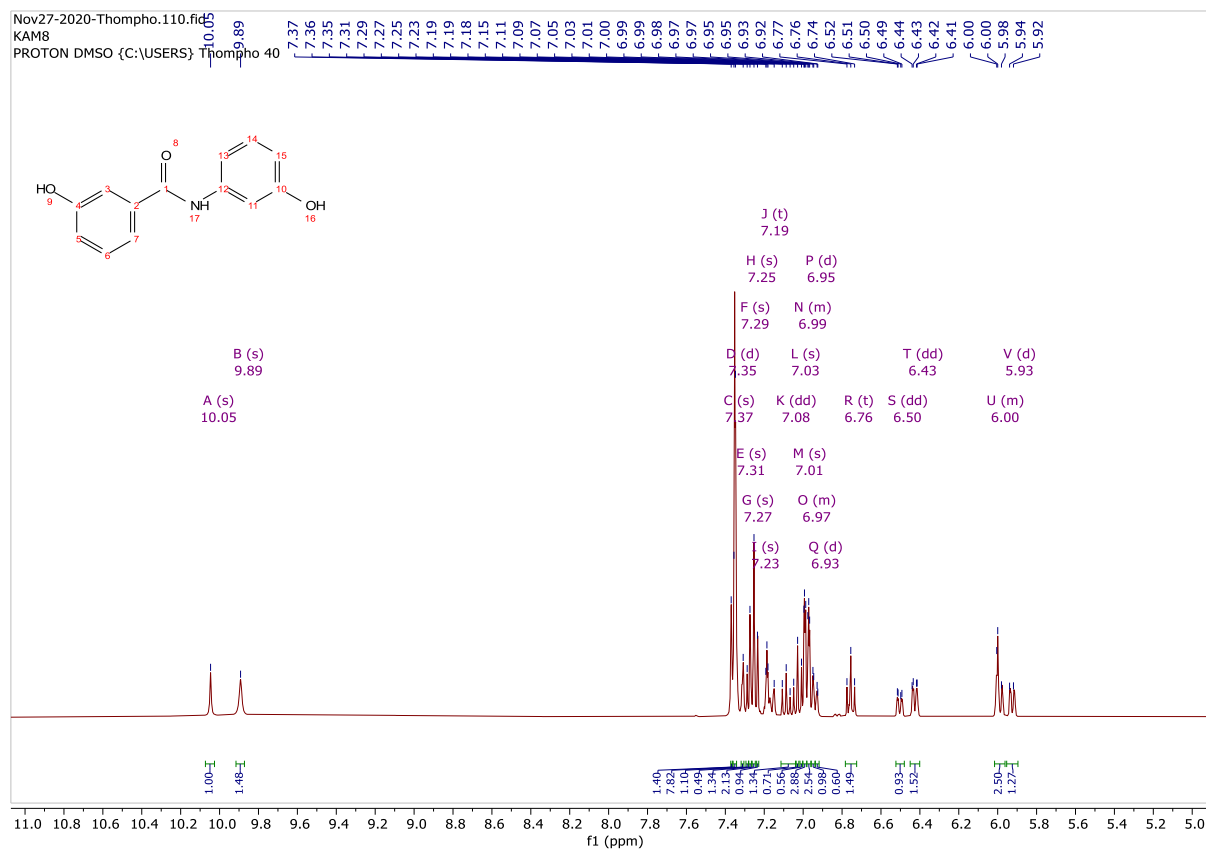


**HRMS of *N,N'*-(1,3-phenylene)bis(3,4,5-trihydroxybenzamide) (KAM 6)**

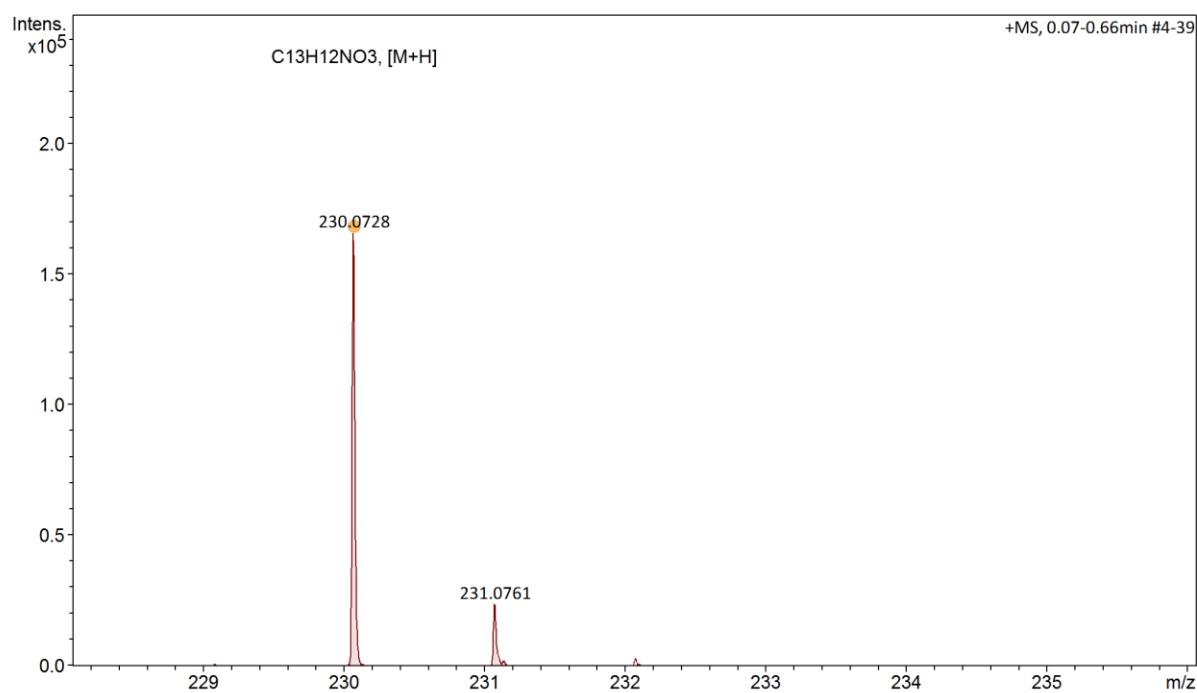
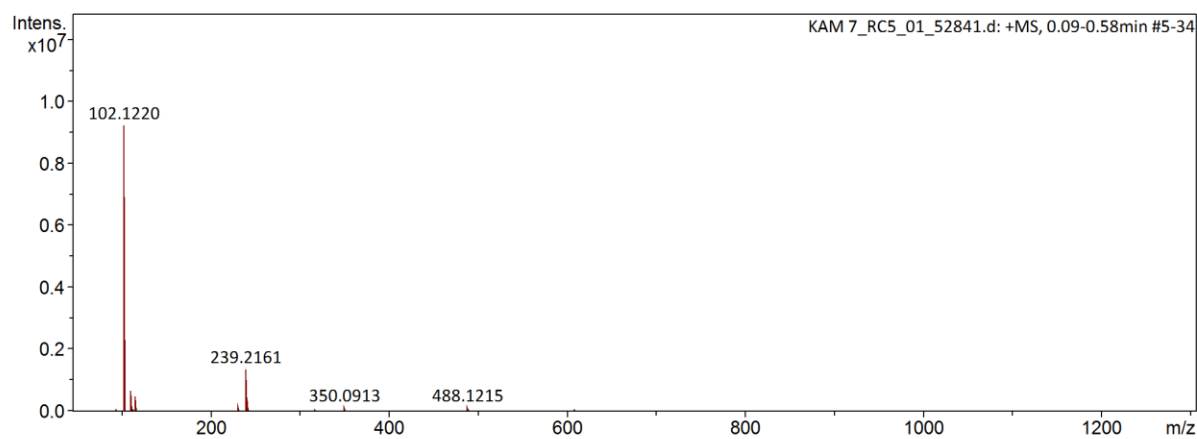




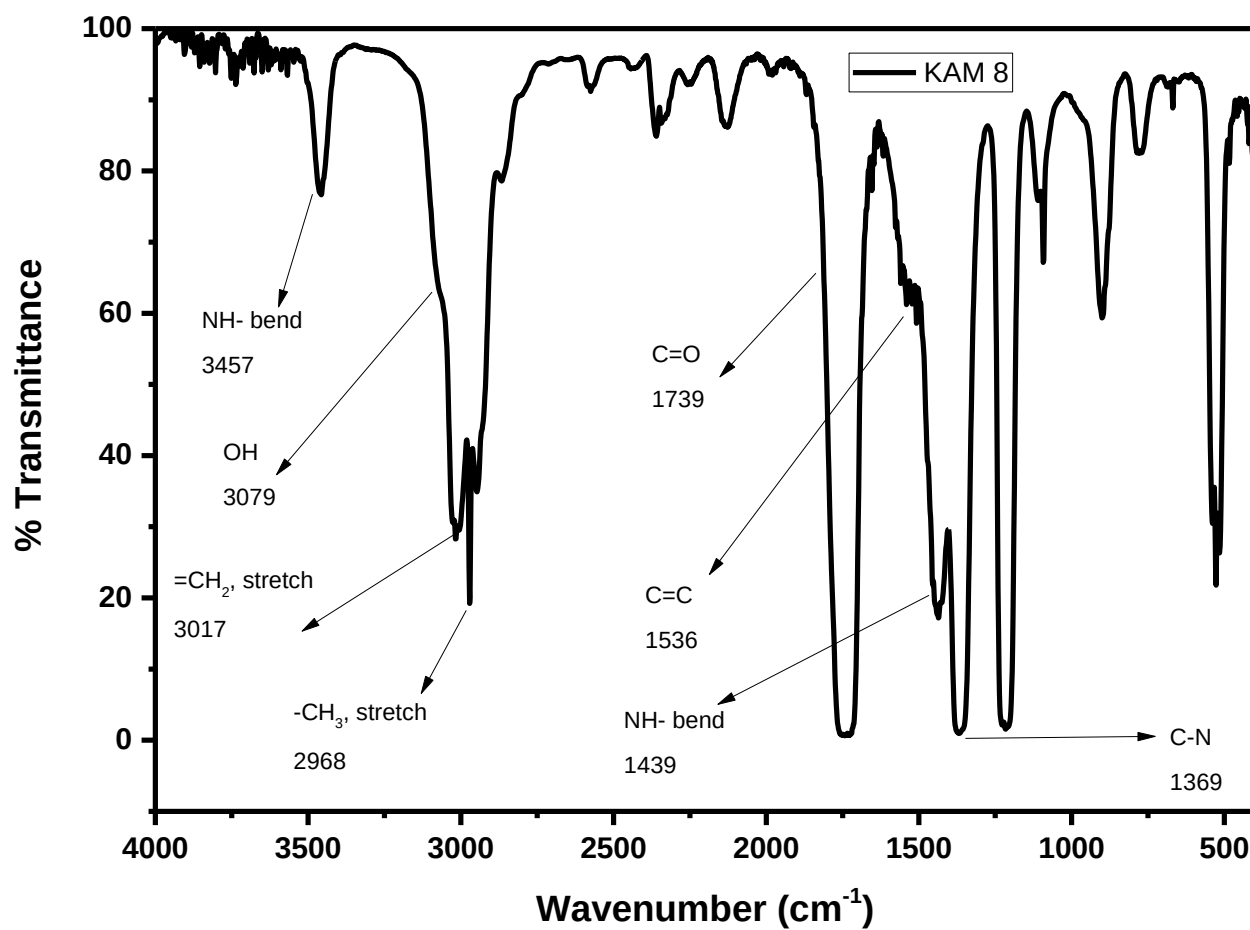
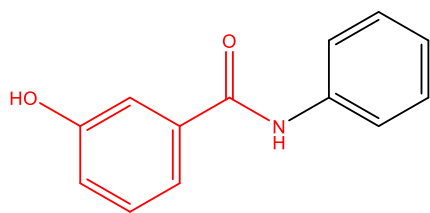
FTIR spectrum of 3-hydroxy-N-(3-hydroxyphenyl)benzamide (KAM 7)



