An Optimal Polyherbal Formulation with Antimicrobial and Anti-oxidant Properties

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

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10 May 2023

Common culinary herbs and spices used in this study

DECLARATION

I, Tsholofelo Mavline Mapeka, student number [1247148], declare that this thesis is my own work and that I contributed amply towards research findings published in the article(s) which are included in my thesis. This thesis is being submitted in fulfilment for the degree of Doctor of Philosophy (PhD) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signature of Student  **Date** 10 May 2023

ABSTRACT

Introduction: Since ancient times, spices and herbs have been traded, valued, and used for the enhancement of taste, flavour, and colour of food and beverages. Their role in preservation has been investigated in several *in vitro* and *in vivo* studies. Many herbs and spices, other than their culinary uses, have health-promoting properties and have been used by different cultures and in various traditions to prevent and manage illnesses and diseases. Such health benefits among others include antibacterial, antifungal, antiviral, anti-oxidant, anti-inflammatory, antidiabetic, and anticancer activities.

A number of studies have demonstrated synergism as an effective strategy to enhance the bio-activity of plant extracts and essential oils. Thus, using this framework, this study was conducted to effectively determine the ideal combination of crude extracts or neat essential oils of some common culinary herbs and spices that would exhibit the most favourable anti-oxidant and antimicrobial activities, and incorporate these into an optimal polyherbal formulation.

Materials and methods: A total of 51 crude extracts (methanol, dichloromethane, water) and 15 neat essential oils were chemically profiled using ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS) and gas chromatography coupled with mass spectrometry (GC-MS), respectively. The major constituents in the extracts and essential oils were identified based on the available data from the literature.

The anti-oxidant activities of the extracts and essential oils were evaluated independently, and in combination (1:1) using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and ferric reducing anti-oxidant power (FRAP) assays. Whereas the minimum inhibitory concentration (MIC) assay was used to determine the antibacterial activities of the individual extracts and essential oils and their combinations (1:1) against three Gram-positive and three Gram-negative bacteria. The

antimicrobial and anti-oxidant interactions were determined by calculating the sum of fractional inhibitory concentration index (ΣFICI). The Design of Experiments (DOE) (MODDE 9.1®) software analysis was used to optimize synergy potential of the herbal extracts by generating a statistically significant model that predicted the most effective ratio required for the polyherbal combination, as validated experimentally. A polyherbal capsule was formulated from blending various spice extracts based on DOE model predictions. The formulation was evaluated for toxicity using Brine shrimp lethality assay (BSLA), and the anti-oxidant and antibacterial activity of the formulation was also determined.

Results: The major compounds that were identified in the analysed essential oils were limonene, linalool, 1,8-cineole, β-caryophyllene, α-pinene, β-pinene, eugenol, estragole, cinnamaldehyde, thymol, citral, terpinene-4-ol, *trans*-sabinene hydrate, γ- terpinene, thujone (α, β), menthone and menthol.

A total of 45 bioactive compounds , mainly phenolic acids and flavonoids were identified in the analysed crude extracts by comparing mass spectra and retention time (Rt) with those of commercially available reference standards. Where standards were not available, the compounds were tentatively identified by comparing mass spectra in terms of *m/z* fragments with data reported in the literature. Compounds such as apigenin, caffeic acid, proanthocyanidins, catechin, and rosmarinic acid were common in numerous analysed extracts.

Each extract and essential oil displayed variable anti-oxidant activity. A total of 27 combinations were assessed for anti-oxidant interactions using the DPPH, ABTS, and FRAP assays. From these combinations, 3.8% was synergistic in the DPPH assay, 7.7% in the FRAP and ABTS assays, whilst 50.0% of the combinations were additive using the DPPH and FRAP assays, and 19.2% with the ABTS assay. Antagonism was shown in 11.1% with the DPPH assay, and 3.7% with ABTS and FRAP assays. Many of these combinations had an indifferent effect (37.0% with DPPH and FRAP assays, and 66.7% with ABTS assay).

Out of the 14 combinations of the extracts assessed for antibacterial interactions, 16.7% were synergistic, additive, and antagonistic, whilst 50.0% of the combinations had an indifferent effect. A total of 21 essential oil combinations were evaluated for antibacterial interactions. of these combinations, 66.7% were antagonistic, whereas 33.3% were indifferent. None of the essential oils combinations showed additive or synergy interactions.

The optimum combination for anti-oxidant properties was predicted by DOE to be the mixture containing crude methanol extracts of *Mentha piperita L.* (55.0%), *Thymus vulgaris L.*(44.0%), and *Zingiber officinale* Roscoe (1.0%). The optimal antimicrobial mixture contained crude methanol extracts of *Rosmarinus officinalis L.*(59.5%), *Salvia officinalis L.*(40.0%), and *1.8* (0.5%).

A polyherbal capsule containing a mixture of *R. officinalis*, *S. officinalis*, and *S. aromaticum* methanol extracts was formulated successfully. The formulation demonstrated a dose-dependent mortality to the brine shrimps. The formulation exhibited less than 50% mortality at concentrations below 500 µg/mL. The formulation demonstrated notable anti-oxidant and antibacterial activity.

Conclusion: An optimal polyherbal capsule containing *R. officinalis* (59.5%), *S. officinalis* (40.0%), and *S. aromaticum* (0.5%) methanol extracts, with antimicrobial and anti-oxidant properties was formulated successfully by calculating ΣFICI and using Design of Experiment (DOE).

DEDICATION

I would like to dedicate this thesis to my late beloved Mom Mrs CM Mapeka and Dad Mr SM Mapeka. You could have been so proud to see me reach this milestone.

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LIST OF PUBLICATIONS

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LISTOF ACRONYMS AND SYMBOLS

AAPH: 2,2′-azobis (2-amidinopropane) dihydrochloride
ABTS: 2,2-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid
ANOVA: One way analysis of variance
BSLA: Brine shrimp lethality assay
℃: degrees Celsius
C_6H_3OH: Phenol
CI: Combination index
COA: Certificate of analysis
Cu+: Cuprous ion
Cu2+: Cupric ion
CUPRAC: Cupric ion reducing anti-oxidant capacity
Da: Daltons
DMSO: Dimethyl sulfoxide
DPPH: 2,2-diphenyl-1-picrylhydrazyl
EC_{50}: Half maximal effective concentration
ESI: Electrospray ionisation
FDA: Food and drug administration
Fe2+: Ferrous ion
Fe3+: Ferrous ion
FIC: Fractional inhibitory concentration
ΣFIC index: Sum of fractional inhibitory index
FRAP: Ferric iron reducing anti-oxidant power
g: gram
GC-MS: Gas chromatography couple with mass spectrometry
GRAS: Generally recognised as safe
hrs.: hours
IC_{50}: Half maximal inhibitory concentration
INT: ρ-iodonitrotetrazolium chloride
L: litre
LC_{50}: Half maximal lethal concentration
m/z: mass to charge ratio
mg: milligram
MIC: Minimum inhibitory concentration
min: minutes
mL: millilitre
mm: millimetre
mg/mL: milligram per millilitre
µg/mL: microgram per millilitre
µL/mL: microliters per millilitre
OH: Hydroxyl group
ORAC: Oxygen radical scavenging capacity
PDA: Photodiode array
PLS: Partial least square
Q2: Model predictability
***r*:** Pearson correlation
R2: Model fit
s: seconds
TEAC: Trolox equivalent anti-oxidant capacity
UPLC-MS: Ultra-performance liquid chromatography coupled with mass spectrometry
USP: United states pharmacopoeia
V: voltage
α: alpha
β: beta
γ: gamma
λ: lambda
ρ: Rho

CHAPTER 1

General introduction and literature review

1.1 Introduction

The use of spices and herbs dates back to long before recorded history, when human ancestors first added strongly-flavoured leaves to their cooking pots. Ancient hunters and gatherers experimented with leaves, roots, flowers and seeds, and accidentally discovered that these plant parts can enhance the taste of food. The knowledge gathered on the use of spices and herbs was later passed down from one generation to the other. Today the use of spices and herbs for culinary preparations has developed into a large industry as it is common practice to add herbs and spices to food to impart colour and enhance the taste and aroma.

The role of culinary herbs and spices in the safety and preservation of food has become increasingly important as many studies are now focusing on the use of these plant parts to prevent lipid oxidation and spoilage by micro-organisms, taking advantage of their anti-oxidant and antimicrobial properties (Shahidi and Ambigaipalan, 2015; Gottardi *et al*., 2016; García–Díez *et al*., 2017; Van Hecke *et al*., 2017; Pop *et al*., 2019). Furthermore, spices and herbs are generally regarded as safe (GRAS) by the USA, Food and Drug Administration (FDA), and recommended to be used as food additives to replace synthetic agents because of the undesirable health problems that may arise from the use of synthetic chemicals. Other beneficial effects of adding spices and herbs in food preparations is that they stimulate the secretion of saliva, promote digestion and absorption of dietary fats, and reduce nausea and vomiting (Marx *et al*., 2013; Jiang, 2019).

However, spices and herbs are more useful than just for culinary purposes. In ancient times spices and herbs were used as folk medicine to promote health, well before modern day science

focused on their bio-active properties. For example, culinary herbs and spices were used back in the time of Hippocrates in Ancient Greece to ease digestion and treat digestive disorders (Opara and Chohan, 2021). Furthermore, many cultural practices such as the Chinese and Indian Ayurveda systems of medicine still rely on the use of these medicinal plants to promote health and prevent illnesses and diseases. For example, a herbal mixture containing equal parts of *Piper longum* L (long pepper), *Piper nigrum* L (black pepper) fruits, and *Z. officinale* (ginger) root powder is often used in many Ayurvedic medicines commonly prescribed for treatments of gastric and abdominal disorders, colic, dysentery and other intestinal infections, coughs, asthma, bronchitis, pyrexia, and insomnia (Kaushik *et al*., 2018).

A renewed interest in the health benefits of spices and herbs has emerged, and many recent scientific studies supports the anecdotal health benefits of spices and herbs first explored by our ancestors (Jiang, 2019; Singh *et al*., 2020; Chaudhari *et al*., 2021). Some of these health benefits include lowering of blood pressure, lowering the risk of cardiovascular disease and stroke (Driscoll *et al*., 2019), control of blood sugar and cholesterol levels (Bi *et al*., 2017; Jiang, 2019), anticancer (Butt *et al*., 2013; Prasad and Tyagi, 2015), anti-inflammatory effects (Garnier and Shahidi, 2021), antibacterial, antifungal (Liu *et al*., 2017) and anti-oxidant activities (Yashin *et al*., 2017), to name a few.

The challenge lies in the fact that when tested *in vitro*, spice and herb extracts have shown promising antimicrobial and anti-oxidant effects, but to achieve comparable results in food and medicine, higher concentrations of the extracts will be required. Therefore, a combination of the extracts may provide greater efficacy at sufficiently lower concentrations and with reduced side effects.

1.2 Definition of herbs and spices

The terms spices and herbs are often used interchangeably though they are made from different parts of plants and processed in different ways. Herbs are green, leafy parts of herbaceous, non-woody plants, which are available in dried or fresh form, however, they are more efficacious and flavoursome when used fresh. Spices can be defined as aromatic or pungent vegetable substances, derived from a plant part other than the leaves; such as buds, bark, root, berries, seeds, fruits, stigmas, pods and gums. Spices are usually used in smaller amounts and are best used dry because it is believed that the drying process enhances the flavour (Peter and Babu, 2012).

1.3 Description and classification of common culinary herbs and spices

Plants used as herbs and spices can be classified based on taxonomy (botany), on the basis of season of growth which can be annual, biennial or perennial, on the basis of growth habit such as shrubs, trees, rhizomes and climbers or their geographical distribution. Spices and herbs can be conventionally classified on the basis of taste as hot, mild and aromatic spices, aromatic herbs and vegetables or on the basis of plant part used (Peter and Babu, 2012). Furthermore, spices can be classified by economic importance as major and minor spices (https://www.encyclopedia.com/plants and animals/botany/botany general/herbs and spices). The botanical species, family, and common name of plants used as spices and herbs in this study are shown in Table 1.1.