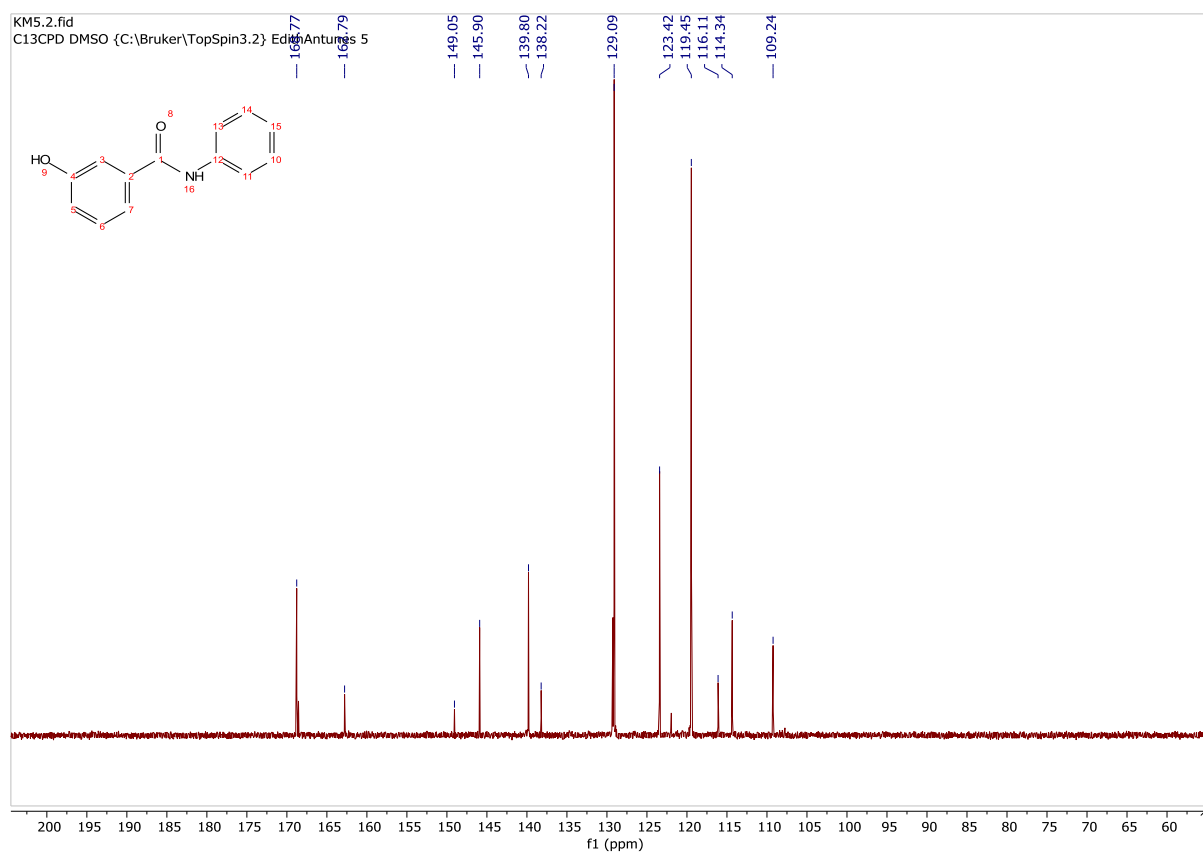
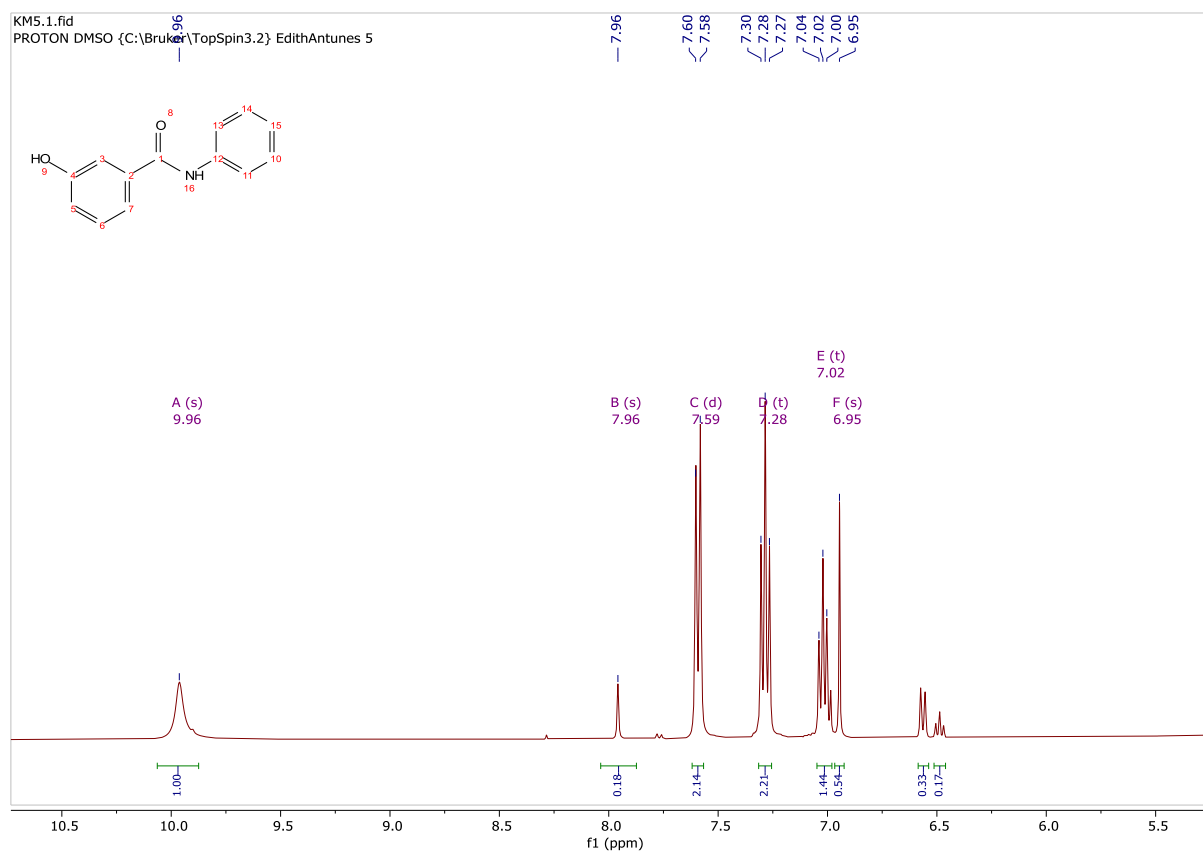
**<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3-hydroxy-N-(3-hydroxyphenyl)benzamide (KAM 7)**



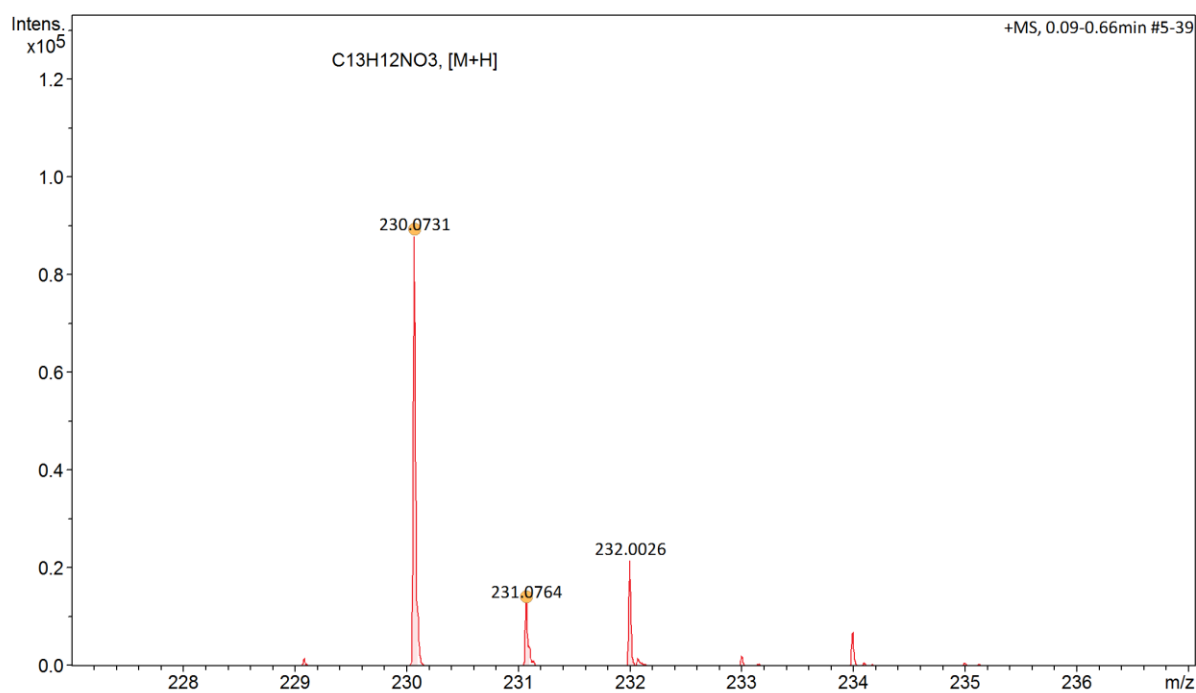
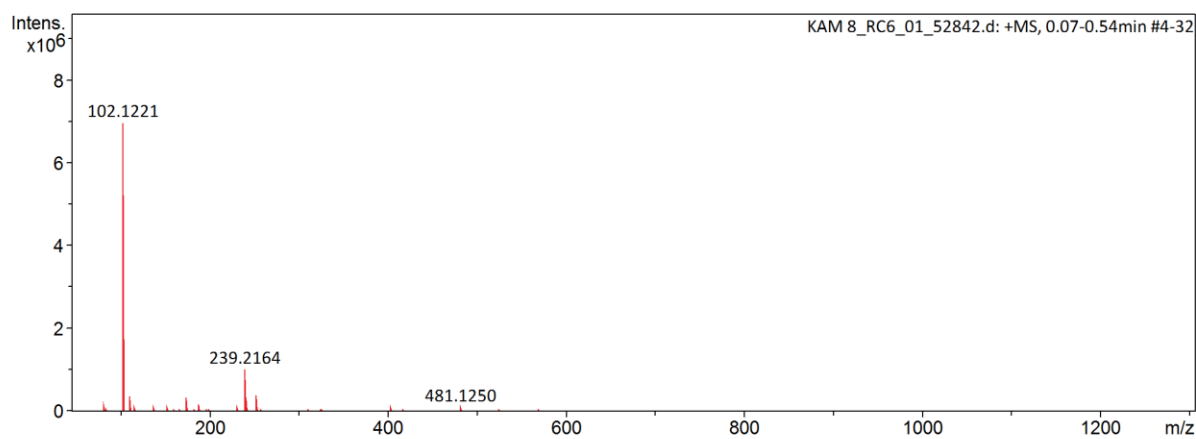
**HRMS of 3-hydroxy-*N*-(3-hydroxyphenyl)benzamide (KAM 7)**



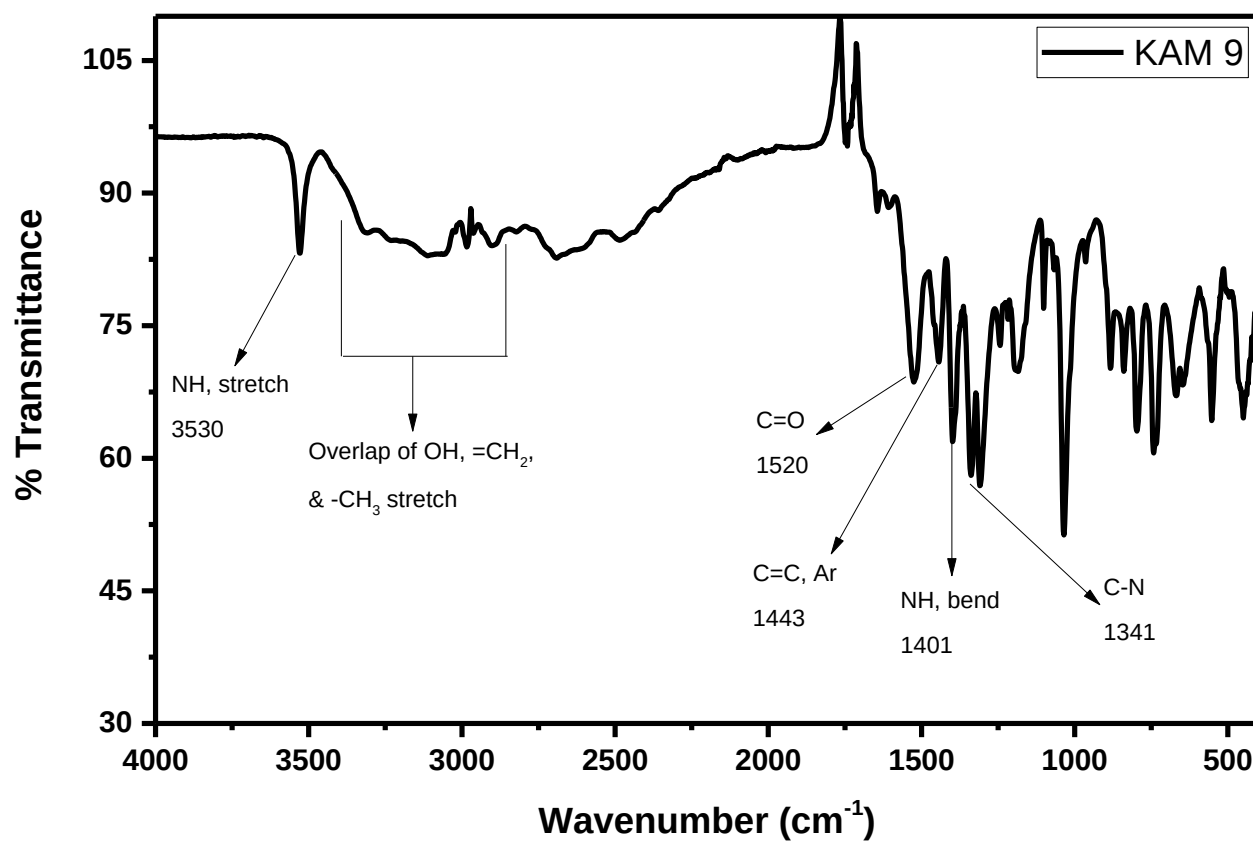
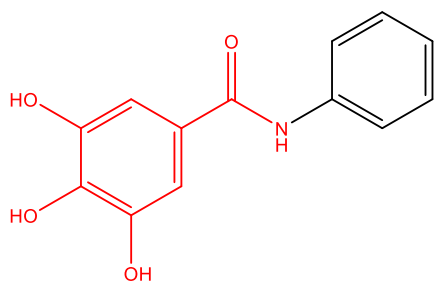
FTIR spectrum of 3-hydroxy-N-phenylbenzamide (KAM 8)



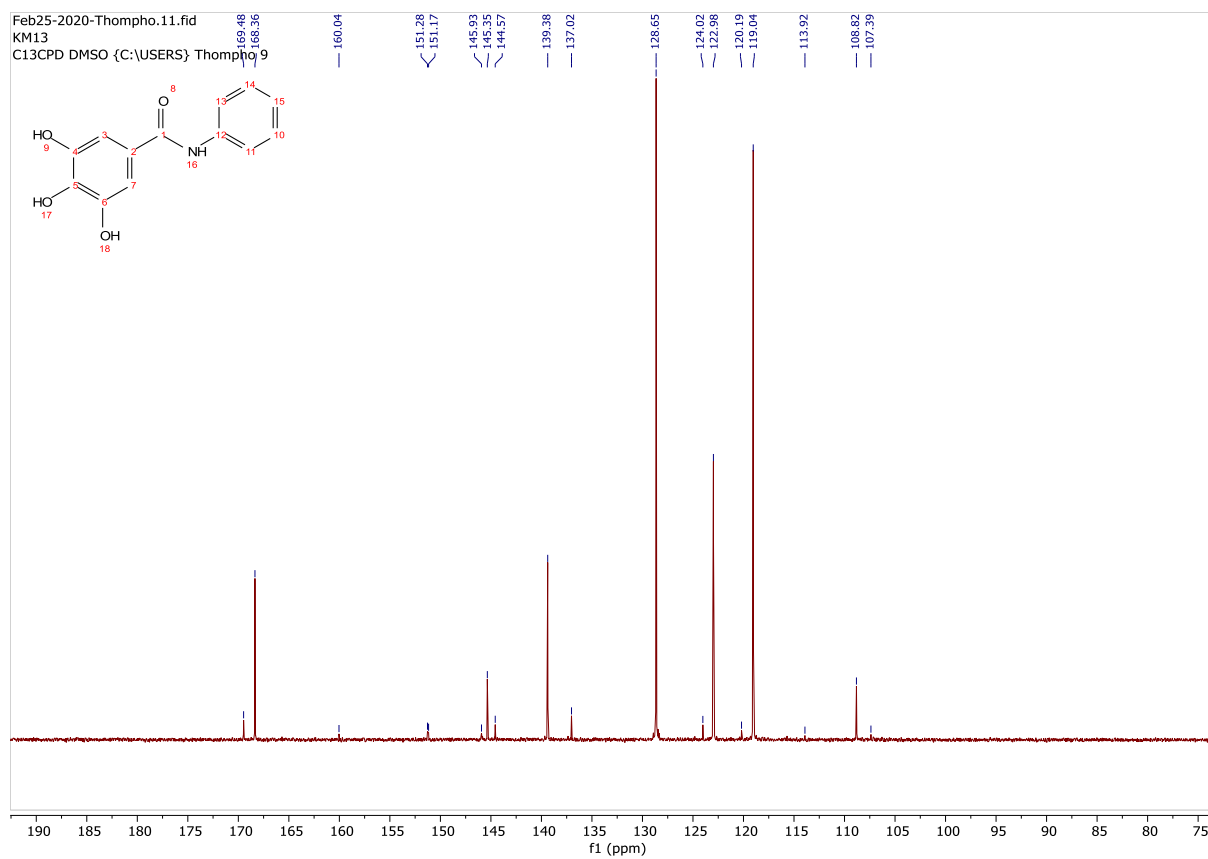
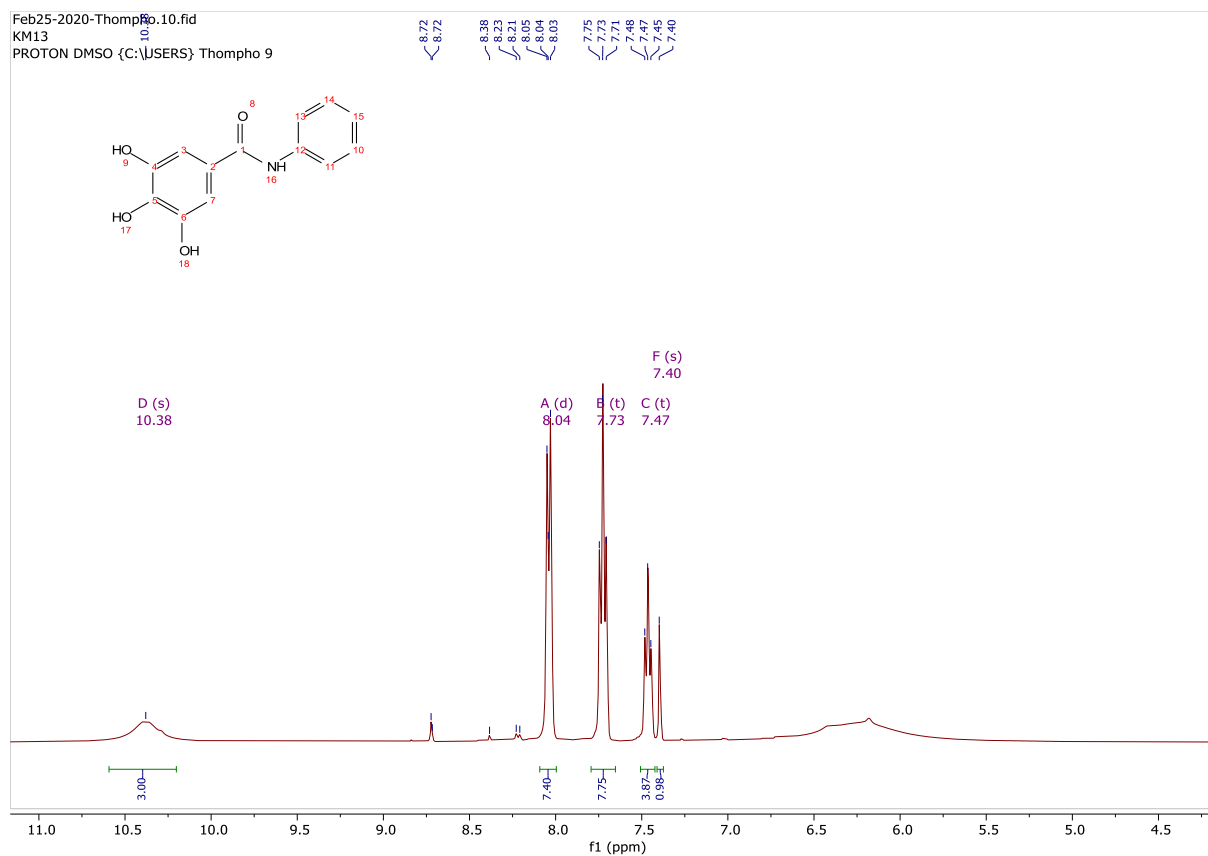
**$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-hydroxy-N-phenylbenzamide (KAM 8)**