Table 1.1 Botanical classification of plants commonly used as herbs and spices

Plant species	Family name	Common name	Herb/ spice
Allium sativum L.	Alliaceae	Garlic	Spice
Anethum graveolens L.	Apiaceae	Dill	Herb
Apium graveolens L.	Apiaceae	Celery	Herb
Coriandrum sativum L.	Apiaceae	Coriander	herb/spice
Cymbopogon citratus (hort. ex DC.) Stapf.	Poaceae	Lemongrass	Herb
Cinnamomum zeylanicum Blume	Lauraceae	Cinnamon	Spice
Laurus nobilis L.	Lauraceae	Bay laurel	Herb
Melissa officinalis L.	Lamiaceae	Lemon balm	Herb
Mentha piperita L.	Lamiaceae	Peppermint	Herb
Ocimum basilicum L.	Lamiaceae	Basil	Herb
Origanum marjorana L.	Lamiaceae	Marjoram	Herb
Petroselinum crispum L.	Apiaceae	Parsley	Herb
*Rosmarinus officinalis** L.	Lamiaceae	Rosemary	Herb
Salvia officinalis L.	Lamiaceae	Sage	Herb
Syzygium aromaticum (L.) Merr. & L.M Perry	Myrtaceae	Clove	Spice
Thymus vulgaris L.	Lamiaceae	Thyme	Herb
Zingiber officinale Roscoe	Zingiberaceae	Ginger	Spice

*Although rosemary has recently been reclassified as *Salvia rosmarinus*, the previous name *Rosmarinus officinalis* will be used for ease of reference

1.4 Bio-active constituents in herbs and spices

Spices and herbs provide a wide range of bio-active phytochemicals. Phytochemicals are derived from primary or secondary metabolites depending on their role in plant metabolism. Secondary metabolites are produced by plants as defence mechanisms against predation by micro-organisms, insects, and herbivores. Examples of these secondary metabolites include flavonoids, alkaloids, phenolic acids, essential oils, terpenoids, saponins, tannins, steroids, and glycosides among others. These plant secondary metabolites are widely recognized for their noticeable potential to regulate and modulate human health and to treat diseases. Many of functional properties of herbs and spices are associated with the presence, type and concentration of phenolic compounds. Phenolics are hydroxyl group (–OH) containing class of chemicals where the (–OH) group is bonded directly to an aromatic hydrocarbon group. Phenol (C_6H_5OH) is considered the simplest class of this group of natural compounds.

The predominant class of polyphenols in herbs and spices are the phenolic acids and flavonoids (mainly flavones and flavonols). Other groups of polyphenols are present in spices and herbs such as furanocoumarins in *P. crispum*, hydroxyphenylpropenes in *Z. officinale*, curcuminoids in *Curcuma longa* L (turmeric), and hydroxycoumarins in *Cinnamomum aromaticum* Nees (Chinese cinnamon) (Shan *et al*., 2005; Pérez–Jiménez *et al*., 2010). These bio-active compounds are often identified using chromatographic techniques such as liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) applicable for the extracts and essential oils respectively. Table 1.2 provides a summary of the pharmacological properties of the major constituents of spices and herbs used in this study.

Table 1.2 Chemical composition of herbs and spices and their related biological activities

Scientific name	Bio-active constituents	Biological activities	References
Allium sativum	Allicin, diallyl sulfide, diallyl disulfide, diallyl trisulfide, allyl isothiocyanate, s-allyl cysteine	Antimicrobial, anti-oxidant, anti-inflammatory, immunoregulatory, antidiabetic, anticancer, cardioprotective, hepatoprotective effect	Batiha *et al*., 2020
Anethum graveolens	Quercetin, kaempferol, myricetin, catechins, isorhamnetin, carvone, limonene	Antibacterial, antifungal, anti-oxidant, anti-inflammatory, antiulcer, mucosal protective effect, analgesic effect	Altameme *et al*., 2017
Apium graveolens	Flavonoids, terpenes, phenols, anhydrides, furocoumarins	Antibacterial, antifungal, anti-oxidant, anti-inflammatory, antidiabetic, hepatoprotective, anticancer, antiplatelet activity, antispasmolytic activity	Kooti and Daraei, 2017; Khairullah *et al*., 2021
Cinnamomum zeylanicum	Eugenol, limonene, terpineol, catechins, proanthocyanidins, tannins, linalool, safrole, pinene, methyleugenol, benzaldehyde	Anti-inflammatory, antimicrobial, anti-oxidant, antitumor, cardiovascular, cholesterol and lipid lowering properties, and hypoglycaemic properties	Gruenwald *et al*. 2010; Rao and Gan 2014; Gülçin *et al*., 2019; Kumar *et al*., 2019

Scientific name	Bio-active constituents	Biological activities	References
Coriandrum sativum	Linalool, borneol, geraniol, rutin terpineol, cumene, pinene, terpinene, quercetin, kaempferol, caffeic acid, ferulic acid, n-coumaric acid, and vanillic acids, tocopherols	Anti-oxidant, anthelmintic, antibacterial, antimutagenic, diuretic, hypnotic, sedative, anticonvulsant effect	Nadeem *et al*., 2013; Laribi *et al*., 2015
Cymbopogon citratus	Flavonoids, phenols, alkaloids, saponins, tannins, essential oil	Antimicrobial, anti-oxidant, antinociceptive, antidiabetic, anti-inflammatory, anticarcinogenic, cardioprotective effect	Haque *et al*., 2018
Laurus nobilis	Terpenoids, anthocyanin, glycosides essential oil	Anti-oxidant, antibacterial, antiviral, antimutagenic anti-inflammatory effect	Taban *et al*., 2018; Mssillou *et al*., 2020
Melissa officinalis	alkaloids, tannins, flavonoids, saponins, phenolic compounds, essential oil	Anti-angiogenic, anti-oxidant, antimicrobial, anticancer, antiviral, anti-anxiolytic, antitumor, anti-Alzheimer, antidiabetic, anti-inflammatory, antidepressant	Sharifi *et al*., 2021
Mentha piperita	Menthol, menthone, limonene, luteolin, hesperidin, isomenthone, caffeic acid, rutin, carotenes, apigenin, tocopherols,	Anti-oxidant, antimicrobial, anti-inflammatory, biopesticidal, larvicidal, anticancer, radioprotective	Eftekhari, 2021; Mahendran and Rahman, 2021

Scientific name	Bio-active constituents	Biological activities	References
	rosmarinic acid, chlorogenic acid	effect, genotoxicity, and antidiabetic activity	
Ocimum basilicum	Apigenin, catechins, quercetin, rutin, kaempferol, anthocyanins, eugenol, limonene, terpinene, carvacrol, geraniol, menthol, safrole, tannins, ursolic acid, p-coumaric acid, rosmarinic acids	Antimicrobial, anti-oxidant, anti-cancer activity, radioprotective activity, antimicrobial activity, anti-inflammatory effects, immunomodulatory, anti-diabetic activity, antipyretic activity	Miraj and Kani, 2016; Shahrajabian *et al*., 2020
Origanum marjorana	Limonene, pinene, terpinene, apigenin, ferulic acid, sinapinic acid, caffeic acid, syringic acid, rosmarinic acid, vanillin acid, 4-hydroxybenzoic acid, and *p*-cymene	Antibacterial, antifungal, anti-oxidant, antiparasitic, nephrotoxicity protective properties, hepatoprotective , analgesic, anti-inflammatory, antimutagenic, anticancer effect	Bouyahya *et al*., 2021
Petroselinum crispum	Apigenin, luteolin, kaempferol, myricetin, quercetin, caffeic acid	Antimicrobial, anti-oxidant, antiparasitic, spasmolytic anti-inflammatory, antidiabetic, effects on gastrointestinal tract, diuretic	Silva *et al*., 2021

Scientific name	Bio-active constituents	Biological activities	References
Rosmarinus officinalis	Rosmarinic acid, camphor, caffeic acid, ursolic acid, betulinic acid, carnosic acid, carnosol, oleanolic acid, betulinic acid	Antimicrobial, anti-oxidant, anti-ageing, analgesic, anticancer effect, improve memory, improve digestion, stimulate blood flow, expectorant, diuretic, antispasmodic	Andrade *et al*., 2018; Aziz *et al*., 2021
Salvia officinalis	Geraniol, pinene, limonene, carnosol, saponin, catechins, apigenin, luteolin, rosmarinic acid, carnosic acid, vanillic acid, caffeic acids	Anti-inflammatory, antibacterial, antiviral, anti-oxidant, antimutagenic, anticancer, antidiabetic effect	Miraj and Kani, 2016; Poulios *et al*., 2020
Syzygium aromaticum	Eugenol, isoeugenol, acetyleugenol, sesquiterpene, pinene, vanillic acid, gallic acid, flavonoids, phenolic acids	Antimicrobial, anti-oxidant, antiseptic, anti-inflammatory, anticancer, antidepressant effect	Devkota and Adhikari, 2020
Thymus vulgaris	Thymol, carvacrol, β-myrcene, ρ-cymene, α-pinene, γ-terpinene, camphor, camphene, β-caryophellene	Antimicrobial, anti-oxidant, anti-inflammatory, anticancer effect	Miraj and Kani, 2016; Silva *et al*., 2021
Zingiber Officinale	Gingerol, turmeric, paradol, geraniol, geranial, borneol, linalool, camphene, zingerol, zingiberene	Anti-oxidant, anti-inflammatory, antimicrobial, antidiabetic, anticancer, antiemetic, neuroprotective effect	Mao *et al*. 2019

1.4.1 Flavones, flavonoids and flavonols

In their study, Shan *et al*. (2005), measured the phenolic contents in herbs and spices and found that *S. aromaticum* had the highest quantities of flavonoids (366.50 mg/100 g), followed by *A. graveolens* (241.20 mg/100 g), and *C. sativum* (167.20 mg/100 g) from the selection of herbs and spices studied.

The flavonoid content of *R. officinalis*, *M. piperita*, *O. basilicum*, and *S. officinalis* was shown to be 37.80, 23.20, 21.00 and 20.50 mg/100 g respectively. Furthermore, it was revealed that herbal plants such as *P. crispum* , *R. officinalis*, *S. officinalis*, and *T. vulgaris* were important sources of flavonoids, which were related to the anti-oxidant activity of these plants (Embuscado, 2015).

Wojdylo and colleagues assessed the phenolic compounds in 32 selected medicinal herbs and found that the most predominant flavonoids were quercetin, luteolin and apigenin (Wojdylo, *et al*., 2007). Liu *et al*. (2008), studied the flavonoid content in food sources. Their results indicated that *Cuminum cyminum* L (cumin) and *M. piperita* were good sources of flavanones, whilst *P. crispum* and *T. vulgaris* contained flavones. Shan *et al*. (2007), reviewed the flavonoid content of selected foods. It was demonstrated that *S. aromaticum* had the highest flavonoid content (366.50 mg/100 g), followed by *Anethum graveolens* (241.20 mg/100 g), *Carum carvi* L (caraway) (171.90 mg/100 g), *C. sativum* (167.20 mg/100 g), *Origanum vulgare* L (oregano) (51.30 mg/100 g), *R. officinalis* (37.80 mg/100 g), *M. piperita* (23.20 mg/100 g), *O. basilicum* (21.00 mg/100 g), and *S. officinalis* (20.50 mg/100 g).

1.4.2 Phenolic acids

Spices such as *R. officinalis*, *S. officinalis*, *T. vulgaris*, and *O. vulgare* have a high content of rosmarinic acid (Benedec *et al*., 2015). Zheng and Wang (2001), in their study found that

rosmarinic acid was the most common compound, with high contents found in *S. officinalis*, *O. vulgare*, *T. vulgaris* and *R. officinalis* (at 117.80, 124.80–54.6, and 91.80 mg/100 g of fresh weight respectively). The phenolic content of 26 common spice extracts were evaluated by Shan and co-workers (2005). In their study, major phenolic compounds were identified as rosmarinic acid, which was found in *M. piperita*, *O. vulgare*, *R. officinalis*, *S. officinalis*, and *T. vulgaris*. Vallverdú-Queralt *et al*. (2014), studied the phenolic profile of some commonly used culinary herbs and spices. They found that rosmarinic acid was dominant in *R. officinalis*, *T. vulgaris*, and *O. vulgare*, at concentrations of 52.02, 84.04 and 156.90 µg/mL respectively.