**HRMS of 3-hydroxy-*N*-phenylbenzamide (KAM 8)**

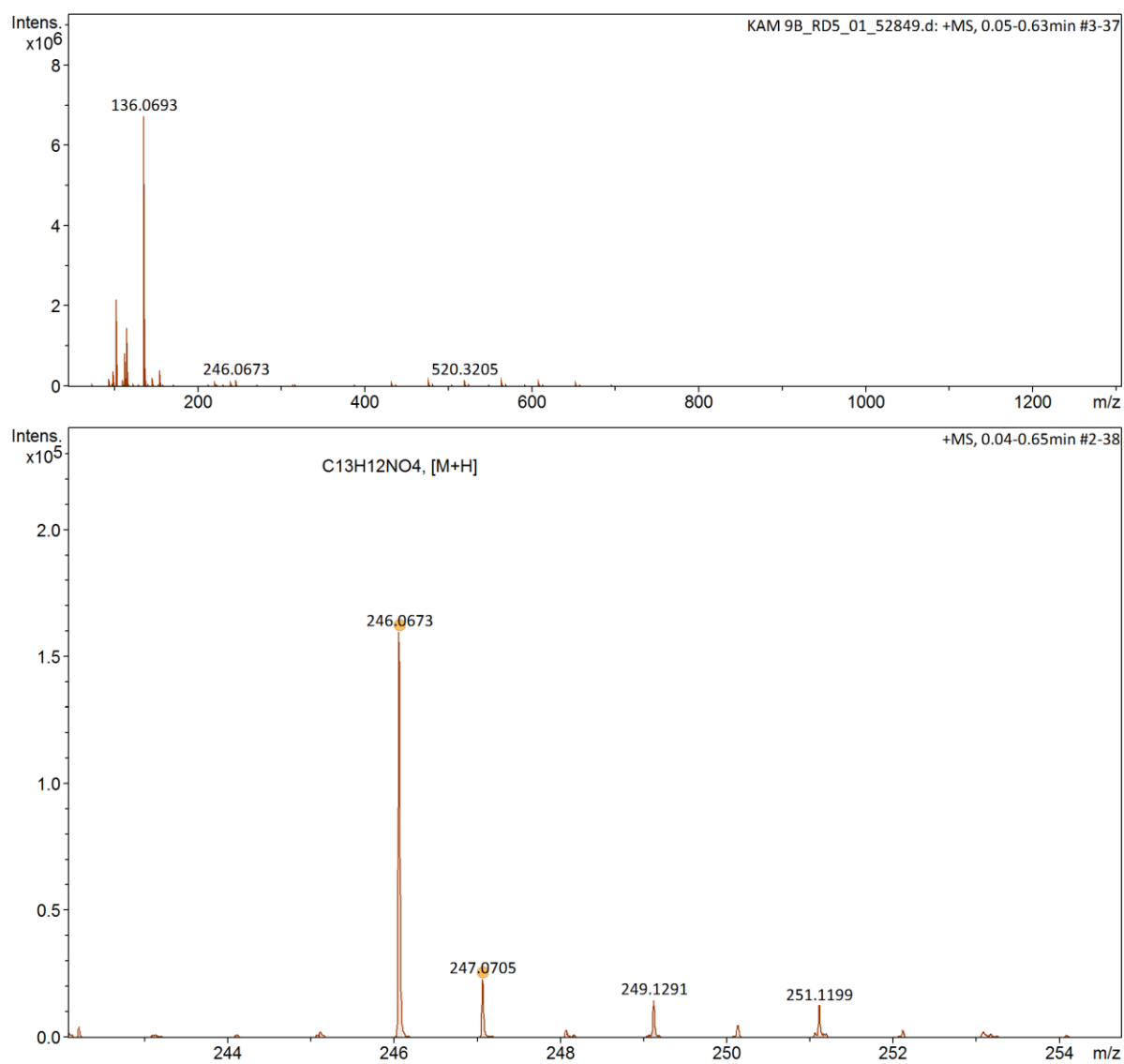


FTIR spectrum of 3,4,5-trihydroxy-*N*-phenylbenzamide (KAM 9)

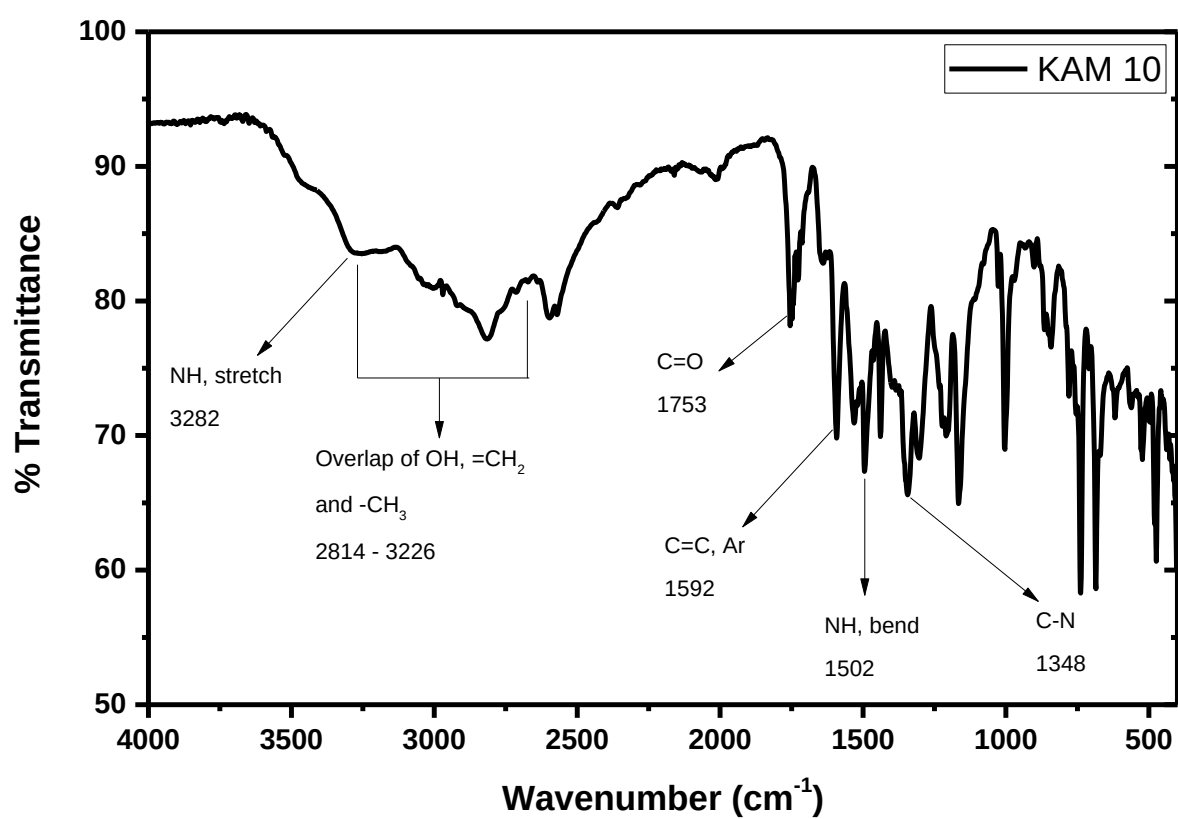
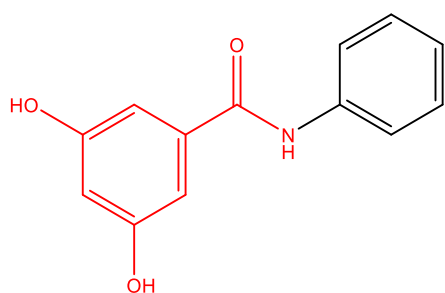




### **<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3,4,5-trihydroxy-*N*-phenylbenzamide (KAM 9)**



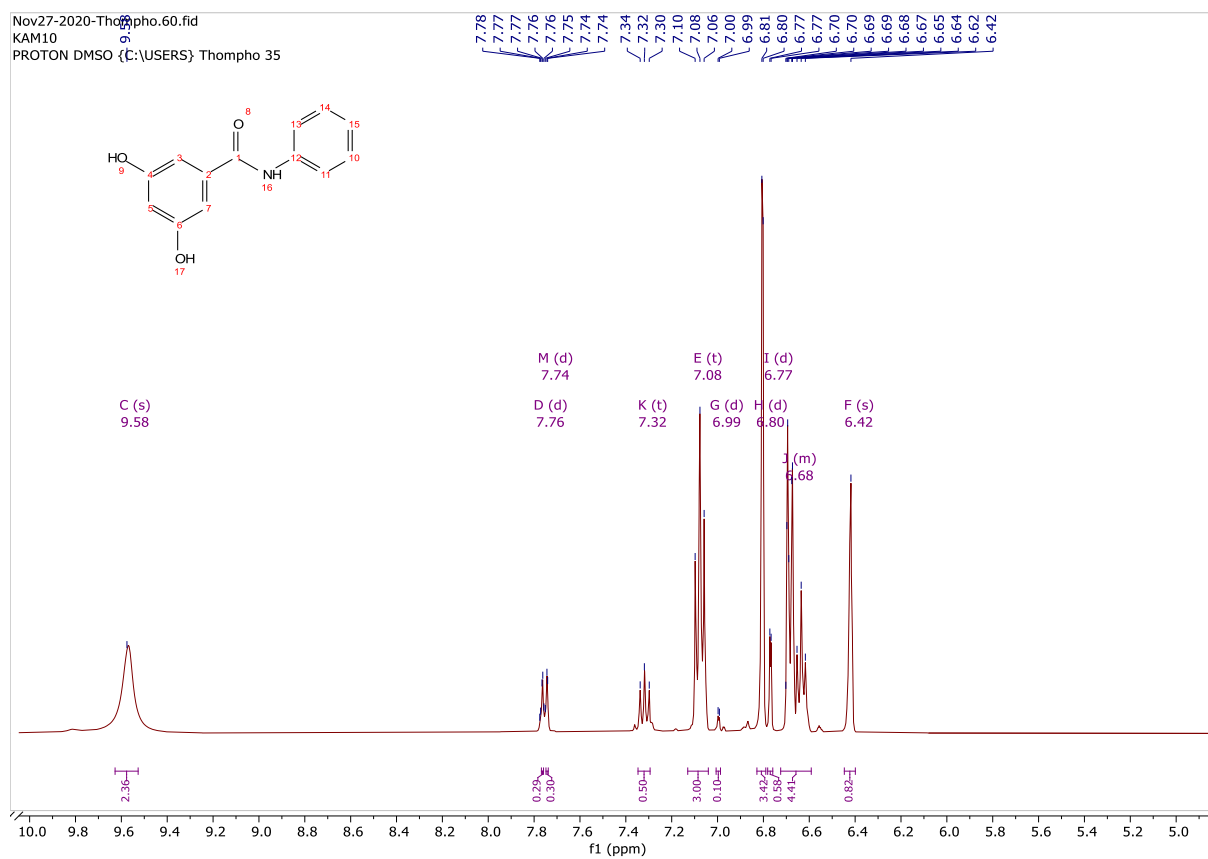
### **HRMS of 3,4,5-trihydroxy-*N*-phenylbenzamide (KAM 9)**

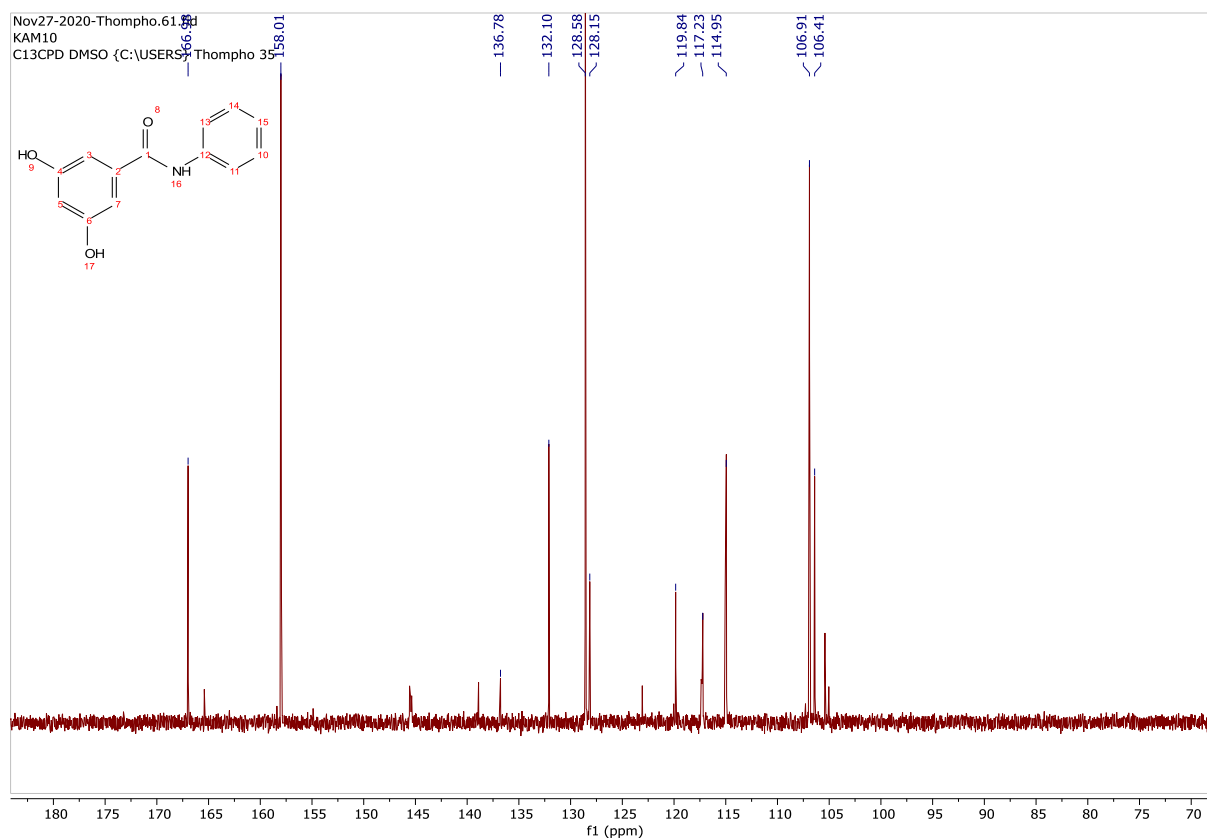


FTIR spectrum of 3,5-dihydroxy-N-phenylbenzamide (KAM 10)

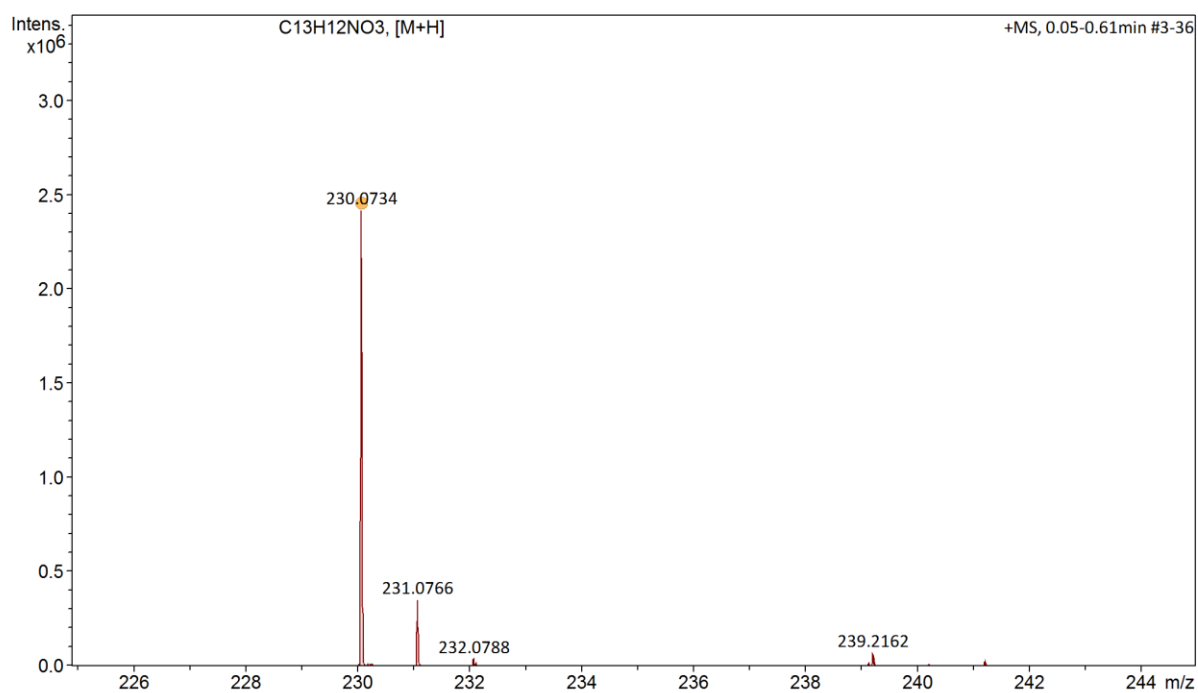
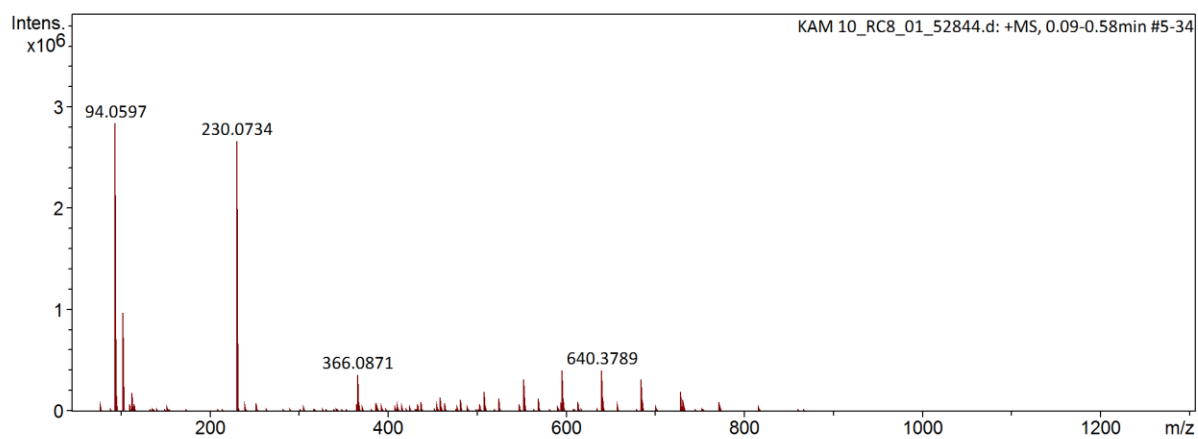
Nov27-2020-Thompho.60.fid  
KAM10

PROTON DMSO {C:\USERS\ Thompho 35

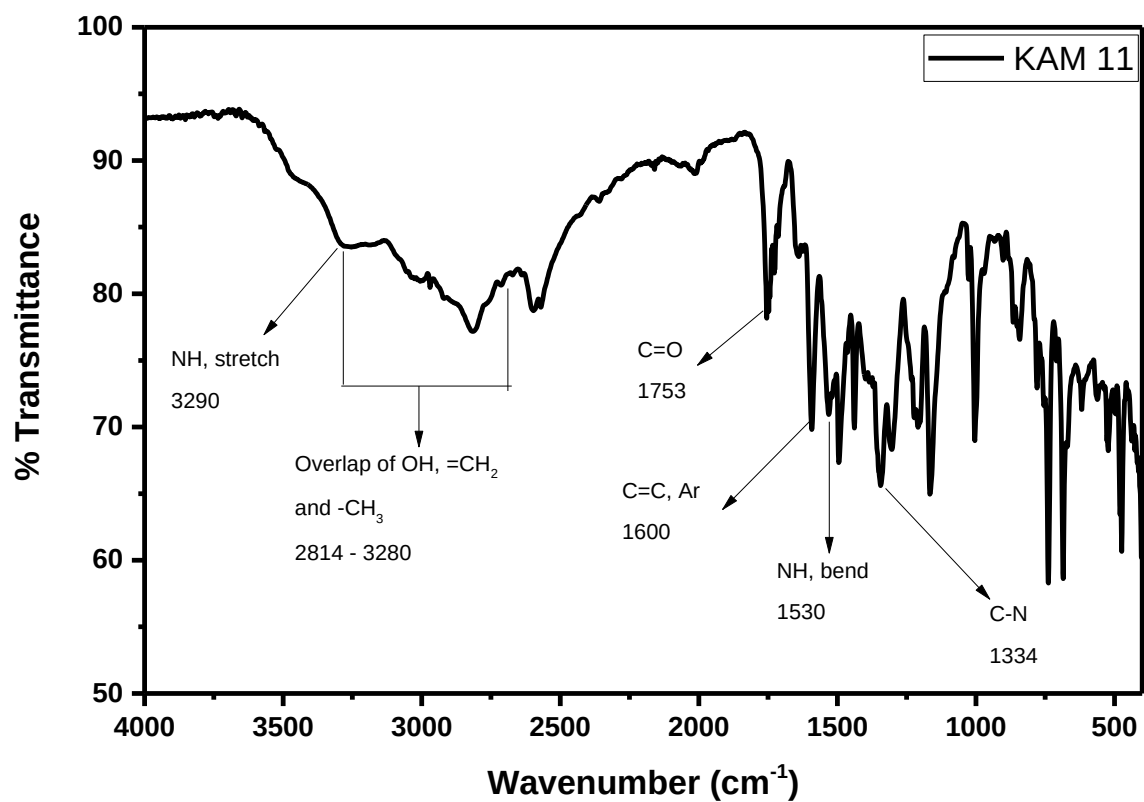
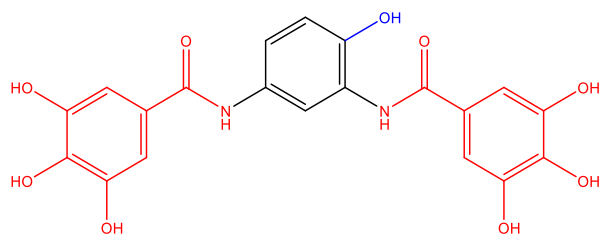




**$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3,5-dihydroxy-*N*-phenylbenzamide (KAM 10)**

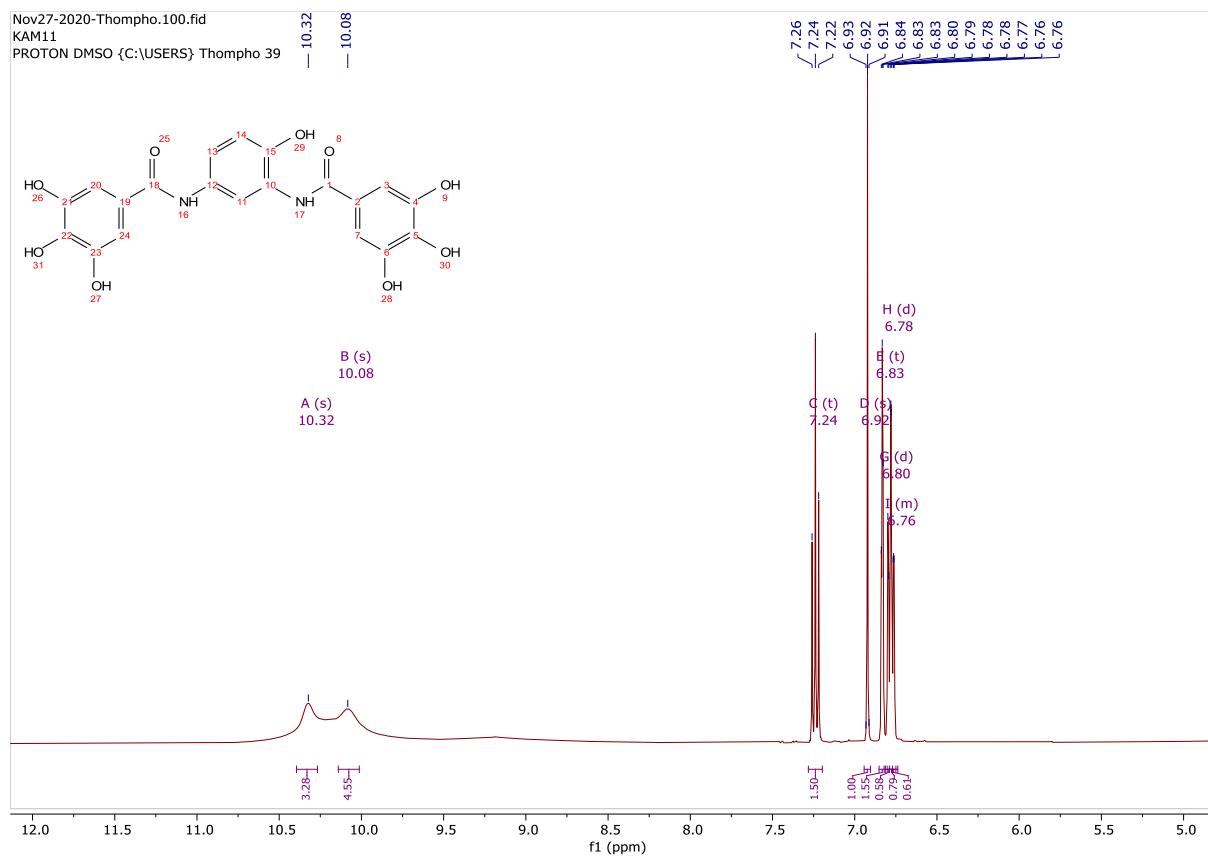


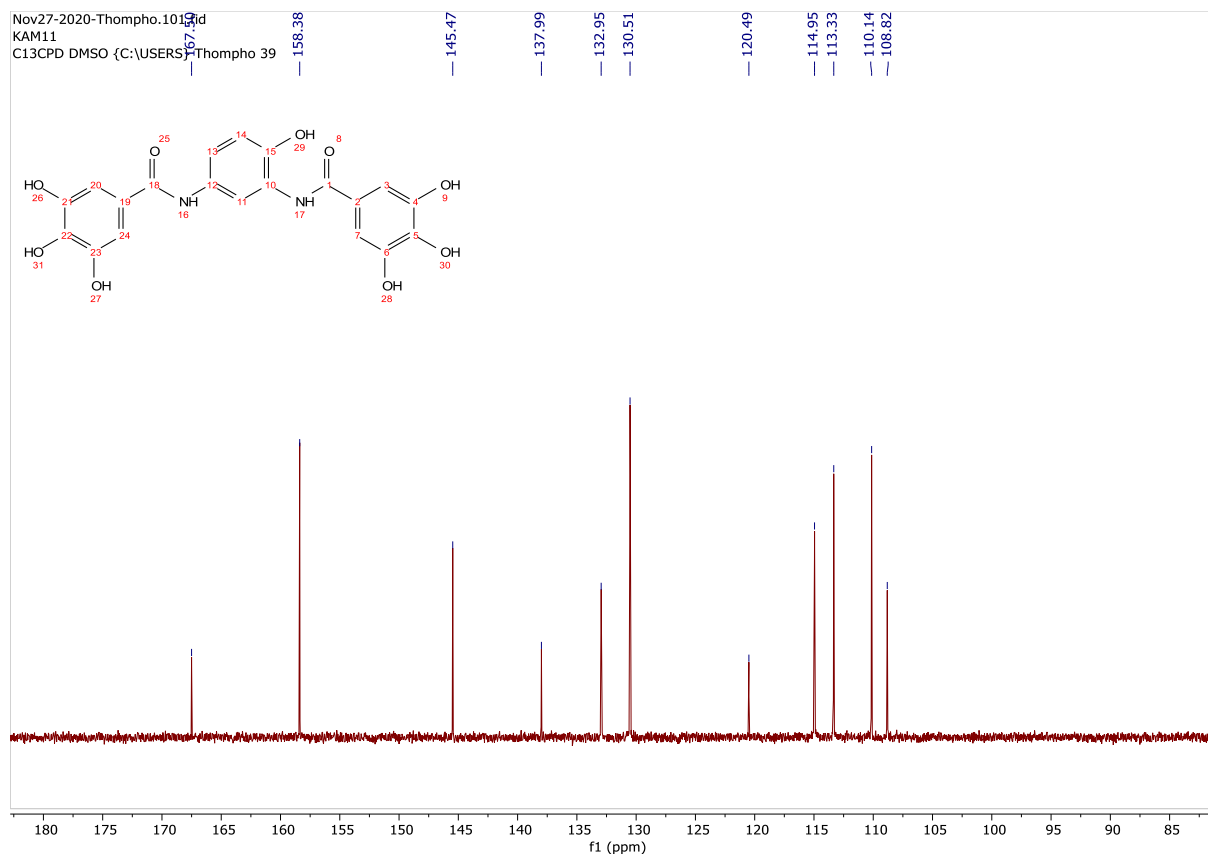
**HRMS of 3,5-dihydroxy-*N*-phenylbenzamide (KAM 10)**