1.4.3 Essential oils

Essential oils from medicinal and aromatic herbs and spices are oily substances containing volatile compounds obtained from different parts of the plant. Each essential oil is composed of different compounds. The main group of compounds include terpenes and terpenoids, aromatic and aliphatic constituents. Some of the compounds with proven biological activities include cinnamaldehyde, menthol, eugenol, thymol and carvacrol amongst others. Thymol, carvacrol, eugenol, and cinnamaldehyde are phenolic compounds found in the members of the genus *Thymus* and *Origanum*, or in *O. basilicum* and *C. zeylanicum* respectively. These compounds have shown to possess antibacterial and antifungal activities (Kalemba and Kunicka, 2003; Burt, 2004; Cordery *et al*., 2018), anti-oxidant effects (Freitas and Cattelan, 2018), anti-angiogenic, antitumoral potential, and anti-inflammatory and wound healing properties, anaesthetic, and analgesic effects (Jungbauer and Medjakovic, 2012; Nieto, 2017; Haro-González *et al*., 2021).

1.5 Anti-oxidant properties of culinary herbs and spices

Anti-oxidants act as radical scavengers and thus inhibit lipid peroxidation and other free radical mediated processes, protecting the human body from several diseases attributed to the reactions

of radicals. It has been demonstrated that the anti-oxidants from spices and herbs could play a role in preventing cancer, heart diseases, inflammation, and other pathological conditions responsible for the early stages of multiple pathologies. Furthermore, anti-oxidants are added to food to prevent deterioration through the oxidative processes (Vasanthi and Parameswari 2010; Jungbauer and Medjakovic, 2012; Huang *et al*., 2018).

The anti-oxidant activity of culinary herbs and spices has been well reported in several studies (Opara and Chohan, 2014; Embuscado, 2015; Yashin *et al*., 2017). Spices and herbs such as *S. aromaticum*, *M. piperita*, *C. zeylanicum*, *T.* vulgaris, *S. officinalis*, and *R. officinalis.* to name a few contain high anti-oxidant levels compared to other spices and herbs, with mean anti-oxidant values ranging from 44 to 277 mmol/100 g determined using ferric anti-oxidant power (FRAP) assay (Carlsen *et al.,* 2010).

1.5.1 Methods applied to determine the anti-oxidant properties of spices and herbs

Various *in vitro* assays have been developed to determine the anti-oxidant capacity of culinary herbs and spices. The reported methods include 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid (ABTS), ferric iron reducing anti-oxidant power (FRAP), cupric ion reducing anti-oxidant capacity (CUPRAC), and oxygen radical scavenging capacity (ORAC). The DPPH and ABTS methods are based on diverse strategies such as to evaluate the consumption of stable free radicals by anti-oxidants. The FRAP and CUPRAC assays measure the capacity of anti-oxidants to reduce ferric or cupric ions respectively, whereas the ORAC assay measures the ability of an anti-oxidant to protect a target molecule exposed to a free radical source.

These chemical-based methods are widely used because they are less costly, rapid, and simple to conduct. It has been demonstrated that a great variation exists between the methods, whereby different protocols for determining and interpreting the anti-oxidant activity of analysed

samples are followed. Authors often express the anti-oxidant activity differently, with some authors using Trolox or Ascorbic acid equivalent whilst others expressed the activity as either percentage inhibition or as a concentration (mg/mL or µg/mL) per 100 g of sample analysed.

1.5.1.1 Free radical 2,2 diphenyl-1-picrylhydrazyl (DPPH) scavenging assay

The DPPH radical cation is a stable and coloured free radical that have been widely used to evaluate the anti-oxidant activity of spices and herbs. The DPPH assay is based on donating electrons from the anti-oxidants in order to neutralise the DPPH free radical. The reaction is accompanied by a change in colour (from deep purple to pale yellow) measured at 517 nm, and the discolouration acts as an indicator of anti-oxidant activity. Anti-oxidant activity by the DPPH neutralisation method is often reported as an EC_{50}, which is the concentration of the anti-oxidant necessary to reduce the initial concentration of DPPH by 50%. The lower EC_{50} values indicate stronger anti-oxidant effects (Foti, 2015). Other forms of reporting radical scavenging capacity is percentage inhibition, whereby a higher percentage DPPH radical inhibition means the sample has higher anti-oxidant activity.

1.5.1.2 Free radical 2,2-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid (ABTS)

In this assay, the ABTS cation is generated from oxidation of ABTS using the oxidising agent, potassium persulphate ($K_2S_2O_8$). The ABTS radical is a stable free radical which is soluble in water and organic solvents and can therefore determine the anti-oxidant capacity of both hydrophilic and lipophilic compounds. The assay measures the relative ability of the anti-oxidant to scavenge ABTS radical. The reduction of a deep blue-green radical by hydrogen donating anti-oxidant is measured at 734 nm. The method is usually expressed as Trolox equivalent anti-oxidant capacity (TEAC), however, in some studies the anti-oxidant activity is reported as EC_{50} , with values expressed as either µg/mL or mg/mL. The method is rapid and simple and can be used over a wide range of pH values, hence it is used in numerous studies.

1.5.1.3 Ferric anti-oxidant reduction power (FRAP) assay

The FRAP mechanism involves transfer of electrons instead of hydrogen atoms. The method measures the reduction of ferric ions (Fe^{3+}) to intensely blue ferrous complex (Fe^{2+}) by the anti-oxidant. The reaction takes place under acidic pH conditions (pH of 3.6). The anti-oxidant activity is measured by an increase in absorbance at a single wavelength of 593 nm. The results are usually expressed as micromolar equivalents of Fe^{2+} or in relation to standard anti-oxidant. The FRAP method is simple, fast and cost effective.

1.5.1.4 Cupric ion reducing anti-oxidant capacity (CUPRAC) assay

The CUPRAC assay measures the anti-oxidant activity based on the reduction of cupric (Cu^{2+}) to cuprous (Cu^{+}) ion in the presence of a chromogenic oxidising agent, neocuproine (Nc; 2,9-dimethyl-1,10-phenanthroline). The copper (II)-neocuproine is able to oxidise the anti-oxidant, and in the process gets reduced to a chelating complex of copper (I)-neocuproine resulting in the change in colour of the reaction mixture from light blue to yellow-orange measurable at 450 nm (Özyürek *et al*., 2011). The degree of colour change correlates with the amount of anti-oxidant present in the sample (Apak *et al*., 2007).

1.5.1.5 Oxygen radical scavenging capacity (ORAC) assay

The ORAC method measures hydrogen atom donating ability of anti-oxidants. The assay uses the ability of 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) to form peroxyl radicals when heated in the presence of sufficient oxygen. The peroxyl radical produced by a generator reacts with a fluorescent probe resulting in the loss of fluorescence, which is recorded using a fluorometer. In this assay, the peroxyl radical scavenging capacity of the anti-oxidant is evaluated. A set of fluorescence decay curves are constructed in the absence or presence of anti-oxidants, and the net integrated area under the decay curves (area gain in the presence of anti-oxidants compared to that of a blank run without anti-oxidants) is calculated as an indicator

of the peroxyl radical scavenging capacity. A standard anti-oxidant, usually Trolox, is used as reference, and ORAC values of the tested anti-oxidants are reported as Trolox equivalents.

1.5.2 Anti-oxidant activity of crude extracts of culinary herbs and spices

Numerous *in vitro* studies indicated that crude extracts from culinary herbs and spices have anti-oxidant activity (Table 1.3). Spices and herbs such as *S. aromaticum*, *M. piperita*, *C. zeylanicum*, *T. vulgaris*, *S. officinalis*, and *R. officinalis* to name a few, were reported to be strong anti-oxidants due to their high levels of phenolic compounds (Shahidi and Ambigaipalan, 2015; Ulewicz-Magulska, 2019). Mariutti *et al*. (2008), studied the free radical scavenging activity of ethanolic extracts from some common culinary herbs and spices using DPPH and ABTS assays. Based on their results, the *S. officinalis* extract showed the highest DPPH radical scavenging effect (EC_{50} value of 1.65 mg/mL) followed by *L. nobilis* and *R. officinalis* extracts at EC_{50} values of 3.85 and 3.86 mg/mL, respectively. The *C. sativum* extract showed the highest ABTS radical scavenging effect at TEAC value of 2.21 Mm/g of spice, whilst the *R. officinalis* and *S. officinalis* presented TEAC values of 89 and 202 respectively.

Lagouri and Alexandri (2013), studied the anti-oxidant activity of *R. officinalis* and *Origanum dictamus* L (dittany of Crete) methanol and aqueous extracts using DPPH and FRAP assays. The methanol extracts showed higher radical scavenging activity than the aqueous extracts. *Rosmarinus officinalis* was superior in activity to *O. dictamus*, with mean EC_{50} values of 0.26 and 1.04 mg/mL for the methanol and aqueous extracts, respectively. However, *R. officinalis* aqueous extract showed higher ferric reducing anti-oxidant activity than the methanol extract (EC_{50} values of 0.04 and 0.11 mg/mL, respectively).

The anti-oxidant activity of *Apium graveolens* extracts (methanol, ethanol, hexane, methylated spirit) was evaluated in a study by Ud Din *et al*. (2015), using DPPH, ABTS, and FRAP assays. With the DPPH assay, the anti-oxidant activity was highest in the methanol fractions (IC_{50}

values of 1.41± 0.27), followed by ethanol extract (IC_{50} value of 1.09± 0.26), and methylated spirit extract (IC_{50} value of 0.92± 0.18) when compared to the standard trolox and quercetin (IC_{50} values of 1.05± 0.05 and 0.05 respectively). The data regarding the ABTS assay revealed that ethanol extract had highest scavenging activity (IC_{50} value of 0.71± 0.15) followed by methanol extract (IC_{50} value of 0.49± 0.06), hexane extract (IC_{50} value of 0.47± 0.07), and methylated spirit (IC_{50} value of 0.42± 0.09). Similarly, the standard Trolox and quercetin had IC_{50} values of 1.08± 0.14 and 0.30 respectively. The IC_{50} values for the FRAP assay ranged from 8.64–12.48. Methanol extract exhibited the highest anti-oxidant activity (IC_{50} value of 12.48± 1.06), followed by hexane extract (IC_{50} value of 10.45± 0.83), and methylated spirit (IC_{50} value of 10.40± 0.74).

Chan and his co-workers evaluated the activity of aqueous extracts of 15 anti-oxidant rich spices using the DPPH and FRAP assays. The EC_{50} values ranged from 0.25–19.94 mg/mL for DPPH assay, and 0.11–15.87 mg/mL for the FRAP assay. The *C. zeylanicum* extract showed the highest radical scavenging activity (EC_{50} value of 0.25 mg/mL), whilst *S. aromaticum* was the strongest ferric iron reducer at EC_{50} value of 0.11 mg/mL (Chan *et al*., 2012). Kim *et al*. (2011), reported DPPH radical scavenging activity of *S. aromaticum* hot water extract to be highest (84.2%) when compared to the positive control, followed by *T. vulgaris* and *R. officinalis* at 70.8% and 57.0% respectively.

The DPPH radical scavenging effects of methanol extracts prepared from six plant species (*M. piperita*, *T. vulgaris*, *M. officinalis*, *O. basilicum*, *R. officinalis* and *S. officinalis*) were evaluated in the study by Albayrak *et al*. (2013). The highest radical scavenging activity was demonstrated by *S. officinalis, R. officinalis, and M. piperita* methanol extracts (IC_{50} value of 15.04, 15.15 and 17.91 µg/mL, respectively). Furthermore, *M. officinalis*, *T. vulgaris*, and *O. basilicum* methanol extracts demonstrated DPPH radical scavenging activity at IC_{50} values of 20.16, 21.91 and 41.80, respectively.

Another study was done to compare the DPPH and ABTS radicals scavenging activity of methanol extracts of 28 commercially available spices and herbs. The *S. aromaticum* extract showed higher DPPH radical scavenging activity (94.9 %), followed by *O. basilicum* (94.6%), *O vulgare* (94.5%), and *C. zeylanicum* (92.5%). Furthermore, *S. aromaticum* extract had highest TEAC value (52.50) than other extracts, followed by *O. basilicum* and *O vulgare* (52.30), and *C. zeylanicum* (51.20) (Slowianek and Leszczyńska, 2016).

Khadhri *et al*. (2019), studied the anti-oxidant activity of *O. marjorana* crude ethanol extracts and neat essential oil using various anti-oxidant assays (DPPH, iron chelating, reducing power). The essential oil was more active compared to the methanol extract with the DPPH scavenging activity ranging from 1.20–2.10 mg/mL, iron chelating activity of 23.00–72.00 mg/mL, and iron reducing power of 0.40–1.40 mg/mL. For the methanol extract, the activity ranged from 13.01–22.05 mg/mL for DPPH assay, 67.20–133.00 mg/mL for iron chelating assay, and iron reducing power of 5.60–7.20 mg/mL. Roby *et al*. (2013), assessed the anti-oxidant activity of *T. vulgaris*, *S. officinalis*, and *O. marjorana* extracts (methanol, ethanol, diethyl ether, and hexane) using DPPH assay. According to this study, the EC_{50} values were in the order of 1.10–2.20 µg/mL for *T. vulgaris* extracts, 1.20–2.70 µg/mL for *S. officinalis* extracts, and 1.30–3.50 µg/mL for *O. marjorana* extracts.