FTIR spectrum of *N,N'*-(4-hydroxy-1,3-phenylene)bis(3,4,5-trihydroxybenzamide)

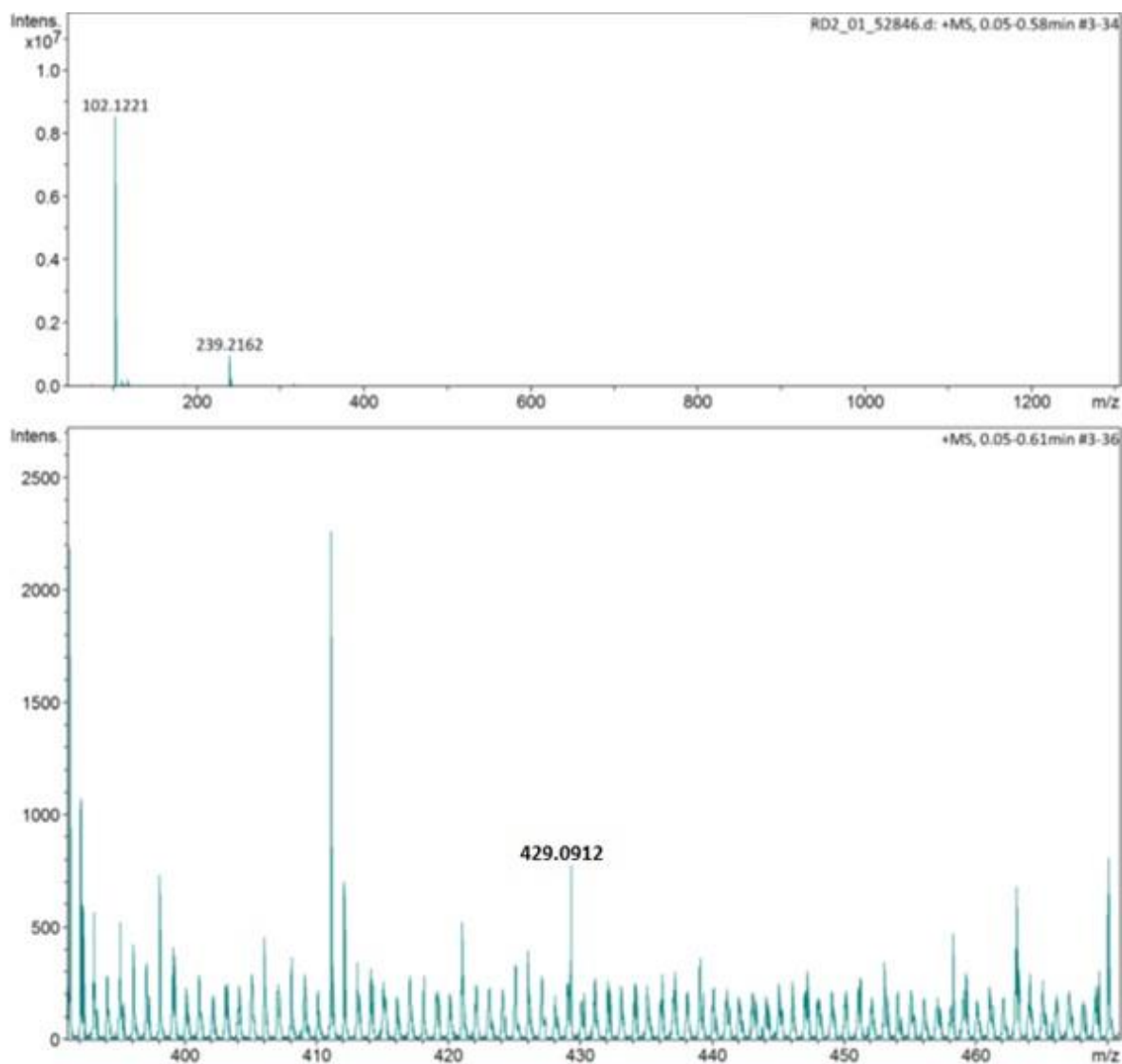
Nov27-2020-Thompho.100.fid  
KAM11  
PROTON DMSO {C:\USERS\ Thompho 39



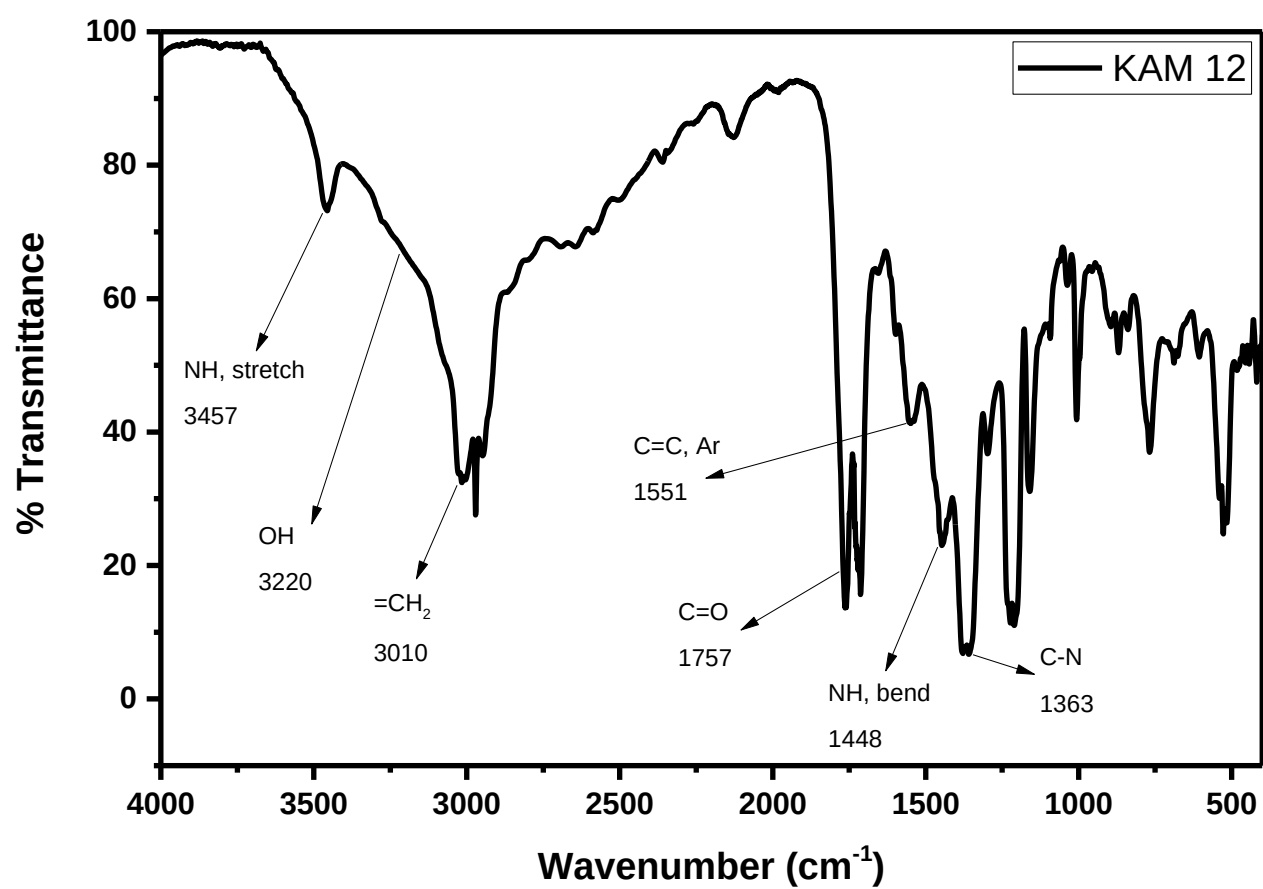
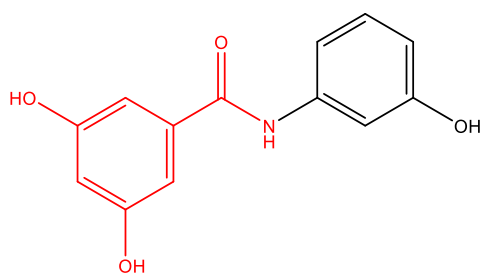


**<sup>1</sup>H and <sup>13</sup>C NMR spectra of *N,N'*-(4-hydroxy-1,3-phenylene)bis(3,4,5-trihydroxybenzamide) (KAM 11)**





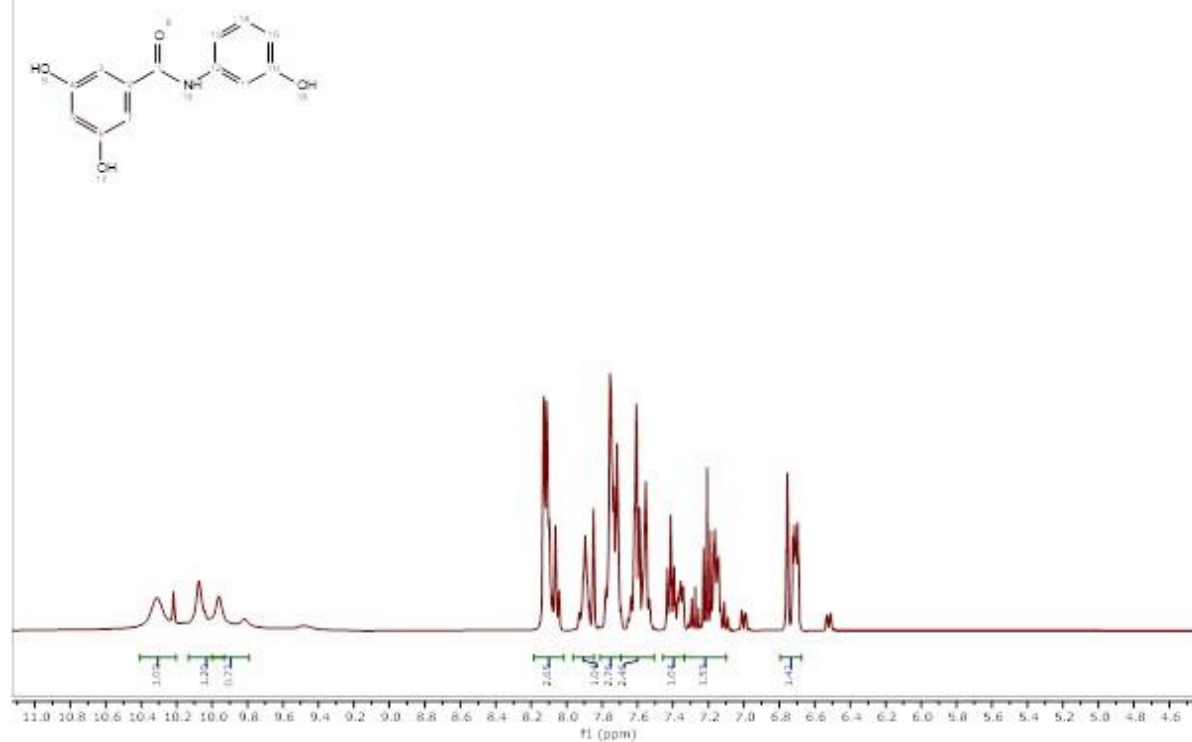
**HRMS of *N,N'*-(4-hydroxy-1,3-phenylene)bis(3,4,5-trihydroxybenzamide) (KAM 11)**



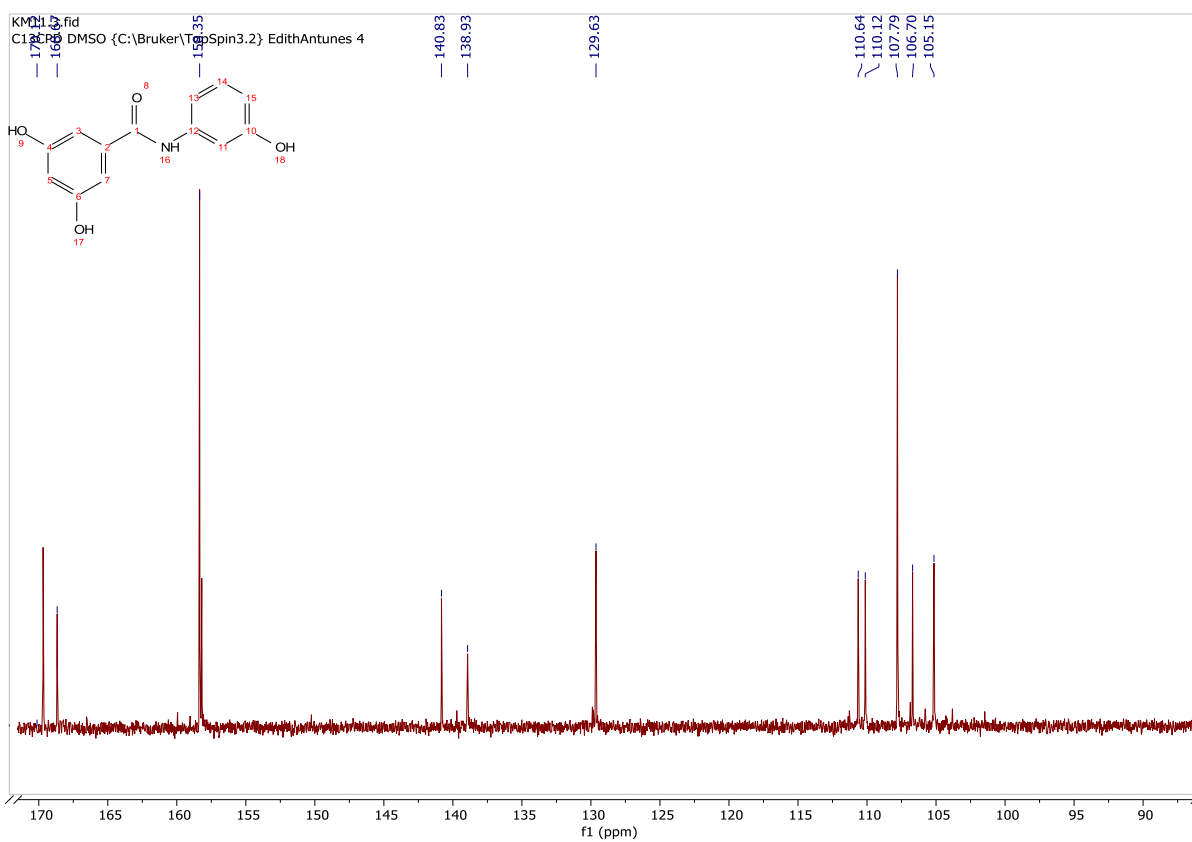
FTIR spectrum of 3,5-dihydroxy-*N*-(3-hydroxyphenyl)benzamide (KAM 12)