1.5.3 Anti-oxidant activity of individual neat essential oils of culinary herbs and spices

El-baroty and colleagues assessed the anti-oxidant activity of *C. zeylanicum* and *Z. officinale* essential oils in comparison to synthetic anti-oxidants: butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and α-Tocopherol, using DPPH and β-carotene bleaching assays. The *C. zeylanicum* oil exhibited higher DPPH radical scavenging activity with IC_{50} value of 13.1 µg/mL compared to the values of 12.2 , 13.1 and 14.4 µg/mL for BHT, BHA and α-Tocopherol, respectively.

Table 1.3 Common culinary herbs and spices with documented anti-oxidant activities

Spice/herb	Plant sample	Justification for inclusion in this study	References
Allium sativum	Ethanol	DPPH and ABTS radical scavenger at EC_{50} value of 41.00 ± 1.00 (g spice / Kg DPPH), and 8.50 ± 0.20 (mM /g spice)	Mariutti *et al*., 2008; Choi *et al*., 2014; Jang *et al*., 2018
	Aqueous, chloroform	Demonstrated DPPH and ABTS radical scavenging activities at 99.56–13.44%	
Anethum graveolens	Methanol, hexane, ethanol, dichloromethane	Demonstrated DPPH, hydroxyl, nitric oxide and superoxide radical scavenging activity at IC_{50} values ranging from 0.24–1.43 mg/ mL	Mariutti *et al*., 2008; Peerakam *et al*., 2014; Kaur *et al*., 2018
	Essential oil	Demonstrated DPPH and ABTS radical scavenging activities and FRAP reducing power at 52.25, 1.59, 0.55 mg/mL Trolox equivalent respectively	
Apium graveolens	Methanol, ethanol, hexane	DPPH and ABTS radical scavengers (IC_{50} values ranging from 0.47–1.41 TEAC, and FRAP values ranging from 8.64–12.48 mmol/L	Hassanen *et al*., 2015; Ud Din *et al*., 2015
	Essential oil	Demonstrated DPPH radical scavenging activity at 56.7% for the seed and 69.3% for the leaves	
Cinnamomum zeylanicum	Methanol, aqueous	Show ability to scavenge DPPH radical at EC_{50} values of 0.25 mg/mL	El-baroty *et al*., 2010; Wang *et al*., 2010; Kim *et al*., 2011; Chan *et al*., 2012; Sontakke *et al*., 2019
	Essential oil	Demonstrated DPPH, hydrogen peroxide and nitric oxide scavenging activities at 96.0%, 30.7% and 15.2%, respectively. ABTS radical scavenger (EC_{50} value of 12 µg/mL)	
Coriandrum sativum	Ethanol	Demonstrated radical scavenging activity at EC_{50} values value of 235.00 ± 5.00 (g spice / Kg DPPH)	Mariutti *et al*., 2008; Dias *et al*., 2012

Spice/herb	Plant sample	Justification for inclusion in this study	References
Cymbopogon citratus	Essential oil	Showed ability to inhibit DPPH radical at 78.9%–89.0% and free radical scavenging effect at 44.06 mg Trolox/100 mL	Selim, 2011; Mirghani *et al*., 2012; del Carmen *et al*., 2015
Laurus nobilis	Ethanol	DPPH free radical scavenger at EC_{50} value ranging from 3.90 ± 0.20 (g spice / Kg DPPH)	Mariutti *et al*., 2008; Taban *et al*., 2018
	Essential oil	Demonstrate DPPH radical scavenging activity at IC_{50} value ranging from 0.84–3.70 µL/mL	
Melissa officinalis	Methanol	DPPH radical scavenger at IC_{50} value of 20.16 µg/mL	Albayrak *et al*., 2013; Mabrouki *et al*., 2018; Ulewicz–Magulska, and Wesolowski, 2019; Rădulescu *et al*., 2021
	Aqueous	DPPH radical scavenger at 51.6% and 48.1% for the methanol and aqueous extracts, respectively	
	Essential oil	DPPH radical scavenging activity with IC_{50} value of 14.02 µg/mL	
Mentha piperita.	Methanol	DPPH radical scavenger at IC_{50} value of 17.19 µg/mL	Albayrak, *et al*., 2013; Singh *et al*., 2015; Ulewicz-Magulska, and Wesolowski, 2019; Wu *et al*., 2019
	Aqueous	DPPH radical scavenger at 39.90%	
	Essential oil	Demonstrated 96.00% DPPH radical inhibition and FRAP reducing power (EC_{50} value of 22.7 mg/mL)	
Ocimum basilicum	Ethanol, methanol	DPPH free radical scavenger at EC_{50} values of 43.00 ± 0.20 (g spice / Kg DPPH) for the ethanol extract, and IC_{50} value of 41.80 µg/mL for the methanol extract	Mariutti *et al*., 2008; Albayrak, *et al*., 2013; Stanojevic *et al*., 2017; Sundararajan *et al*., 2018
	Essential oil	Demonstrate DPPH radical scavenging activity at IC_{50} values of 0.013 and 2.38 mg/mL	
Origanum marjorana	Aqueous, methanol, ethanol, hexane	Demonstrated DPPH radical scavenging activity at EC_{50} values ranging from 0.001–17.84 mg/mL	Mariutti *et al*., 2008; Hussain *et al*., 2011; Mossa *et al*., 2011; Roby *et al*., 2013;

Spice/herb	Plant sample	Justification for inclusion in this study	References
	diethyl ether,		Guerra-Boone *et al*., 2015; Makrane *et al*., 2018
	Essential oil	Demonstrated DPPH, OH, H_2O_2 radicals scavenging activity, ferric iron reducing power (IC_{5O} values ranging from 58.67–250 µg/mL	
Petroselinum crispum	Methanol, aqueous, hexane, dichloromethane ethyl acetate	Demonstrated DPPH radical scavenging activity with IC_{50} values of 3.31, 4.46, and 4.71 mg/mL and ferric reducing power with values ranging from 0.01 – 0.36 mmol/g	Tang *et al*., 2015; de Menezes Epifanio *et al*., 2020
Rosmarinus officinalis.	Essential oil	Demonstrated DPPH radical scavenging activity with EC_{50} values ranging from 0.52–3.20 mg/mL, and ferric iron reducing power (IC_{50} value of 0.09 mg/mL)	Mariutti *et al*., 2008; Albayrak, *et al*., 2013; Lagouri and Alexandri, 2013; Bajalan *et al*., 2017; Bouyahya *et al*., 2017
	Methanol, aqueous, ethanol	Demonstrated DPPH radical scavenging activity at EC_{50} values of ranging from 0.02-3.86 mg/mL, and ferric reducing power at EC_{50} values of 0.03- 0.11 mg/mL	
Salvia officinalis	Aqueous, ethanol, methanol	DPPH radical scavenger at EC_{50} values ranging from 0.02–1.65 mg/mL	Mariutti *et al*., 2008; Albayrak, *et al*., 2013
Syzygium aromaticum	Aqueous, essential oil, ethanol, hexane	DPPH radical scavenger with EC_{50} values of ranging from 0.13 – 0.55 µg/mL for the extract and 21.50 µg/mL for the essential oil	Chan *et al*., 2012; Gülçin *et al*., 2012; Selles *et al*., 2020; Mahmood *et al*., 2021
Thymus vulgaris	Ethanol, methanol	Demonstrated DPPH radical scavenging activity with EC_{50} value of 6.4 ± 0.3 (g spice / Kg DPPH) for the ethanol extracts, and IC_{50} value of 21.91 µg/mL for the methanol extracts	Mariutti *et al*., 2008; Albayrak, *et al*., 2013,

Spice/herb	Plant sample	Justification for inclusion in this study	References
	Essential oil	Demonstrated DPPH and ABTS radical scavenging activity at IC_{50} values of 149.80–192.40 µmol of TE/g, (IC_{50} = 0.30 ± 0.003 mg/mL	Aljabeili *et al*., 2018; Golkar *et al*., 2020
Zingiber officinale	Methanol	Demonstrated free radical scavenging activity with IC_{50} values of 14.00 and 67.50 µg/mL with DPPH and ABTS assays, respectively	Mariutti *et al*., 2008; Ansari *et al*., 2016

The *Z. officinalis* oil displayed lower activity (IC_{50} value of 65.5 µg/mL) compared to *C. zeylanicum* oil. For the β-carotene bleaching assay, *Z. officinalis* oil, *C. zeylanicum* oil, α-Tocopherol, BHT and BHA had activity of 66.5%, 82.3%, 75.4%, 74.4% and 81.2%, respectively (El-baroty *et al*., 2010).

The anti-oxidant activity of *A. graveolens* essential oil was studied by Peerakam *et al*. (2014), using DPPH, ABTS and FRAP assays. The oil exhibited antioxidant activities on DPPH (TEAC value of 52.54 mg/mL), ABTS (TEAC value of 1.59 mg/mL) and FRAP assay (TEAC value of 0.55 mg/mL). Wang *et al*. (2010), screened 45 commonly used essential oils and their major components for anti-oxidant activity using ABTS radical scavenging assay. Both the *C. zeylanicum* and *S. aromaticum* essential oils demonstrated strongest radical scavenging activity compared to the other oils with EC_{50} values of 12.00 µg/mL and 10.00 µg/mL respectively.

The anti-oxidant activity of *L. nobilis* essential oils obtained through four different extraction methods (hydrodistillation, hydro-steam distillation, microwave-assisted hydrodistillation and Ohmic-assisted hydrodistillation) were evaluated using DPPH assay. The IC_{50} values of the oils ranged from 0.84–3.70 µl/mL (Taban *et al*., 2018). Saab *et al*. (2012), reported on the anti-oxidant activity of *L. nobilis* essential oil. The oil exhibited DPPH radical scavenging activity (IC_{50} values ranging 53.50–66.10 µg/mL) and linoleic acid oxidation activity (IC_{50} values ranging 41.10–45.90 µg/mL).

Singh *et al*. (2015), comparatively evaluated the anti-oxidant activity of *M. piperita* essential oil and crude extracts using DPPH assay. The oil exhibited stronger radical scavenging activity (92.6%) than the extracts. The anti-oxidant activity of three *Mentha* species (*M. piperita*, *M. spicata*, and *M. gracilis)* were evaluated by Wu *et al*. (2019), using DPPH, ABTS and FRAP assays. The *M. piperita* oil displayed highest anti-oxidant activity in all the three assays (EC_{50} values of 79.29, 29.51 and 22.70 mg/mL for DPPH, ABTS and FRAP assay, respectively.

Golkar *et al*. (2018), studied the anti-oxidant activity of *O. basilicum* essential oil using the DPPH assay. The IC_{50} value for the oil was 13.21 µg/mL. Stanojevic *et al*. (2017), reported an EC_{50} value of 2.38 mg/mL for *O. basilicum* oil using DDPH assay. In their study, Bouyahya *et al*. (2017), evaluated the anti-oxidant activity of *R. officinalis* oil using DPPH and FRAP assay. The activity of the oil was better with FRAP assay compared to DPPH assay, whereby the IC_{50} values were 85.74 for FRAP assay and 523.41 µg/mL for DPPH assay.

Aljabeili *et al.* (2018), assessed *T. vulgaris* oil using DPPH and ABTS assays. The oil showed DPPH and ABTS radical scavenging activity at IC_{50} values of 149.80 and 192.40 µmol of TEAC/g, respectively. Another study was done to assess the DPPH free radical scavenging ability of *T. vulgaris* essential oil. The oil demonstrated notable activity (IC_{50} value of 0.30 mg/mL) (Golkar *et al*., 2020). In their study, Haljoui *et al*. (2016), investigated the anti-oxidant activity of *O. marjorana* leaf oil using various *in vitro* methods. The IC_{50} values were 1.66, 2.5, 12.83, and 62.66 µg/mL for the superoxide quenching activity, reducing power, β-carotene-linoleic acid, and DPPH assays respectively. Chaves *et al*. (2019), reported the IC_{50} value of 418.00 µg/mL for *O. marjorana* leaf oil using DPPH radical scavenging assay, whilst Salha *et al*. (2017) reported better DPPH radical scavenging activity at IC_{50} value of 16.83 µg/mL.

1.5.4 Anti-oxidant interactions from combinations of crude extracts of culinary herbs and spices

The anti-oxidant activity of spices and herbs has been related to the presence of phenolic compounds and flavonoids (Manda *et al*., 2010; Henning *et al*., 2011; Kim *et al*., 2011). However, based on the complexity of the extracts, it is possible that the overall anti-oxidant activity of herbs and spices is due to a synergistic effect of the total phenolic acids and other anti-oxidant components present. Therefore, combination of these highly complex extracts may

result in synergistic and /or additive anti-oxidant activities, thereby enhancing their anti-oxidant efficacy. Anti-oxidant interactions were reported in some studies as a combination index (CI), where the CI value below 1.00 was regarded as synergy and a value of 1.00 was regarded as additive. For example, Ramadan *et al.* (2017), reported anti-oxidant synergy (CI: 0.85) from combining *C. zeylanicum* with *S. aromaticum* water extracts using the DPPH assay. Mansour *et al*. (2017), also reported synergistic anti-oxidant interaction from combining *C. zeylanicum* and *S. aromaticum* methanol extracts using DPPH assay. The CI value for the combination was 0.87.

1.5.5 Anti-oxidant interactions from combinations of neat essential oils of culinary herbs and spices

The anti-oxidant efficacy from combinations of culinary herbs and spices essential oils were also evaluated in some studies. Bag and Chattopadhyay (2015), evaluated the anti-oxidant interaction from a combination of *C. sativum* and *C. cyminum* essential oils using the DPPH assay. This combination demonstrated synergy with a CI value of 0.79, whilst the combination of *Brassica nigra* L (black mustard) oil with either *C. sativum* or *C. cyminum* oils showed additive anti-oxidant activity (CI value of 1.00).

The anti-oxidant effect from combination of C. *zeylanicum* and *S. aromaticum* oils by DPPH method was evaluated in a study by García-Diez *et al*. (2017). The combination yielded a synergistic effect at CI values of 0.82. Similar observations were made in a study by Purkait *et al.* (2020), whereby the combination of *C. zeylanicum* and *S. aromaticum* oils were synergistic with CI value of 0.82. However, an additive anti-oxidant interaction (CI value of 1.00) was observed when *C. zeylanicum* or *S. aromaticum* oils were combined with *P. nigrum* oil using the DPPH method.

The use of combinations of different extracts and essential oils of spices and herbs is a promising strategy to increase the anti-oxidant efficacy of spices and herbs as applied in food and in medicine. However, there are limited studies in the literature reporting on synergistic anti-oxidant interactions from combinations of crude extracts and neat essential oils of culinary herbs and spices, hence this study was undertaken to further investigate anti-oxidant interactions resulting from various combinations of crude extracts and neat essential oils of some commonly used culinary herbs and spices.

1.6 Antimicrobial activity of culinary herbs and spices

Foodborne diseases and gastrointestinal tract infections are a major threat to human health worldwide. Bacteria such as *Bacillus cereus*, *Escherichia coli*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Shigella sonnei*, *Shigella flexneri*, and *Shigella dysenteriae* are often associated with foodborne illnesses and gastrointestinal tract infections (Bintsis, 2017). Many of these pathogens are reported to be increasingly resistant to different classes of antibiotics and disinfectants, leading to increased morbidity and mortality (Rozman *et al*., 2021).

Furthermore, these micro-organisms deteriorate the organoleptic quality of food and produce harmful toxins in food. The microbial safety of foods continues to be a major concern to consumers, food industries, and regulatory agencies throughout the world. Many food preservative strategies such as chilling, freezing, water activity reduction, nutrient restriction, acidification, fermentation, pasteurisation or use of synthetic antimicrobials have been applied traditionally to control microbial spoilage in foods (Davidson, 2012). Other technologies such as packaging in controlled atmosphere, activated films, non-thermal treatments, irradiation, and modified atmosphere packaging have been used to avoid or delay microbial growth in food. However, most of these procedures may cause loss of organoleptic properties of foods, thus reduce consumer's acceptability.

Chemical preservatives are commonly used to control food spoilage and pathogenic micro-organisms (García and Searle, 2015). However, these can also result in development of microbiological resistance (Xu *et al*., 2022). Furthermore, chemical preservatives cannot completely eliminate or delay the growth of several pathogenic and spoilage bacteria (Tajkarimi *et al*., 2010). As a consequence of microbial resistance to chemical preservatives, several studies are focusing on the application of natural products as antimicrobials, such as those from herbs and spices, as these have been proven to inhibit the growth of Gram-positive and Gram-negative bacteria, some fungi and moulds (Pavithra, 2014; Liu *et al*., 2017). Furthermore, herbs and spices are being considered as substitutes for synthetic chemical preservatives by the food industry because of their safety, and that they are better tolerated by the human body (Silva and Domingues, 2017).

1.6.1 Methods used to evaluate antimicrobial activity of culinary herbs and spices

Various *in vitro* methods can be used to evaluate the antimicrobial activity of plant extracts. The most widely used methods include the disc diffusion method, and broth or agar dilution methods. The effectiveness of these techniques were investigated by Valgas *et al*. (2007), and Seow *et al*. (2014). These studies reported that agar diffusion analysis was the most suitable procedure. However, the method is not suitable to be used for volatile substances including essential oils and non-polar compounds. Therefore, other assays such as microdilution and bioautography can be applied (Golus *et al*., 2016; Owen *et al*., 2019). The broth microdilution method was used in this study to screen crude extracts and neat essential oils of selected herbs and spices for antimicrobial activity.

1.6.1.1 Agar and broth micro dilution methods

The two methods (agar and broth micro dilution) aim to determine the minimum inhibitory concentration (MIC), which is the lowest concentration of the assayed antimicrobial agent that

under defined test conditions can inhibit the visible growth of the micro-organism being investigated. The MIC values are used to determine susceptibilities of bacteria to an antimicrobial agent. A 96-well microtiter plate format is often used, and the test micro-organism is inoculated into a liquid growth medium in the presence of different concentrations of an antimicrobial agent. Whereas, agar dilution involves the incorporation of different concentrations of the antimicrobial substance into a nutrient agar medium followed by the application of a standardized number of cells to the surface of the agar plate. In both assays, growth is assessed after incubation for a defined period of time (24 hrs) and the MIC value is recorded (Balouiri *et al*., 2016).

1.6.2 Antibacterial activity of crude extracts of culinary herbs and spices

Culinary herbs and spices such as *A. sativum*, *Anethum graveolens*, *Apium graveolens*, *C. zeylanicum*, *C. sativum*, *C. citratus*, *L. nobilis*, *M. piperita*, *M. officinalis*, *O. basilicum*, *O. marjorana*, *P. crispum*, *R. officinalis*, *S. aromaticum*, *S. officinalis*, *T. vulgaris*, and *Z. officinalis* were confirmed to possess antibacterial activity against different types of foodborne and spoilage bacteria. A summary of the antibacterial activities of some common culinary herbs and spices used in this study is given in Table 1.4.

Meriga *et al*.(2012), assessed the antibacterial activity of *A. sativum* methanol and aqueous extracts against *E. coli*, *S. aureus*, *Bacillus subtilis*, and *Klebsiella pneumoniae* . The MIC values ranged from 0.10–0.15 mg/mL, with *S. aureus* being resistant to the methanol extract. In their study, Geremew *et al*. (2015), reported the activity of six spice crude extracts (acetone, ethanol and hexane extracts) against four bacteria (*E. coli*, *S. flexneri*, *S. aureus* and *Streptococcus pneumoniae*) using the disc diffusion method. *Allium sativum* extracts showed better activity compared to other spices extracts.

Petropoulos *et al*. (2018) showed that freeze-dried fresh extract of various *A. sativum* genotypes could inhibit the growth of various bacteria including antibiotic resistant strains. The MIC values ranged from 0.04–40 mg/mL. The antibacterial effects of *A. graveolens* (dill) crude extracts were reported in several *in vitro* studies.

Kaur and Arora (2009), reported on the inhibitory effect of *Anethum graveolens* acetone and hot water extracts on *S. aureus*, *E. faecalis*, *E. coli*, *S. flexneri*, *S. typhimurium* and *Pseudomonas aeruginosa*. The acetone extract demonstrated better efficacy against *S. aureus* and *S. typhimurium* (MIC value of 5.00 mg/mL) than the hot water extract. Ud Din *et al*. (2015), investigated the effect of the various crude extracts (ethanol, methanol, hexane and methylated spirit) of *A. graveolens* on *E. coli*, *S. aureus*, *S. typhimurium*, *B. subtilis* and *P. aeruginosa*. The ethanol fraction demonstrated notable activity against *S. aureus* (MIC value of 0.12 µg/mL) and *S. typhi* (0.50 µg/mL).

The antibacterial activity of crude ethanol extracts of some common spices against *E. coli* and *S. typhimurium* were reported by Nanasombat and Lohasupthawee (2005). Among all the ethanolic extracts, *S. aromaticum* showed better activity against the test pathogens (MIC value of 2.60 mg/mL). However, the activity was lower compared to the positive control, penicillin G (MIC values of 0.5–500 units/mL). Teshale *et al*. (2013), demonstrated the activity of methanol and ethyl acetate extracts of *C. sativum* on *S. aureus* and *S. typhimurium* with MIC values of 45.00 µg/mL and 40.00 µg/mL respectively.

The antibacterial activity of *O. basilicum* methanol extract was tested against foodborne bacteria (*Bacillus megaterium, B. cereus, B. subtilis, E. coli, L. monocytogenes, S. aureus, S. typhimurium, Shigella boydii, S. dysenteriae, and Vibrio parahaemolyticus*) The MIC values ranged from 62.50–500.00 µg/mL (Hossain *et al*., 2010).

Table 1.4 Common culinary herbs and spices with documented antibacterial activities

Spice/herb	Plant sample	Justification for inclusion in this study	References
Allium sativum	Methanol, ethanol, aqueous	Inhibited the growth of *E. coli*, *S. aureus*, *B. cereus*, *B. subtilis*, *S. typhimurium*, *Shigella* species (MIC ranging from 0.03–20.00 mg/mL) , and MIC values ranging from 0.12–0.50 µg/mL against, *S. aureus*, *S. typhimurium*	Nedorostova *et al*., 2009; Pundir *et al*., 2010; Meriga *et al*., 2012; Mikaili *et al*., 2013; Petropoulos *et al*., 2018
	Essential oil	Inhibited the growth of *S. aureus* and *E. coli* at MIC values of 0.01 and 0.53 µL/mL respectively	
Anethum graveolens	Aqueous, acetone	Demonstrated antibacterial activity against *E. faecalis*, *E. coli*, *S. aureus*, *S. typhimurium* at MIC values ranging from 5.00–25.00 mg/mL	Kaur and Arora, 2009; Isopencu and Ferdes 2012; Sharopov *et al*. 2013, Peerakam *et al*., 2014
	Essential oil	Inhibited the growth of *S. aureus* and *E. coli* at MIC values of 0.06 and 0.73 mg/mL respectively	
Apium graveolens	Methanol, ethanol, hexane, aqueous	Showed antibacterial activity towards *B. subtilis*, *E. coli*, *S. typhimurium*, *S. aureus* (MIC ranging from 0.12–1.17 µg/mL), and MIC values of 0.01–1.25 mg/mL	Edziri *et al*., 2012; Baananou *et al*., 2013; Ud Din *et al*., 2015
	Essential oil	Inhibited the growth of *B. cereus*, *E. coli*, *E. faecalis*, *S. aureus* at MIC values ranging from 5.00–40.00 µg/mL	