Nov27-2020-Thompho.90.fid

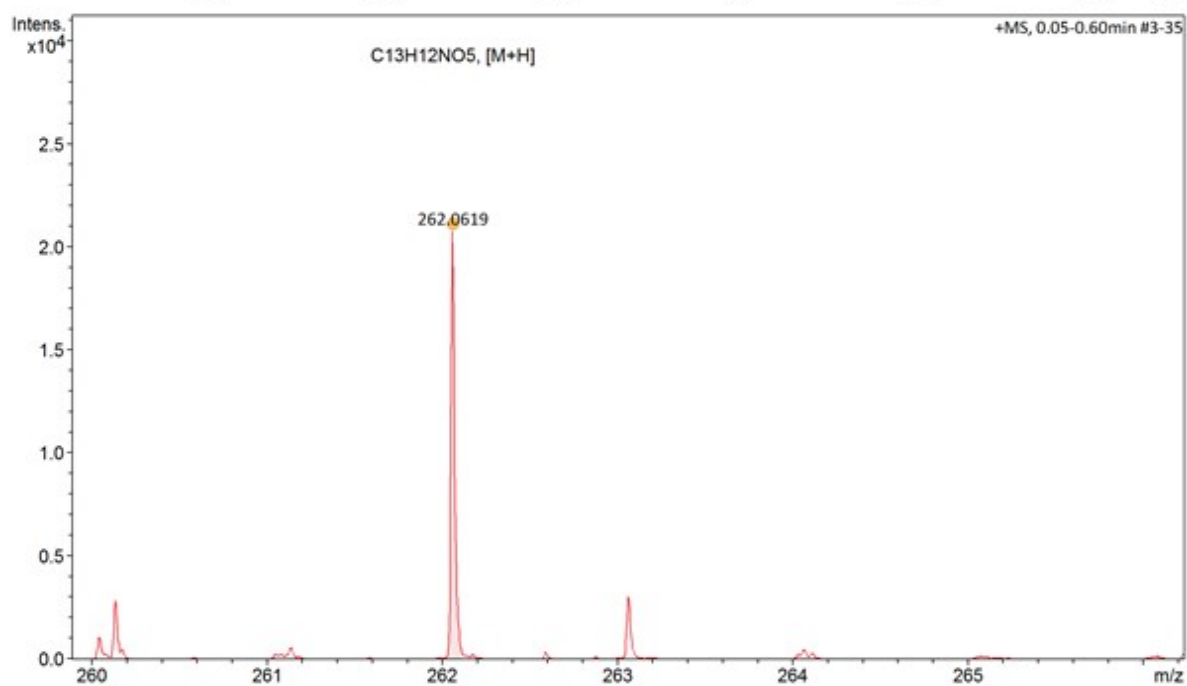
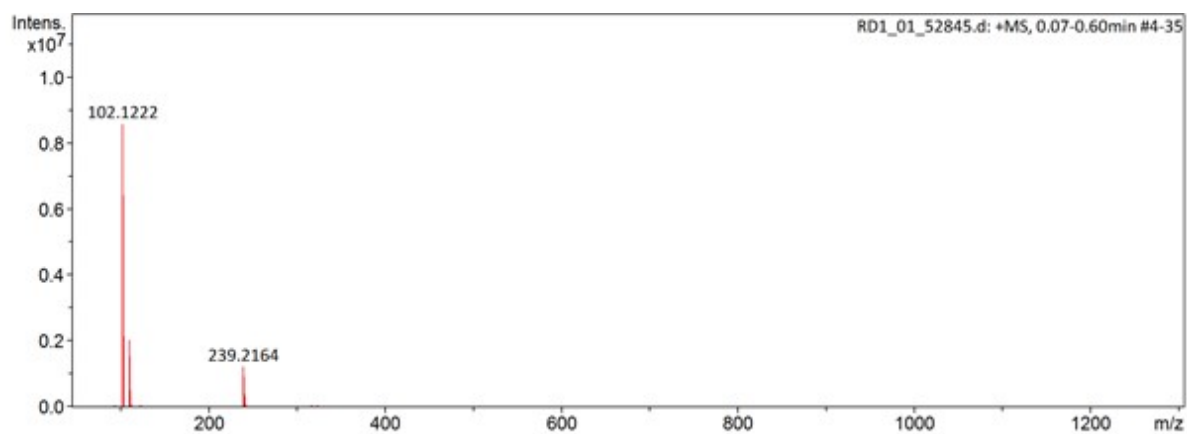
PROTON DMSO (C:\USERS\ Thompho.38



KAM 12.fid  
C13CPD DMSO (C:\Bruker\TopSpin3.2) EdithAntunes 4



**$^1\text{H}$  and  $^{13}\text{C}$  NMR of 3,5-dihydroxy-N-(3-hydroxyphenyl)benzamide (KAM 12)**



**HRMS of 3,5-dihydroxy-*N*-(3-hydroxyphenyl)benzamide (KAM 12)**

## Appendix D1: Tables summarizing NMR results of Monoester compounds

**Table 5.3:**  $^1\text{H}$  NMR data for compounds that are conjugated to 3-hydroxyacetophenone (**C1**, **C2**, **C7** and **C11**)

| Proton          | Chemical shift/ $\delta$ (ppm) (multiplicity, coupling constant (Hz), integration) |                         |                       |                             |
|-----------------|------------------------------------------------------------------------------------|-------------------------|-----------------------|-----------------------------|
|                 | <b>C1</b>                                                                          | <b>C2</b>               | <b>C7</b>             | <b>C11</b>                  |
| H <sub>2</sub>  | 8.0 - 8.14<br>(dd, J=6.1, 1)                                                       | 7.99<br>(td, J=7.9, 1)  | 7.40<br>(d, J=7.8, 4) | 7.38<br>(d, J= 2.5, 1)      |
| H <sub>3</sub>  | 7.67 – 7.94<br>(m, 3)                                                              | 7.77<br>(dd, J=7.8, 2)  | 7.26 – 7.32<br>(m, 8) | 7.30<br>(t, J=4.7, 2)       |
| H <sub>4</sub>  | 7.59<br>(tt, J=6.6, 1)                                                             | 7.48<br>(ddd, J=8.8, 2) | 7.02<br>(d, J=8.1, 5) | 7.00<br>(d, J=7.90, 1)      |
| H <sub>10</sub> | 7.67 – 7.94<br>(m, 3)                                                              | 7.38<br>(dq, J=8.1, 4)  | 6.81 (s, 2)           | 6.93 (s, 2)                 |
| CH <sub>3</sub> | 2.48 (s, 3)                                                                        | –                       | 1.73 (s, 3)           | –                           |
| OH              | 9.96/9.74<br>(s, 1)/(s, 4)                                                         | 9.76 (s, 3)             | –                     | 9.74/ 9.16<br>(s, 1)/(s, 1) |

**Table 5.4:**  $^{13}\text{C}$  NMR data for compounds that are conjugated to 3-hydroxyacetophenone (**C1**, **C2**, **C7** and **C11**)

| Carbon atoms   | Chemical shift/ $\delta$ (ppm) |           |           |            |
|----------------|--------------------------------|-----------|-----------|------------|
|                | <b>C1</b>                      | <b>C2</b> | <b>C7</b> | <b>C11</b> |
| C <sub>1</sub> | 138.23                         | 130.84    | 132.40    | 129.81     |
| C <sub>2</sub> | 127.01                         | -         | 119.30    | 120.34     |
| C <sub>3</sub> | 129.76                         | 109.58    | 120.30    | 120.34     |
| C <sub>4</sub> | 123.30                         | -         | 119.30    | 119.34     |
| C <sub>5</sub> | 150.54                         | 141.31    | 138.10    | 157.64     |
| C <sub>6</sub> | 120.25                         | -         | 106.80    | 114.31     |

|                     |        |        |        |        |
|---------------------|--------|--------|--------|--------|
| C <sub>7</sub> =O   | 197.80 | 180.92 | 198.20 | 197.90 |
| C <sub>8</sub> =O   | 167.34 | 172.09 | 167.40 | 167.57 |
| C <sub>9</sub>      | 132.03 | -      | 129.80 | 120.34 |
| C <sub>10</sub>     | 114.25 | 112.08 | 114.10 | 108.84 |
| C <sub>11</sub> -OH | 157.58 | 161.07 | 157.30 | 145.50 |
| C <sub>12</sub>     | 115.86 | 113.47 | 67.00  | 138.31 |
| C <sub>13</sub>     | 129.96 | -      | 157.30 | 145.50 |
| C <sub>14</sub>     | 119.24 | -      | 114.10 | 108.84 |
| CH <sub>3</sub>     | 26.77  | 24.78  | 26.60  | 26.80  |

**Table 5.5:** <sup>1</sup>H NMR data for compounds that are conjugated to *N*-3-hydroxyphenylacetamide (**C6**, **C10**, **C14** and **C15**)

| Proton          | Chemical shift/ $\delta$ (ppm) (multiplicity, coupling constant (Hz), integration) |                       |                       |                       |
|-----------------|------------------------------------------------------------------------------------|-----------------------|-----------------------|-----------------------|
|                 | <b>C6</b>                                                                          | <b>C10</b>            | <b>C14</b>            | <b>C15</b>            |
| H <sub>2</sub>  | 9.86 (s, 3)                                                                        | 6.83 (s, 2)           | 7,38<br>(d, J=7.7, 5) | 6.98 (s, 2)           |
| H <sub>8</sub>  | 9.61 (s, 1)                                                                        | 7.18 (s, 2)           | 7.31 (s, 1)           | 7.23 (s, 1)           |
| H <sub>10</sub> | 9.70<br>(d, J=8.1, 6)                                                              | 6.92<br>(d, J=8.0, 1) | 7.47 – 7.64<br>(m, 1) | 6.95<br>(d, J=8.0, 1) |
| H <sub>11</sub> | 10.03<br>(t, J=7.9, 16)                                                            | 7.03<br>(t, J=8.0, 1) | 7.27 – 7.29<br>(m, 4) | 7.07<br>(t, J=7.7, 1) |
| H <sub>12</sub> | 9.31<br>(d, J=8.0, 5)                                                              | 6.66<br>(d, J=7.8, 2) | 7.00 (m, 4)           | 6.45<br>(d, J=7.1, 2) |
| CH <sub>3</sub> | 4.71 (s, 2)                                                                        | 2.01 (s, 3)           | 3.38 (s, 3)           | 2.04 (s, 3)           |
| OH              | 10.18 (s, 3)                                                                       | 9.47 (s, 2)           | 9.96 (s, 3)           | 9,19 (s, 3)           |
| NH              | 10.55 (s, 1)                                                                       | 9.73 (s, 1)           | 9.76 (s, 1)           | 9.81 (s, 1)           |

**Table 5.6:**  $^{13}\text{C}$  NMR data for compounds that are conjugated to *N*-3hydroxyphenylacetamide (C6, C10, C14 and C15)

| Carbon atoms       | Chemical shift/ $\delta$ (ppm) |        |        |        |
|--------------------|--------------------------------|--------|--------|--------|
|                    | C6                             | C10    | C14    | C15    |
| C <sub>1</sub>     | 139.79                         | 134.95 | 138.24 | 120.45 |
| C <sub>2</sub>     | 114.90                         | 107.32 | 119.26 | 106.19 |
| C <sub>3</sub>     | 169.28                         | 158.27 | 164.52 | 145.38 |
| C <sub>4</sub>     | 117.26                         | 106.21 | 120.59 | 140.27 |
| C <sub>5</sub>     | 132.79                         | 158.27 | 132.40 | 145.38 |
| C <sub>6</sub>     | 121.52                         | 107.32 | 126.68 | 106.19 |
| C <sub>7</sub>     | 159.97                         | 157.48 | 157.59 | 157.50 |
| C <sub>8</sub>     | 110.28                         | 113.54 | 114.26 | 108.74 |
| C <sub>9</sub>     | 146.47                         | 140.25 | 150.86 | 137.96 |
| C <sub>10</sub>    | 111.32                         | 129.27 | 115.83 | 109.80 |
| C <sub>11</sub>    | 131.98                         | 132.47 | 129.77 | 129.28 |
| C <sub>12</sub>    | 114.90                         | 130.49 | 119.26 | 110.15 |
| C <sub>13</sub> =O | 170.60                         | 167.38 | 167.35 | 167.47 |
| C <sub>14</sub> =O | 174.05                         | 168.31 | 197.82 | 168.26 |
| CH <sub>3</sub>    | 20.74                          | 23.96  | 26.78  | 23.98  |

## Appendix D2: Tables summarizing NMR results of di-ester compounds

**Table 5.6:**  $^1\text{H}$  NMR data for compounds that are conjugated to 3,5-dihydroxy acetophenone (C3, C8 and C12)

| Proton              | Chemical shift/ $\delta$ (ppm) (multiplicity, coupling constant (Hz), integration) |                        |                            |
|---------------------|------------------------------------------------------------------------------------|------------------------|----------------------------|
|                     | C3                                                                                 | C8                     | C12                        |
| H <sub>12/12'</sub> | 7.98 – 8.10<br>(m, 1)                                                              | 6.76 – 6.86<br>(m, 5)  | 6.50<br>(s, 1)             |
| H <sub>13/13'</sub> | 7.72 – 7.86<br>(m, 2)                                                              | –                      | –                          |
| H <sub>14/14'</sub> | 6.92 – 7.03<br>(m, 4)                                                              | 6.40 – 6.50<br>(m, 3)  | –                          |
| H <sub>16/16'</sub> | 7.40 – 7.66<br>(m, 3)                                                              | 4.28<br>(q, J=7.1, 2)  | 6.50<br>(s, 1)             |
| CH <sub>3</sub>     | 2.69 – 3.06<br>(m, 1)                                                              | 1.32 (s, 3)            | 2.54<br>(s, 2)             |
| OH                  | 9.64 (s, 2)                                                                        | 9.65<br>(d, J=67.7, 1) | 9.64 (s, 4)/<br>9.23(s, 2) |