Spice/herb	Plant sample	Justification for inclusion in this study	References
Cinnamomum zeylanicum	Ethanol, methanol	Inhibited the growth of *E. coli*, *S. aureus*, *B. cereus* at MIC ranging from 0.63–6.25 mg/mL	Nanasombat and Wimuttigosol, 2011; Naveed *et al*., 2013, Aumeeruddy-Elalfi *et al*., 2015; Madiha *et al*., 2017; Salma *et al*., 2019.
	Essential oil	Inhibited the growth of *B. cereus*, *B. subtilis*, *E. coli*, *S. aureus*, *S. typhimurium*, *E. faecalis* (MIC ranging from 0.10–4.80 mg/mL)	
Coriandrum sativum	Methanol	Inhibited the growth of *B. subtilis*, *E. coli*, *S. aureus*, *S. typhimurium*, *S. dysenteriae* (MIC ranging from 0.05–0.50 mg/mL)	Casetti *et al*., 2012; Jastaniah, 2014
	Essential oil	Showed antibacterial activity against *S. aureus* at MIC values ranging from 0.04–0.25% v/v	
Cymbopogon citratus	Methanol, chloroform, ethanol	Inhibited the growth of *B. cereus*, *B. subtilis*, *E. coli*, *S. aureus*, *S. typhimurium*. (MIC values ranging from 0.01–2.50 mg/mL against	Bassolé *et al*., 2011; Danlami *et al*. 2011; Ewansiha *et al*., 2012 Zulfa *et al*., 2016
	Essential oil	Inhibited the growth of *E. coli*, *E. faecalis*, *S. aureus*, *S. typhimurium*, *S. dysenteriae*, *S. enterica* at MIC values ranging from 1.00–2.50 µL/mL	

Spice/herb	Plant sample	Justification for inclusion in this study	References
Laurus nobilis	Ethanol, aqueous	Inhibited the growth of *B. cereus*, *E. coli*, *S. aureus* at MIC values ranging from 0.78–3.12 mg/mL	Ceyhan *et al*., 2012; Caputo *et al*., 2017; Tomar *et al*., 2020; Nabila *et al*., 2022
	Essential oil	Inhibited the growth of *B. cereus*, *E. coli*, *S. aureus*, *S. faecalis* at MIC values ranging from 0.01–1.00 mg/mL	
Melissa officinalis	Aqueous, aqueous, ethanol	Inhibited the growth of *E. coli* and *S. aureus* at MIC values of 0.78–3.12 mg/mL	Hussain *et al*., 2011; Ceyhan *et al*., 2012; Albayrak *et al.*, 2013*;* Abdellatif *et al*., 2014
	Essential oil	Inhibited the growth of *E. coli*, *S. aureus*, *B. cereus* at MIC values of 0.48–3.00 µL/mL	
Mentha piperita	Methanol	Inhibited the growth of *S. aureus*, *E. coli* , *S. typhimurium* (MIC ranging from 0.01–0.03 mg/mL	Hussain et al., 2010; Albayrak *et al.*, 2013; Ceylan *et al.*, 2014; Mahboubi and Kazempur, 2014; Singh *et al*., 2015
	Essential oil	Inhibited the growth of *B. cereus, B. subtilis, S. aureus, S. typhimurium, E. coli* at MIC values ranging from 0.01–30 mg/mL	
Ocimum basilicum	Methanol, ethyl acetate, chloroform	Inhibited the growth of *B. cereus*, *E. coli*, *B. subtilis*, *S. aureus*, *S. typhimurium* with MIC values ranging from 0.06–0.25 mg/mL	Hossain *et al*., 2010; Beatovic *et al*., 2015

Spice/herb	Plant sample	Justification for inclusion in this study	References
	Essential oil	Showed antibacterial activity with MIC values ranging from 0.01–23.48 µg/mL for *B. cereus*, *E. coli*, *E. faecalis*, *S. typhimurium* and *L. monocytogenes*	
Origanum marjorana	Methanol, aqueous, dichloromethane	Showed antibacterial activity with MIC values ranging from 0.05–1.00 mg/mL for *E. coli*, and *S. aureus*	Samy *et al*., 2013; Hajlaoui *et al*., 2016; Al-Fatimi *et al*., 2018
	Essential oil	Inhibited the growth of *B. cereus*, *S. aureus E. faecalis*, *E. coli* , *S. typhimurium* (MIC values ranging from 0.08–1.56 mg/mL)	
Petroselinum crispum	Methanol	Inhibited the growth of *B. subtilis*, *S. aureus* at MIC value of 0.037 mg/mL	Alsaiqali *et al*., 2016; Linde *et al*., 2016
	Essential oil	Inhibited the growth of *B. cereus*, *S. aureus*, *E. coli* (MIC ranging from 0.04–1.00 mg/mL)	
Rosmarinus officinalis	Aqueous, methanol, ethanol, hexane	Inhibited the growth of *B. cereus*, *E. coli*, *E. faecalis*, *S. aureus*, *S. typhimurium* with MIC ranging from 0.78–3.12 mg/mL	Wang *et al*., 2010; Weerakkody *et al*., 2010; Ceyhan *et al*., 2012; Kozlowska, 2015

Spice/herb	Plant sample	Justification for inclusion in this study	References
	Essential oil	Inhibited the growth of *B. subtilis*, *S. aureus* and *E. coli* at MIC values ranging from 0.03–0.06% v/v	
Salvia officinalis	Essential oil	Showed antibacterial activities with MIC values in the range of 0.01–0.30 mg/mL against *E. coli* and *S. aureus*	Generalić Mekinić *et al*., 2014; Saricaoglu and Turhan, 2018; Poulios *et al*., 2020
	Ethanol	Inhibited the growth of MIC ranging from 0.30–6.20 mg/mL against *B. cereus*, *E. coli* and *S. aureus*	
Syzygium aromaticum	Aqueous, methanol	Showed antibacterial activities with MIC ranging from 31.25–250.00 µg/mL against *S. aureus* and *S. enterica*	Fei *et al*., 2011; Pandey et al., 2011; Ivanovic *et al*., 2013; Nassan *et al*., 2015; Thielmann *et al*., 2019
	Essential oil	Showed antibacterial activities with MIC values in the range of 0.40–0.80 mg/mL against *B. cereus*, *B. subtilis*, *E. coli*, *S. aureus*, *S. typhimurium*	
Thymus vulgaris	Essential oil	Inhibited the growth of *B. cereus*, *B. subtilis*, *E. coli*, *S. aureus*, *S. typhimurium* at MIC values ranging from 0.40–0.80 µL/mL	Al Maqtari *et al*., 2011; Fei *et al*., 2011; Aliakbarlu and Shameli, 2013; Vaillancourt *et al*., 2018

Spice/herb	Plant sample	Justification for inclusion in this study	References
	Methanol	Inhibited the growth of *B. cereus, E. coli, S. aureus, S. sonnei,* at MIC values ranging from 0.07–1.10 mg/mL	
Zingiber officinale	Methanol, ethanol, aqueous	Inhibited the growth of *B. cereus, B. subtilis, E. coli, S. enterica, S. typhimurium* at MIC values ranging from 0.02–20.00 µg/mL	Sivasothy *et al*., 2011; López *et al*., 2017; Yusuf *et al*., 2018; Wang *et al*., 2020
	Essential oil	Inhibited the growth of *E. coli, S. aureus, S. typhimurium* and *S. sonnei, B. cereus* (MIC values ranging from 0.25–1.00 mg/mL)	

Generalić *et al*. (2012), studied the antibacterial effects of *S. officinalis* ethanol extracts against *B. cereus, S. aureus, E. coli* and *Salmonella infatis* using broth microdilution method. The MIC values ranged between 0.02 and 0.99 mg/mL, and *B. cereus* was the most susceptible bacteria.

The antibacterial effect of *S. aromaticum* extracts (ethanol, methanol) were evaluated against some foodborne pathogens (*S. aureus, E. coli, P. aeruginosa*). Compared with ethanol extract, methanol showed better activity against the test pathogens. The MIC values for the methanol extract was reported to be 2.31 mg/mL for *E. coli*, 0.39 mg/mL for *S. aureus* and 0.01 mg/mL for *P. aeruginosa* (Pandey and Singh, 2011). Yusuf *et al*. (2018), studied the antibacterial activity of *Z. officinalis* methanol extract against five pathogens (*S. aureus, E. coli, S. typhi, P. aeruginosa, Klebsiella pneumoniae*). The MIC value was 25.00 µg/mL for all the test pathogens except *K. pneumoniae* with MIC value of 12.50 µg/mL.

1.6.3 Antibacterial activity of essential oils of culinary herbs and spices

Baananou *et al*. (2013), studied the inhibitory effect of *Apium graveolens* essential oil on *E. coli, P. aeruginosa* and *S. aureus.* The oil demonstrated a better antibacterial activity against *E.coli* (MIC value of 0.01 mg/mL) than *S. aureus* (MIC value of 0.06 mg/mL) and *P. aeruginosa* (MIC value of 0.12 mg/mL).

Delaquis *et al*. (2002), examined the antibacterial activity of neat essential oils of *C. sativum* and *A. graveolens* against some Gram-positive and Gram-negative food spoiling bacteria. The results revealed the effectiveness of the oils against *L. monocytogenes, S. aureus* and *E. coli* with MIC values ranging from 0.10–0.40 (%vol/vol). Silva *et al*. (2011), assessed the effects of *C. sativum* oil against some Gram-positive and Gram-negative bacterial strains. The results showed bactericidal activities against all the test strains except for *B. cereus* and *E. faecalis*, which were found to be resistant. The MIC values ranged between 0.10% and 1.60% v/v.

Trajano *et al*. (2010), evaluated the antibacterial activity of *C. zeylanicum* oil against food contaminating bacteria. The oil was able to inhibit the growth of all the assayed bacterial strains with MIC values ranging from 1.25–10.00 µL/mL. Furthermore, Vazirian *et al*. (2015), studied the activity of *C. zeylanicum* essential oil against some food borne bacterial pathogens (*B. cereus, E. coli, S. aureus* and *S. typhimurium*). The oil demonstrated MIC value of 0.50 µL/disc

against all the pathogens, except for *S. aureus* for which the oil was not effective at tested concentration.

Nedorostova *et al*. (2009), evaluated the antimicrobial properties of spice essential oils against food borne pathogens (*E. coli, L. monocytogenes*, *P. aeruginosa*, *S. enteritidis*, *S. aureus*). Based on their results, the *A. sativum* oil was more active against Gram-positive bacteria (MIC values of 0.01 µL/mL) than Gram-negative bacteria (MIC values ranging from 0.26–0.53 µL/mL). All the bacterial strains were susceptible to *T. vulgaris* oil (MIC values ranging from 0.02–0.26 µL/mL, whilst *O. marjorana* oil inhibited *E.coli* and *S. aureus* with MIC values of 0.26 and 0.53, respectively. Nanasombat and Wimuttigosol, (2011) studied the antimicrobial activity of essential oils of different spices against some food borne pathogens. Their results showed that *C. zeylanicum* oil inhibited *B. cereus* (MIC value of 0.50 mg/mL).

The antibacterial activity of *M. piperita* oil against various micro-organisms was reported in several studies (Bassolé *et al*., 2010; Hussain *et al*., 2010; Ceylan *et al*., 2014; Zaidi and Dahiya, 2015; Desam *et al*., 2019). Hussain *et al*. (2010), reported on the activity of *M. piperita* oil against *S. aureus* and *B. subtilis* (MIC values of 0.01 and 30 mg/mL, respectively). Ceylan *et al*. (2014), evaluated the antibacterial activity of *M. piperita* oil against *S. aureus* and *P. aeruginosa* using broth microdilution method. The MIC values ranged from 0.05–25.00 µL/mL. Furthermore, *M. piperita* oil has shown to inhibit the growth of *B. cereus* and *B. subtilis*, with MIC values of 0.25 and 0.5 µL/mL, respectively (Mahboubi and Kazempur, 2014).

According to Ayoola *et al*. (2008), *S. aromaticum* essential oil was active against Gram-positive and Gram-negative bacteria, with *S. aureus* and *E. coli* MIC values ranging from 0.23–2.40 mg/mL. Thielmann *et al*. (2019), reported on the activity of *C. zeylanicum* , *C. citratus*, *C. sativum*, *L. nobilis*, *M. piperita*, *M. officinalis*, *O. basilicum*, *O. marjorana* and *S. aromaticum*, *S. officinalis*, and *Z. officinalis* essential oils against *E. coli* and *S. aureus* . The MIC values for the oils ranged from 0.05–6.4 mg/mL. The highest inhibition was shown by *C. zeylanicum* oil (MIC value of 0.05 mg/mL).

1.6.4 Methods used to evaluate antimicrobial interactions from combinations of culinary herbs and spices

A number of different methods can be used to explain the interactions between combinations of plant extracts and their essential oils. These methods include diffusion assay, minimum inhibitory concentration assay, measuring synergy interaction by calculating the sum of fractional concentration index (ΣFICI), isobole method, and the time-kill assay.

The diffusion assay is the simplest form of determining synergistic interactions whereby each independent test sample is placed in an individual well or disc. The combination of the test samples is placed on a separate well or disc, and the inhibition zone of the combination is compared with those of the individual test samples. Synergy interaction is noted when the zone of inhibition for the combination is larger than the zone of inhibition for the individual test samples, whereas antagonism is noted when the zone of inhibition for the combination is smaller than the zone of inhibition for the individual test samples (Van Vuuren and Viljoen, 2011). Although they are simple to conduct, these assays are subject to many variables that may influence the results and should therefore be used as a qualitative guide only (Hewitt and Vincent, 2003; Van Vuuren, 2007). For essential oils, the results may be influenced by the ability of the oil to diffuse through the agar medium. This may give variable results, false negatives, or a reduction in antimicrobial activity since essential oils are hydrophobic and thus poorly diffuse through an aqueous medium (Nakatsu *et al*., 2000).

The broth microdilution method may be undertaken, where the combinations are comparatively examined by combining the individual inhibitors at selected concentrations. This arrangement of combinations formed by multiple dilutions is referred to as the "checkerboard method". Synergy can be extrapolated by comparing growth as determined by optical density readings of single entities and comparing these growth rates with that found when exposed to test substances in combination. The limitation of using this method is that it can be difficult to determine the MIC due to interference of turbid or highly coloured samples, making visual or spectrophotometric readings inaccurate (Tan *et al*., 2015).

1.6.4.1 Sum of fractional inhibitory concentration index (ΣFICI)

The use of an algebraic equation to determine synergy using the fractional inhibitory concentration (FIC), is a widely accepted means of measuring interactions by calculating the ΣFICI. The ΣFICI is expressed as the interaction of two agents where the concentration of each test agent in combination is expressed as a fraction of the concentration that would produce the same effect when used independently. The ΣFICI is interpreted as synergy if it is less than or equal to 0.5, additive effects are greater than 0.5 but less than or equal to 1.0. For indifference, the ΣFICI must be greater than 1.0 but less than or equal to 4.0 and for antagonism an ΣFICI is greater than 4.0 (Van Vuuren and Viljoen, 2011).

1.6.4.2 The isobole method

The isobole method involves the combination of two inhibitors at various ratios, whereby their interaction may vary depending on the ratio at which they are combined. The MIC value for each inhibitor is determined independently and comparatively assessed against the MIC value obtained in the ratio combination. This is expressed as a dose ratio response on an isobole graph. The adjoining line of the two axes indicates the individual doses and the isobologram can be interpreted by examining the data points of the ratios.

The classical interpretation of the isobole is where the data points fall below the 1:1 line; synergy is expressed. Antagonism is noted for data points falling above the 1:1 line, and an additive response is given when ratio points fall in the vicinity closest to or on the line (Van Vuuren and Viljoen, 2011). This method is complicated; however, it provides more accurate assessment of each agent when studied in various combinations. Furthermore, the results can be validated using mathematical equations (Boucher and Tam, 2006; Tallarida, 2006).

1.6.4.3 Time-kill method

The time-kill method provides descriptive information on the relationship between the bactericidal activity of the test sample and its concentration. The time-kill method involves exposing the inhibitor to a selected pathogen, and at selected time intervals, aliquots are sampled and serially diluted. The dilutions are plated out, incubated at optimum conditions for

the test organism, and the colony forming units (CFU) are counted and plotted logarithmically against time.

Depending on the curve of the dose response, either an additive, synergistic, or antagonistic effect is noted. Antagonism in time-kill methods may be defined as at least a100-fold increase in colony counts whereas synergism, is a 100-fold decrease in colony counts. The time-kill method has been recommended as one of the best methodologies for studying synergy. However, this method is not frequently used in plant-based studies due to the laborious nature of repetitive dilution sampling. An advantage of this method is the possibility of identifying a direct correlation between exposure time to a plant test material and the extent of pathogen death (Saiman, 2007). A cidal effect is monitored over time which is not possible with the frequently used MIC assays.

1.6.5 Antimicrobial interactions from combinations of crude extracts of culinary herbs and spices

The antibacterial interactions from combinations of crude extracts of culinary herbs and spices were evaluated in some studies. For example, Das *et al*. (2012), reported synergy from combinations of aqueous extracts of *Trigonella foenum-graecum* L (fenugreek) and *C. cyminum* against *Proteus vulgaris* (ΣFICI of 0.50), and additive against *S. aureus* (ΣFICI of 0.83), whilst the combination of *Nigella sativa* L (black cumin) and *Brassica nigra* L (mustard) were synergistic against *S. aureus* (ΣFICI of 0.42), and additive against *Salmonella enterica*, *P. vulgaris* and *Lactobacillus brevis* at ΣFICI of 1.00. The combination of *C. cyminum* and *Trachyspermum ammi* L. Sprague (ajwain) aqueous extracts were additive against *E. coli* (ΣFICI of 1.00).

Baljeet *et al*., (2015) studied the combination effect of *C. cyminum*, *A. sativum*, and *Z. officinalis* ethanol extracts against four bacterial and two fungal species. Their results showed that all the combinations were additive against all the test pathogens, and this additive effect was more pronounced against *S. typhimurium* and *Pseudomonas fluorescens* (ΣFICI of 0.61). Another study evaluated the antibacterial effect of combined ethanol extracts from five different spices (*Allium cepa* L, *Curcuma longa* L, *Capsicum annuum* L, *A. sativum*, and *Z. officinalis*) were evaluated against *S. aureus* and *Klebsiella pneumoniae.* Synergy was observed for combinations of *C. longa* with *C. annuum*, *C. longa* with *Z. officinalis* with ΣFICI

of 0.28 for both combinations, and *C. annuum* with *Z. officinalis* ethanol extracts, ΣFICI of 0.25) against *S. aureus*. Whereas the combination of *Z. officinalis* and *A. sativum* extracts were additive against *S. aureus* (Maharjan *et al*., 2019).

1.6.6 Antimicrobial interactions from combinations of neat essential oils of culinary herbs and spices

Antimicrobial synergy interactions from the combination of *M. piperita* and *O. basilicum* essential oils were reported by Bassolé and colleagues. The ΣFICI for the combination was 0.29, 0.37, and 0.36 against *E. coli*, *E. faecalis* and *S. aureus* respectively (Bassolé *et al*., 2010). However, they reported an additive effect (ΣFICI of 0.69) against *S. typhimurium*. Goñi *et al*., (2009), reported a synergistic interaction effect from the combination of *C. zeylanicum* and *S. aromaticum* essential oils against *B. cereus* (ΣFICI of 0.50). Bag and Chattopadhyay (2015), studied the antibacterial combination efficacy of essential oils of some selected culinary herbs and spices. The evaluation of different possible combinations of the oils showed that only *C. sativum* in combination with *C. cyminum* seed oil produced synergistic interactions whereby the sum of fractional inhibitory concentration for the combination (ΣFICI was < 0.50).

García–Diez *et al*., (2017) studied antimicrobial interactions from combinations of some spice essential oils against food borne and spoilage bacteria (*E. coli*, *S. aureus*, *L. monocytogenes* and *S. typhimurium*). Their results showed that the *C. zeylanicum* and *S. aromaticum* oil combination had synergistic antibacterial effects against *S. aureus* (ΣFICI of 0.45), *L. monocytogenes* (ΣFICI of 0.42), *S. typhimurium* (ΣFICI of 0.49) and *P. aeruginosa* ΣFICI of 0.50). A combination of *T. vulgaris* and *R. officinalis* essential oils resulted in a synergistic interaction (ΣFICI of 0.28) whilst the ΣFICI for the *A. sativum* and *L. nobilis* essential oil combination was 0.15 against *S. typhimurium*. The combination of *T. vulgaris* essential oil with either *A. sativum* was additive against *S. aureus* (ΣFICI of 0.75).

1.7 Optimization of synergy in polyherbal combinations

The use of combination of multiple anti-oxidant and antibacterial agents in herbal combinations can result in synergistic interactions. Synergy is obtained when two or more agents are combined to produce an effect greater than the sum of the activities of each individual agent

(Van Vuuren and Viljoen, 2011). Due to synergism, combinations of different herbal extracts may provide greater antimicrobial and anti-oxidant efficacy. It could also minimise the toxicity and adverse effects of the extracts since they would be used at lower concentrations in the combination compared to when used individually.

Producing a polyherbal combination with optimal efficacy is a challenge since one cannot just mix the extracts randomly, and many experiments are required to do so. Furthermore, when experiments are based on trial and error, without an experimental design, there is a possibility of low reproducibility, reliability, and versatility as the results are not statistically validated (Das *et al*., 2016). The use of design of experiments (DOE) makes it possible to find the best ratio of components through predictive statistical models, making it possible to determine optimal conditions for the combination. These models aim to produce optimized mixtures, with a limited number of experimental assays, thus saving time and resources. Furthermore, DOE highlights possible interactions between multiple combinations of extracts that might not be observed when using a labour intensive one single factor at a time approach, whereby synergism could otherwise be overlooked.

The DOE provides an insight regarding individual factors (in this case the extracts), or a combination of factors and the effect they may have on the response. By exploring different combinations of high, low and midpoints of all the relevant factors, it is possible to establish their interactions and how these can affect the experimental results (Eriksson *et al*., 2008). Although it has been known since the early 1900s, DOE has found a wide application in pharmaceutical industry (Dennison *et al*., 2016; Beg *et al*., 2019). The DOE has become more accessible to researchers through the advent of user-friendly software options such as MODDE. For antimicrobial applications in natural products, chemometrics has become a useful tool to predict structure activity relationships (Kasote *et al*., 2014; Maree *et al*., 2014; Orchard *et al*., 2017; Leigh-de Rapper *et al*. 2021), however the DOE approach is lacking. Taking into consideration the advantages of using DOE, a herbal formulation can be made based on the outcomes calculated for optimum anti-oxidant and antimicrobial activity.

1.8 Polyherbal formulations

Polyherbal therapy has been used by Chinese, Ayurvedic, Unani, and African traditional healing systems for thousands of years. In these medicinal systems, different chronic diseases are better managed with polyherbal formulations instead of single herbs due to enhanced efficacy and reduced side effects. The concept of polyherbal combinations has also been well established in Western medicine. Drug-drug combination often produce an enhanced effect in the treatment of diseases over a single drug and have been successfully employed for the treatment of critical diseases such as cancer, acquired immunodeficiency syndrome (AIDS), and pulmonary tuberculosis in order to achieve the desired effects (Risberg *et al*., 2011).

A polyherbal formulation contain two or more herbal extracts, which are combined in a specific ratio to elicit a more effective pharmacological activity than could be achieved with a single herbal formulation. This combination may result in either synergistic, additive or antagonistic effects. Due to synergism, polyherbal formulations offers some benefits which lacks in single herb formulations. It has been reported previously that better biological activity can be obtained from a polyherbal combination than with a single herbal formulation (Parasuraman *et al*., 2014; Ramamoorthy *et al*., 2019; Mussarat *et al*., 2021).

In many cases a lower dose of the polyherbal preparation is required to achieve a desirable biological effect, thus reducing the side effects of using large amounts of each herb extract (Wachtel-Galor *et al*., 2011). Furthermore, a polyherbal formulation is more convenient for patients in that it eliminates the need to take more than one different single herb formulation at a given time, thus improving patient compliance and therapeutic effect (Karole *et al*., 2019).
In preparing a polyherbal formulation, various plant parts are used such as leaves, flowers, roots, buds, stems, seeds, barks and fruits (Rawat and Vashistha, 2011; Gurley, 2012), and these can be formulated into various pharmaceutical dosage forms such as Tablets, capsules, gels and liquid dosage forms.

Several polyherbal formulations have been developed and evaluated *in vitro* for their antimicrobial and anti-oxidant efficacy. An example of such a formulation includes a polyherbal gel containing ethanol extracts of *Terminalia arjuna* (Roxb.) Wight & Arn. (arjuna), *Centella asiatica* (L.) Urban (gotu kola), and *Curcuma longa* L. (turmeric). The polyherbal gel

demonstrated better activity against *B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa* compared to the plant extracts when tested independently, with zones of inhibition ranging from 12.00 mm to 19.00 mm (Patel *et al*., 2011). Kandasamy *et al*. (2011), developed a polyherbal formulation with antimicrobial activity against. *S. aureus* and *E. coli*. The formulation contained hydroalcoholic extracts of *S. aromaticum*, *C. cyminum*, *Myristica fragrans* (nutmeg), *Trachyspermum ammi* (ajwain), and *Elettaria cardamomum* (cardamom).