**Table 5.7:**  $^{13}\text{C}$  NMR data for compounds that are conjugated to 3,5-dihydroxyacetophenone (C3, C8 and C12)

| Carbon atoms      | Chemical shift/ $\delta$ (ppm) |        |        |
|-------------------|--------------------------------|--------|--------|
|                   | C3                             | C8     | C12    |
| C <sub>1/3</sub>  | 158.58                         | 158.60 | 158.53 |
| C <sub>2</sub>    | 113.17                         | 131.62 | 120.41 |
| C <sub>4/6</sub>  | 107.12                         | 107.74 | 108.69 |
| C <sub>5</sub>    | 138.81                         | 138.84 | 138.79 |
| C <sub>7</sub> =O | 197.68                         | 197.73 | 197.67 |



|                         |        |        |        |
|-------------------------|--------|--------|--------|
| C <sub>10/10'</sub> =O  | 171.94 | 167.40 | 173.77 |
| C <sub>11/11'</sub>     | 135.67 | 132.53 | 137.95 |
| C <sub>12/12'</sub>     | 119.19 | 106.19 | 106.14 |
| C <sub>15/15'</sub> -OH | 161.14 | 165.79 | 167.43 |
| C <sub>14/14'</sub>     | 116.73 | 60.59  | 145.37 |
| C <sub>13/13'</sub>     | 130.28 | 165.79 | 167.43 |
| C <sub>16/16'</sub>     | 106.17 | 106.19 | 106.14 |
| CH <sub>3</sub>         | 26.75  | 26.78  | 26.70  |

---

### Appendix D3: Tables summarizing NMR results of tri-ester compounds

**Table 5.8:**  $^1\text{H}$  NMR data for compounds that are conjugated to phloroglucinol (**C4**, **C5**, **C9** and **C13**)

| Proton                   | Chemical shift/ $\delta$ (ppm) (multiplicity, coupling constant (Hz), integration) |                       |                       |                        |
|--------------------------|------------------------------------------------------------------------------------|-----------------------|-----------------------|------------------------|
|                          | <b>C4</b>                                                                          | <b>C5</b>             | <b>C9</b>             | <b>C13</b>             |
| $\text{H}_{8/8'}/8''$    | 7.49 – 7.66<br>(dd, J=26.7, 1)                                                     | 7.68 – 7.84<br>(m, 1) | 6.82<br>(d, J=2.3, 2) | 6.93<br>(d, J=10.7, 5) |
| $\text{H}_{9/9'}/9''$    | –                                                                                  | 7.38 – 7.66<br>(m, 1) | –                     | –                      |
| $\text{H}_{10/10'}/10''$ | 7.14<br>(d, J=8.2, 1)                                                              | 7.38 – 7.66<br>(m, 1) | 6.80<br>(d, J=2.3, 4) | –                      |
| $\text{H}_{11/11'}/11''$ | 7.36 – 7.45<br>(m, 1)                                                              | 5.82 – 5.92<br>(m, 6) | –                     | –                      |
| $\text{H}_{12/12'}/12''$ | 7.73<br>(d, J=15.5, 3)                                                             | –                     | 6.82<br>(d, J=2.3, 2) | 6.93<br>(d, J=10.7, 5) |
| $\text{H}_{2/4/6}$       | 5.66 (s, 3)                                                                        | 5.66 (s, 3)           | 5.65 (s, 3)           | 7.14 – 7.00<br>(m, 2)  |
| OH                       | 8.95 (s, 3)                                                                        | 11.29 (s, 2)          | 9.69 (s, 6)           | 8.39 – 10.35<br>(m, 9) |

**Table 5.9:**  $^{13}\text{C}$  NMR data for compounds that are conjugated to phloroglucinol (**C4**, **C5**, **C9** and **C13**)

| Carbon atoms          | Chemical shift/ $\delta$ (ppm) |           |           |            |
|-----------------------|--------------------------------|-----------|-----------|------------|
|                       | <b>C4</b>                      | <b>C5</b> | <b>C9</b> | <b>C13</b> |
| $\text{C}_1$          | 150.81                         | 122.13    | 132.52    | 137.35     |
| $\text{C}_{2/4/6}$    | 94.03                          | 76.89     | 94.07     | 98.68      |
| $\text{C}_{3/5}$      | 150.81                         | 122.13    | 132.52    | 137.35     |
| $\text{C}_{7/7'}/7''$ | 130.10                         | 58.06     | 107.31    | 108.71     |

|                          |        |        |        |        |
|--------------------------|--------|--------|--------|--------|
| C <sub>8/8'/8''</sub>    | 116.21 | 94.28  | 60.55  | 59.50  |
| C <sub>9/9'/9''</sub>    | 157.67 | 83.21  | 158.41 | 145.42 |
| C <sub>10/10'/10''</sub> | 120.60 | 99.69  | 60.55  | 120.43 |
| C <sub>11/11'/11''</sub> | 127.86 | 81.11  | 158.41 | 145.42 |
| C <sub>12/12'/12''</sub> | 122.64 | 125.13 | 62.56  | 59.50  |
| C <sub>13/13'/13''</sub> | 158.89 | 136.36 | 167.38 | 168.66 |

## Appendix E1: Tables summarizing NMR results of Mono-amide compounds

**Table 5.10:**  $^1\text{H}$  NMR data for mono-amide compounds derived from aniline (**KAM 7**, **KAM 9**, and **KAM 10**)

|                    | $\delta$ (ppm) [(multiplicity, coupling constant (Hz), integration)] |                       |                       |
|--------------------|----------------------------------------------------------------------|-----------------------|-----------------------|
| Proton             | <b>KAM 7</b>                                                         | <b>KAM 9</b>          | <b>KAM 10</b>         |
| H <sub>3</sub>     | 6.95 (s, 1)                                                          | 6.88 (s, 4)           | 6.42 (s, 1)           |
| H <sub>4</sub>     | 9.96 (bs, 1)                                                         | 9.95 (bs, 1)          | 9.58 (bs, 2)          |
| H <sub>5</sub>     | 7.59<br>(d, J=8.5, 2)                                                | 9.95 (bs, 1)          | 6.59 – 6.72<br>(m, 4) |
| H <sub>6</sub>     | 7.28<br>(t, J=7.8, 2)                                                | 9.95 (bs, 1)          | 9.58 (bs, 2)          |
| H <sub>7</sub>     | 7.28<br>(t, J=7.8, 2)                                                | 6.88 (s, 4)           | 6.42 (s, 1)           |
| H <sub>10/14</sub> | 7.28<br>(t, J=7.8, 2)                                                | 7.27 (t, 3)           | 7.08 (t, 3)           |
| H <sub>11/13</sub> | 7.59<br>(d, J=8.5, 2)                                                | 7.57<br>(d, J=7.4, 3) | 6.77<br>(d, J=2.2, 1) |
| H <sub>15</sub>    | 7.02 (t, 1)                                                          | 7.01 (t, 2)           | 7.32 (t, 1)           |
| NH <sub>16</sub>   | 9.96 (bs, 1)                                                         | 9.95 (bs, 1)          | 9.58 (bs, 2)          |

**Table 5.11:**  $^{13}\text{C}$  NMR data for mono-amide compounds derived from aniline (**KAM 7**, **KAM 9**, and **KAM 10**)

| Carbon atom        | Chemical shift/ $\delta$ (ppm) |              |               |
|--------------------|--------------------------------|--------------|---------------|
|                    | <b>KAM 7</b>                   | <b>KAM 9</b> | <b>KAM 10</b> |
| C <sub>2</sub>     | 145.90                         | 132.10       | 137.01        |
| C <sub>3</sub>     | 109.24                         | 106.91       | 108.73        |
| OH-C <sub>4</sub>  | 162.79                         | 158.01       | 145.34        |
| C <sub>5</sub>     | 116.11                         | 106.41       | 139.31        |
| C <sub>6</sub>     | 139.80                         | 117.23       | 145.34        |
| C <sub>7</sub>     | 114.34                         | 106.91       | 108.73        |
| C <sub>8</sub>     | 149.05                         | 136.78       | 136.93        |
| C <sub>11/13</sub> | 119.45                         | 118.93       | 114.19        |
| C <sub>10/14</sub> | 129.09                         | 128.58       | 128.61        |
| C <sub>15</sub>    | 123.42                         | 128.15       | 122.92        |
| O=C                | 168.77                         | 166.98       | 168.28        |

**Table 5.12:**  $^1\text{H}$  NMR data for mono-amide compounds derived from 3-aminophenol (**KAM 5**, **KAM 8** and **KAM 12**)

| Proton           | $\delta$ (ppm) [(multiplicity, coupling constant (Hz), integration)] |                        |                       |
|------------------|----------------------------------------------------------------------|------------------------|-----------------------|
|                  | <b>KAM 5</b>                                                         | <b>KAM 8</b>           | <b>KAM 12</b>         |
| H <sub>3</sub>   | 6.92 (s, 1)                                                          | 7.35<br>(d, J=3.1, 4)  | 7.52 – 7.66<br>(m, 2) |
| H <sub>4</sub>   | 9.68 (bs, 1)                                                         | 9.89 (s, 1)            | 9.96 (s, 1)           |
| H <sub>5</sub>   | 6.72 – 6.82<br>(m, 6)                                                | 6.96 – 6.98<br>(m, 3)  | 7.08 – 7.31<br>(m, 1) |
| H <sub>6</sub>   | 9.68 (bs, 1)                                                         | 7.19<br>(t, J=2.2, 1)  | 9.96 (s, 1)           |
| H <sub>7</sub>   | 6.92 (s, 1)                                                          | 7.19<br>(t, J=2.2, 1)  | 7.52 – 7.66<br>(m, 2) |
| H <sub>11</sub>  | 6.72 – 6.82<br>(m, 6)                                                | 6.96 – 6.98<br>(m, 3)  | 7.33 – 7.45<br>(m, 1) |
| H <sub>13</sub>  | 6.72 – 6.82<br>(m, 6)                                                | 7.35<br>(d, J=3.1, 4)  | 7.84 – 7.94<br>(m, 3) |
| H <sub>14</sub>  | 7.24<br>(t, J=7.9, 2)                                                | 6.76<br>(t, J=7.8, 1)  | 6.76<br>(t, J=2.2, 1) |
| H <sub>15</sub>  | 6.72 – 6.82<br>(m, 6)                                                | 6.43<br>(dd, J=8.9, 1) | 6.68 – 6.73<br>(m, 1) |
| NH <sub>16</sub> | 9.68 (bs, 1)                                                         | -                      | 10.31 (s, 1)          |
| OH <sub>17</sub> | 9.68 (bs, 1)                                                         | 10.05 (s, 1)           | 10.07 (s, 1)          |

**Table 5.13:**  $^{13}\text{C}$  NMR data for mono-amide compounds derived from 3-aminophenol (**KAM 5**, **KAM 8** and **KAM 12**)

| Carbon atom        | Chemical shift/ $\delta$ (ppm) |              |               |
|--------------------|--------------------------------|--------------|---------------|
|                    | <b>KAM 5</b>                   | <b>KAM 8</b> | <b>KAM 12</b> |
| C <sub>2</sub>     | 138.40                         | 157.43       | 138.93        |
| C <sub>3</sub>     | 127.81                         | 119.92       | 107.79        |
| OH-C <sub>4</sub>  | 146.82                         | 167.58       | 158.35        |
| C <sub>5</sub>     | 145.92                         | 129.22       | 106.70        |
| C <sub>6</sub>     | 146.82                         | 140.40       | 158.35        |
| C <sub>7</sub>     | 127.81                         | 129.43       | 107.79        |
| C <sub>11</sub>    | 145.92                         | 158.11       | 140.83        |
| C <sub>12</sub>    | 109.34                         | 115.88       | 105.15        |
| OH-C <sub>10</sub> | 158.77                         | 165.58       | 168.67        |
| C <sub>13</sub>    | 120.95                         | 118.15       | 110.64        |
| C <sub>14</sub>    | 142.04                         | 149.83       | 129.63        |
| C <sub>15</sub>    | 115.66                         | 119.59       | 110.12        |
| O=C                | 167.94                         | 168.22       | 170.12        |

## Appendix E2: Tables summarizing NMR results of di-amide compounds

**Table 5.14:**  $^1\text{H}$  NMR data for di-amide compounds derived from 1,3-diaminobenzene (**KAM 1**, **KAM 4** and **KAM 6**)

| Proton               | $\delta$ (ppm) [(multiplicity, coupling constant (Hz), integration)] |                        |                     |
|----------------------|----------------------------------------------------------------------|------------------------|---------------------|
|                      | <b>KAM 1</b>                                                         | <b>KAM 4</b>           | <b>KAM 6</b>        |
| H <sub>1</sub>       | 7.36 (s, 2)                                                          | 9.68 (s, 2)            | 6.93 (s, 1)         |
| H <sub>3/5</sub>     | 7.28<br>(d, J=2.1, 2)                                                | 7.43<br>(dd, J=8.1, 1) | 7.97<br>(d, J=2, 1) |
| H <sub>4</sub>       | 7.38<br>(t, J=1.4, 1)                                                | 7.35 (t, 1)            | 7.18<br>(t, J=2, 1) |
| H <sub>8/8'</sub>    | 7.26 (s, 1)                                                          | 6.70 (s, 1)            | 6.91 (s, 2)         |
| H <sub>9/9'</sub>    | 7.34 (m, 3)                                                          | 9.94 (s, 1)            | 9.19 (s, 3)         |
| H <sub>10/10'</sub>  | 7.00<br>(d, J=2,6, 1)                                                | 6.78<br>(d, J=2.2, 1)  | 8.84 (s, 1)         |
| H <sub>11/11'</sub>  | 9.69 (s, 1)                                                          | 9.94 (s, 1)            | 9.18 (s, 3)         |
| H <sub>12/12'</sub>  | 6.98<br>(d, J=2,6, 1)                                                | 6.70 (s, 1)            | 6.91 (s, 2)         |
| NH <sub>13/13'</sub> | 9.94 (s, 2)                                                          | 10.10 (s, 1)           | 9.96 (s, 4)         |

**Table 5.15:**  $^{13}\text{C}$  NMR data for hydroxybenzoic acids conjugated to 1,3-diaminobenzene (**KAM 1**, **KAM 4** and **KAM 6**)

| Carbon atom            | Chemical shift/ $\delta$ (ppm) |              |              |
|------------------------|--------------------------------|--------------|--------------|
|                        | <b>KAM 1</b>                   | <b>KAM 4</b> | <b>KAM 6</b> |
| C <sub>7/7'</sub>      | 157.30                         | 158.35       | 145.37       |
| C <sub>12/12'</sub>    | 119.94                         | 107.28       | 108.64       |
| OH-C <sub>11/11'</sub> | 167.31                         | 167.43       | 167.47       |
| C <sub>10/10'</sub>    | 129.29                         | 105.84       | 165.45       |



|                   |        |        |        |
|-------------------|--------|--------|--------|
| C <sub>9/9'</sub> | 149.64 | 167.43 | 167.47 |
| C <sub>8/8'</sub> | 132.01 | 107.28 | 108.64 |
| C <sub>2/6</sub>  | 157.41 | 165.72 | 154.07 |
| C <sub>1</sub>    | 115.80 | 137.15 | 137.39 |
| C <sub>4</sub>    | 139.56 | 154.62 | 149.62 |
| C <sub>3/5</sub>  | 129.56 | 149.00 | 120.45 |
| O=C               | 172.00 | 167.97 | 171.98 |

**Table 5.16:** <sup>1</sup>H NMR data for second-class di-amide compounds derived from 2,4-diaminophenol (**KAM 2**, **KAM 3**, and **KAM 11**)

| Proton            | $\delta$ (ppm) [(multiplicity, coupling constant (Hz), integration)] |                       |                       |
|-------------------|----------------------------------------------------------------------|-----------------------|-----------------------|
|                   | KAM 2                                                                | KAM 3                 | KAM 11                |
| H <sub>3/3'</sub> | 6.89 (s, 1)                                                          | 6.79<br>(d, J=2.2, 4) | 6.92 (s, 1)           |
| H <sub>4/4'</sub> | 9.74<br>(d, J=9.2, 4)                                                | 9.55 (s, 4)           | 10.08 (bs, 5)         |
| H <sub>5/5'</sub> | 7.30<br>(d, J=1.4, 2)                                                | 6.40<br>(d, J=2.4, 2) | 10.08 (bs, 5)         |
| H <sub>6/6'</sub> | 7.38<br>(dt, J=3.1, 3)                                               | 9.55 (s, 4)           | 10.08 (bs, 5)         |
| H <sub>7/7'</sub> | 7.38<br>(dt, J=3.1, 3)                                               | 6.79<br>(d, J=2.2, 4) | 6.92 (s, 1)           |
| H <sub>11</sub>   | 8.15<br>(d, J=2.6, 1)                                                | 7.97 (s, 1)           | 6.78<br>(d, J=0.9, 1) |
| H <sub>13</sub>   | 7.26<br>(d, J=2.7, 1)                                                | 8.54 (m, 1)           | 6.80<br>(d, J=2.3, 1) |
| H <sub>14</sub>   | 7.05<br>(d, J=2.5, 1)                                                | 6.79<br>(d, J=2.2, 4) | 7.24<br>(t, J=8.0, 1) |

|                  |              |             |               |
|------------------|--------------|-------------|---------------|
| OH <sub>15</sub> | 9.41 (s, 1)  | 9.88 (s, 1) | 10.32 (bs, 3) |
| NH <sub>8</sub>  | 9.94 (s, 2)  | 9.68 (s, 1) | 10.32 (bs, 3) |
| NH <sub>9</sub>  | 10.04 (s, 1) | 9.94 (s, 1) | 10.32 (bs, 3) |

**Table 5.17:** <sup>13</sup>C NMR data for second-class di-amide compounds derived from 2,4-diaminophenol (**KAM 2**, **KAM 3** and **KAM 11**)

| Carbon atom          | Chemical shift/ $\delta$ (ppm) |              |               |
|----------------------|--------------------------------|--------------|---------------|
|                      | <b>KAM 2</b>                   | <b>KAM 3</b> | <b>KAM 11</b> |
| C <sub>2/2'</sub>    | 157.91                         | 151.66       | 130.51        |
| C <sub>3/3'</sub>    | 120.22                         | 108.30       | 108.82        |
| OH-C <sub>4/4'</sub> | 167.88                         | 159.34       | 158.38        |
| C <sub>5/5'</sub>    | 127.05                         | 107.21       | 145.47        |
| C <sub>6/6'</sub>    | 150.89                         | 159.34       | 158.38        |
| C <sub>7/7'</sub>    | 129.94                         | 108.30       | 108.82        |
| C <sub>12</sub>      | 133.70                         | 150.49       | 132.95        |
| C <sub>11</sub>      | 116.31                         | 113.36       | 110.14        |
| C <sub>10</sub>      | 132.71                         | 134.32       | 120.49        |
| OH-C <sub>15</sub>   | 167.25                         | 167.42       | 137.99        |
| C <sub>14</sub>      | 123.07                         | 120.89       | 113.33        |
| C <sub>13</sub>      | 126.41                         | 133.26       | 114.95        |
| O=C                  | 169.67                         | 169.98       | 167.50        |

## Appendix F1

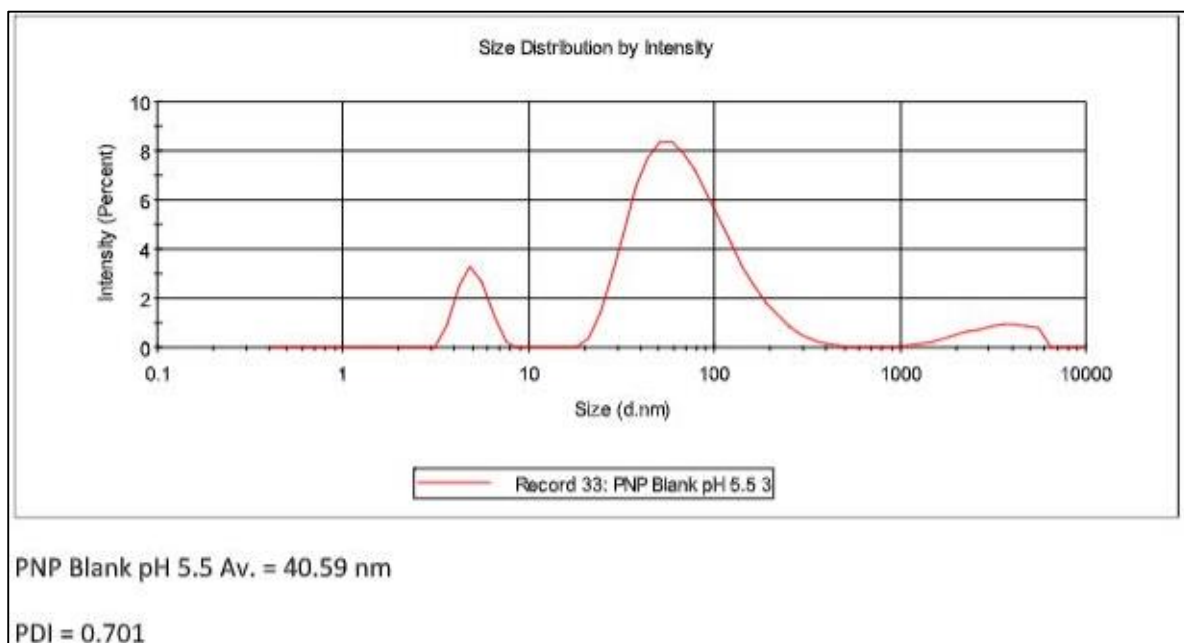
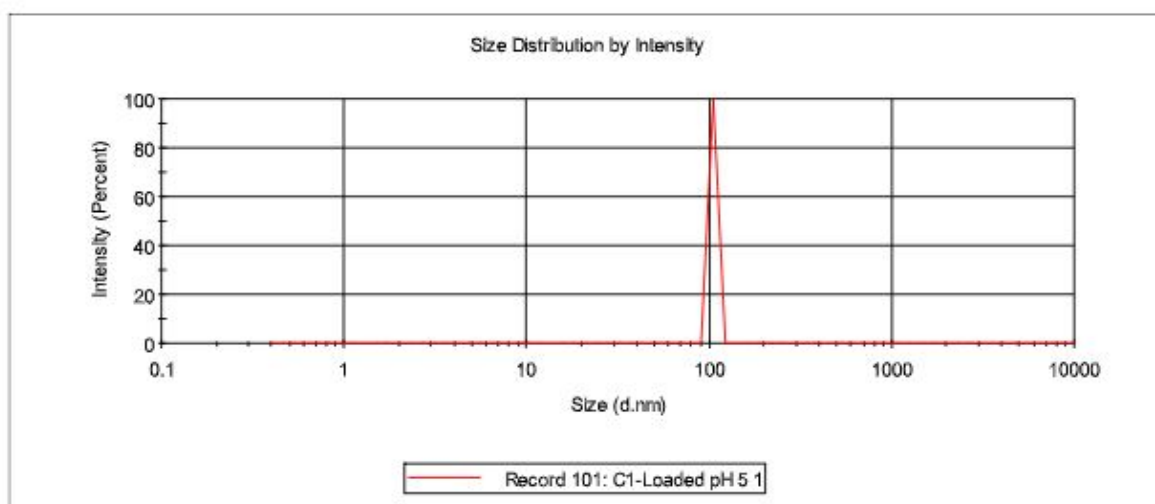


**Fig. 6.7:** Size distribution image of blank PNPs

|                                              |              |                                      |             |
|----------------------------------------------|--------------|--------------------------------------|-------------|
| Sample Name: C1-Loaded 3                     |              |                                      |             |
| SOP Name: mansettings.nano                   |              |                                      |             |
| File Name: Khumbulani WITS.dts               |              | Dispersant Name: PBS                 |             |
| Record Number: 21                            |              | Dispersant RI: 1.330                 |             |
| Date and Time: 22 February 2022 04:10:04 PM  |              | Viscosity (cP): 0.8800               |             |
|                                              |              | Dispersant Dielectric Constant: 79.0 |             |
| Temperature (°C): 25.0                       |              | Zeta Runs: 12                        |             |
| Count Rate (kcps): 3052.1                    |              | Measurement Position (mm): 2.00      |             |
| Cell Description: Clear disposable zeta cell |              | Attenuator: 10                       |             |
|                                              | Mean (mV)    | Area (%)                             | St Dev (mV) |
| Zeta Potential (mV): 16.4                    | Peak 1: 0.00 | 0.0                                  | 0.00        |
| Zeta Deviation (mV): 0.00                    | Peak 2: 0.00 | 0.0                                  | 0.00        |
| Conductivity (mS/cm): 21.2                   | Peak 3: 0.00 | 0.0                                  | 0.00        |
| Result quality : Good                        |              |                                      |             |

**Fig. 6.8:** Zeta potential of compound C1-L nanocomposite



C1-Loaded pH 5 Av. = 98.7 nm

PDI = 0.105

**Fig. 6.8:** Size distribution of C1-L nanocomposite

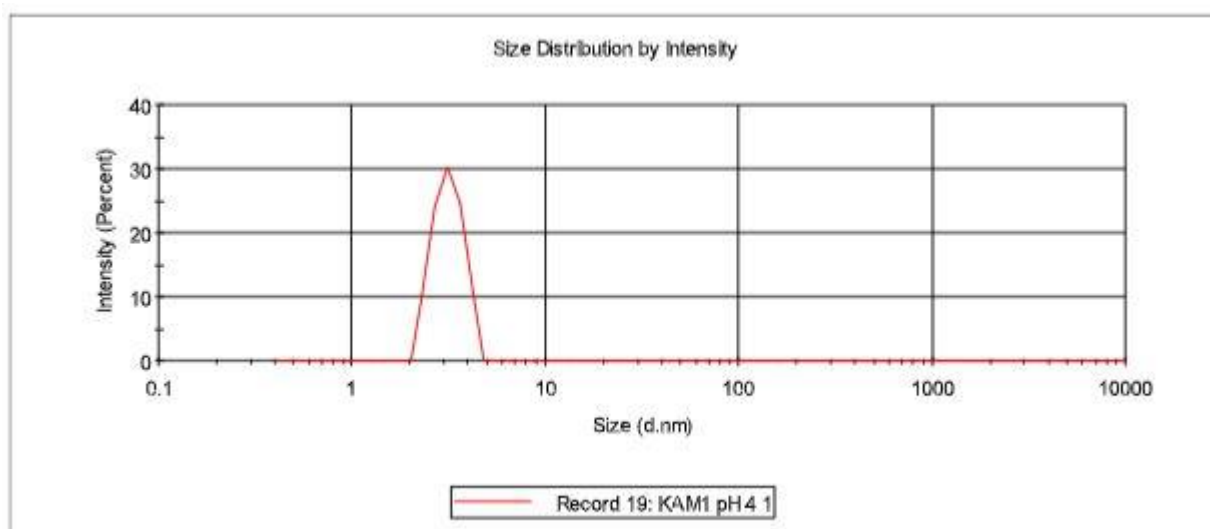


**Fig. 6.9:** Size distribution of C1-L nanocomposite

|                                              |                                 |                                      |             |
|----------------------------------------------|---------------------------------|--------------------------------------|-------------|
| Sample Name: KAM1-Loaded 3                   |                                 |                                      |             |
| SOP Name: mansettings.nano                   |                                 |                                      |             |
| File Name: Khumbulani WITS.dts               | Dispersant Name: PBS            |                                      |             |
| Record Number: 27                            | Dispersant RI: 1.330            |                                      |             |
| Date and Time: 22 February 2022 04:41:29 PM  | Viscosity (cP): 0.8800          |                                      |             |
|                                              |                                 | Dispersant Dielectric Constant: 79.0 |             |
| <hr/>                                        |                                 |                                      |             |
| Temperature (°C): 25.0                       | Zeta Runs: 86                   |                                      |             |
| Count Rate (kcps): 249.8                     | Measurement Position (mm): 2.00 |                                      |             |
| Cell Description: Clear disposable zeta cell | Attenuator: 11                  |                                      |             |
| <hr/>                                        |                                 |                                      |             |
|                                              | Mean (mV)                       | Area (%)                             | St Dev (mV) |
| Zeta Potential (mV): 7.33                    | Peak 1: 0.00                    | 0.0                                  | 0.00        |
| Zeta Deviation (mV): 0.00                    | Peak 2: 0.00                    | 0.0                                  | 0.00        |
| Conductivity (mS/cm): 43.9                   | Peak 3: 0.00                    | 0.0                                  | 0.00        |
| Result quality : Good                        |                                 |                                      |             |

**Fig 6.10:** Zeta potential of compound C1-L nanocomposite

## ANNEXURE A

---

UNIVERSITY OF THE  
WITWATERSRAND,  
JOHANNESBURG



HUMAN RESEARCH ETHICS  
COMMITTEE (MEDICAL)

### Human Research Ethics Committee (Medical)

---

Research Office Secretariat:

Faculty of Health Sciences, Phillip Tobias Building, 3<sup>rd</sup> Floor, Office 301/2/4, 29 Princess of Wales Terrace, Parktown, 2193

Tel +27 (0)11-717-1252 /1234/2656/2700

Private Bag 3, Wits 2050

Office email: [HREC-Medical.ResearchOffice@wits.ac.za](mailto:HREC-Medical.ResearchOffice@wits.ac.za)

Website: [www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/](http://www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/)

Ref: W-CBP-190621-1

21/06/2019

#### ***TO WHOM IT MAY CONCERN:***

**Waiver:** This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)

**Investigator:** Khumblani Mnqiwu (Student Number: 2050388)

**Supervisor:** Dr Sharon Moeno

**Department:** Oral Biological Sciences

**Project title:** Bioactivity of Levuclenic acid analogues and their pH-responsive polymer Nanocomposites

**Reason:** No human participants will be required

A handwritten signature in black ink, appearing to read 'CB Penny'.

---

**Doctor CB Penny**

**Chairperson: Human Research Ethics Committee (Medical)**

Copy – HREC (Medical) Secretariat: Rhulani Mkansi and Zanele Ndlovu.