Choudhari and colleagues developed a polyherbal preparation containing extracts of *Salacia chinensis* L (lolly berry), *Tinospora cordifolia* (Wild.) Hook. f. and Thoms. (guduchi), and *C. longa*. They reported that the formulation had increased DPPH and H_2O_2 radical scavenging activities compared to the individual extracts (Choudhari *et al*., 2020). The polyherbal formulation containing aqueous extracts of *C. citratus*, *C. longa*, *Z. officinalis*, and *Murraya koenigii* (L.) (curry leaf) was evaluated for anti-oxidant activity using DPPH and FRAP assays. The formulation showed increased activity when compared to the activity of the individual extracts (Rahim *et al*., 2020).

1.9 Toxicological effects of selected culinary herbs and spices

Herbs and spices have been consumed with food to enhance their taste and aroma, and as medicine to prevent or cure some diseases. However, depending on the dose taken, the potential health benefits of herbs and spices might be changed and reversed to toxic effects. The amount and frequency of consumption of herb and spice extracts, or essential oils might lead to carcinogenic, neurotoxic, genotoxic, teratogenic, cytotoxic, nephrotoxic, hepatotoxic, and gastrointestinal toxic effects (Guldiken *et al*., 2018). It is evident that overconsumption of herbs and spices is associated with some adverse effects as reported in numerous studies (Chrubasik *et al*., 2005; Ündeğer *et al*., 2009; Kesavanarayanan *et al*., 2012).

The organosulphur compounds in *Allium sativum* have generally been considered to be of great benefit to human health. However, excessive consumption of *A. sativum* could lead to cytotoxicity (Kesavanarayanan *et al*., 2012) and other adverse effects, including miosis, excessive salivation, bronchial constriction, vomiting, diarrhoea, cognitive disturbances,

convulsion, and coma (Alare and Alare, 2020). Furthermore, *A. sativum* at high doses (≥ 500 mg/kg) caused severe damage to the lungs and liver of test animals (Alnaqeeb *et al*., 1996).

The effects of *Apium graveolens* (celery) on abortion has been reported. Too high doses of *A. graveolens* juice stimulates the uterus and is therefore not recommended in pregnancy (Kolarovic *et al*., 2009). Exposure to *A. graveolens* extracts and essential oil has been associated with photodermatitis and contact dermatitis in allergic people (Walter *et al*., 2019). Furthermore, *A. graveolens* can cause damage to the small intestines as reported in one clinical study (Kooti *et al*., 2015).

Although *Cinnamomum zeylanicum* has been regarded as generally safe by the FDA authorities, some studies have indicated that *C. zeylanicum* is toxic at higher doses. For example, Singh *et al*. (2009), reported on the cytotoxic effects of *C. zeylanicum* aqueous extract at concentrations above 0.16 mg/mL. Changes in liver, spleen, lung, or reproductive organs along with a significant increase in sperm count and motility, and decreased haemoglobin levels have been reported in animals treated with *C. zeylanicum* ethanol extract (Shah *et al*., 1998). Furthermore, *C. zeylanicum* and *Ocimum basilicum* oils were reported to be carcinogenic (Bode and Dong, 2015).

With regard to *Coriandrum sativum*, there is currently no acute toxicity associated with the use of *C. sativum* leaves and seed extracts (Pingale *et al*., 2021). However, prolonged exposure to the oil, oleoresins and vapours may lead to skin and eye irritations. These can also be harmful if swallowed (Peter and Babu, 2012). It was discovered that prolonged usage (28 days) of *C. citratus* aqueous and ethanol leaf extracts resulted in liver tissue damage and mild renal toxicity (Tarkang *et al*., 2012). Furthermore, *Cymbopogon citratus* plant extract (30%–80%) has demonstrated hepatoxicity and nephrotoxicity to experimental animals (Martínez *et al*., 2000).

Laurus nobilis is a commonly consumed herbal preparation. A recent study reported toxic hepatitis resulting from the daily consumption of *L. nobilis* tea for one month, which ultimately led to progressive liver failure and death (Hasan *et al*., 2021). Genotoxicity of *L. nobilis* and *O. basilicum* has been indicated in some *in vitro* assays, and *in vivo* models (Mezzoug *et al*., 2016).

The consumption of *Melissa officinalis* extract at higher doses (1200 mg) may lead to increased appetite (Alijaniha *et al*., 2015), headache, changes in electro-encephalogram (EEG) recordings, reduced alertness, increased intra-ocular pressure, palpitation, and thyroid hormone inhibition (Giles *et al*., 2005). Other reported side effects included dizziness, wheezing, agitation, abdominal pain, nausea and vomiting. In topical application, local reddening, burning sensation, residual pigmentation dermal irritation, and contact dermatitis were reported (Giles *et al*., 2005). Furthermore, long term oral administration of *M. officinalis* hydroalcoholic extracts may cause the formation of hepatic and renal lesions (Hashemnia *et al*., 2017).

The intake of *Mentha piperita* oil and extracts at higher than recommended doses could lead to hepatotoxic and nephrotoxic effects (Nair, 2001; Kligler and Chaudhary, 2007). Furthermore, the genotoxic effect of *M. piperita* and *Anethum graveolens* (dill) essential oils were reported in another study (Lazutka *et al*., 2001). Adverse effects related to overuse of *M. piperita* oil include slow or rapid breathing, abdominal pain, diarrhoea, nausea, vomiting and blood in urine, no urine production, convulsions, depression, dizziness, twitching, unconsciousness, uncoordinated movement and flushing (Lazutka *et al*., 2001). Skin and mucous membrane irritation may occur due to menthol content (https://pubmed.ncbi.nlm.nih.gov/compound/1-Menthol).

The use of *Ocimum basilicum* oil in mouthwashes at concentrations above 5.0% can cause some tissue and gastrointestinal tract irritation at higher concentrations (Dhama *et al*., 2021). Furthermore, *O. basilicum* oil was reported to possess hepatocarcinogenic effects when studied *in vitro* (Dhama *et al*., 2021). Contact with *Petroselinum crispum* and its essential oil has been associated with photodermatitis, an allergic reaction that most often occurs among people allergic to selected plants from the Apiaceae family, and among light-skinned individuals (Walter *et al*., 2019). The application of higher doses of *P. crispum* oil can cause poisoning. Symptoms include among others; general weakness, increased smooth muscles contraction, strong irritations to the gastrointestinal and urinary tracts, anuria, blood in stools, haemolysis, and bleeding of mucus membranes (Craft and Setzer, 2017).

Rosmarinus officinalis oil demonstrated genotoxic and mutagenic effects at higher doses when studied *in vitro* (Maistro *et al*., 2010). Another *in vitro* study reported the histopathological changes on the heart, kidneys, and liver after administration *R. officinalis* oil for 30 days (Wang *et al*., 2012). *Syzygium aromaticum* essential oil and buds are generally recognised as safe

(GRAS) by the FDA to be used as additives in food and pharmaceutical preparations, but the oil was reported to be highly cytotoxic to human fibroblast and endothelial cells (Prashar *et al*., 2006).

Prolonged usage or an overdose of *Salvia officinalis* ethanol extract and volatile oils has demonstrated unwanted effects such as vomiting, salivation, tongue swallowing, cyanosis, convulsions, vertigo, tachycardia, and hot flushes (Miraj and Kiani, 2016). Furthermore, the use of *S. officinalis* oil in pregnancy and lactation is not recommended as the oil may induce toxicity in the foetus or new-born (Fu *et al*., 2013; Miraj and Kiani, 2016). It was reported that *S. officinalis* tea induces hepatoxicity in mice (Fu *et al*., 2013; Miraj and Kiani, 2016).

The genotypic effects of *Thymus vulgaris* oil was reported in some studies (Miraj and Kiani, 2016; Patil *et al*., 2021). The gastrointestinal toxicity of *Zingiber officinalis* has been reported in one clinical study, whereby participants complained of diarrhoea, indigestion, and gastric mucosa irritation from consumption of *Z. officinalis* at doses higher than 6.00 g (Ali *et al*., 2008). Furthermore, it was found that *Z. officinalis* ethanol extract had gastrointestinal toxicity in mice, whereby the animals suffered from mild diarrhoea. However, administration of higher doses (3.00 and 3.50 g/kg) of *Z. officinalis* ethanol extract caused mortality (20% and 30% respectively) within 72 hrs. (Chrubasik *et al*., 2005).

1.10 Evaluation of toxicity of herbal plant extracts

Many studies recommended the use of natural product such as herbal plant extracts as antimicrobial and anti-oxidant agents. However, in most of these studies the negative effects of these extracts are often ignored. Plant extracts contain bio-active compounds, which at higher doses may be toxic to the human body. Several *in vitro* methods are employed to assess the toxicity of herbal extracts and essential oils. However, these methods have proven to be financially costly, and time consuming. The use of mammalian models, which provides meaningful human-like predictions have been restricted due to religious, social and ethical dilemmas (Freires *et al*., 2017). For this reason, invertebrate animals are used because of their minimal ethical requirements. *Artemia* species such as *Artemia salina*, commonly known as brine shrimp, are widely used in the evaluation of toxicity of plant extracts, and are regarded

as a convenient preliminary toxicity screening method prior to further experiments in mammalian animal models.

The brine shrimp lethality Assay (BSLA) is a commonly used assay to test for the cytotoxicity of plant extracts and their bio-active chemicals. It is a rapid and comprehensive assay for the evaluation of cytotoxic effects of plants extracts and their bio-active constituents. (Bussmann *et al*., 2011; Rawat and Vashistha, 2011; Risberg *et al*., 2011; Gurley, 2012; Parasuraman *et al*., 2014; Ghosh *et al*., 2015; Kabubii *et al*., 2015; Mwiti and Afolayan, 2016; Ramamoorthy *et al*., 2019; Mussarat *et al*., 2021).

The BSLA is regarded as superior to other cytotoxic procedures because it is rapid, inexpensive, and simple to conduct as it doesn't require any special equipment and no aseptic techniques need to be followed. Furthermore, this assay utilises a large number of organisms for statistical validation and a relatively small amount of sample is needed (Sarah *et al*., 2017). The BLSA is based on testing the killing ability of a sample on a simple zoological organism *Artemia salina,* commonly named brine shrimp.

Although there may be a decrease in the solubility of some chemical substances in the medium, producing false positives due to the toxicity of the solvent itself, this method is still regarded as cost-effective, simple, rapid, reliable, comprehensive and can be used to detect the general toxicity in a broad spectrum of natural products. The wide use of *Artemia* as a test organism is because it is easy to culture and well-studied (Hamidi *et al*., 2014; Ntungwe *et al*., 2020).

1.11 Aim and study objectives

The aim of the study was to formulate a polyherbal capsule with optimal antimicrobial and anti-oxidant effects.
In order to achieve the aim of the study the following objectives were set;

- To perform chemical characterization of a selection of neat essential oils of selected culinary herbs and spices using gas chromatography coupled with mass spectroscopy (GC– MS).

- To perform chemical characterization of crude extracts of selected culinary herbs and spices using ultra-performance liquid chromatography coupled with mass spectroscopy (UPLC–MS).
- To screen crude extracts and neat essential oils of selected culinary herbs and spices for anti-oxidant activity using the DPPH, FRAP and ABTS assays.
- To evaluate the anti-oxidant interactions from a 1:1 combination of the most active crude extracts and neat essential oils by determining ΣFIC for the combinations.
- To screen crude extracts of selected spices and herbs and their essential oils for antimicrobial activity using broth the microdilution method to determine the MIC.
- To evaluate the antimicrobial interaction from various 1:1 combination of the most active crude extracts and neat essential oils by determining the sum of fractional inhibitory concentration (ΣFIC) for the combinations.
- To evaluate the toxic effects of crude extracts independently, at 1:1 combination, and a triple combination (1:1:1) using brine shrimp lethality Assay.
- To optimize the anti-oxidant and antimicrobial synergy potential of the combinations of crude extracts using Design of Experiments (MODDE 9.1®) analysis.
- To formulate a polyherbal capsule with optimal antimicrobial and anti-oxidant effects.
- To evaluate the toxic effects of the polyherbal formulation using brine shrimp lethality Assay.
- To evaluate the formulation for anti-oxidant and antimicrobial activity

A flow diagram representing the outline of the study is provided in Figure 1.1

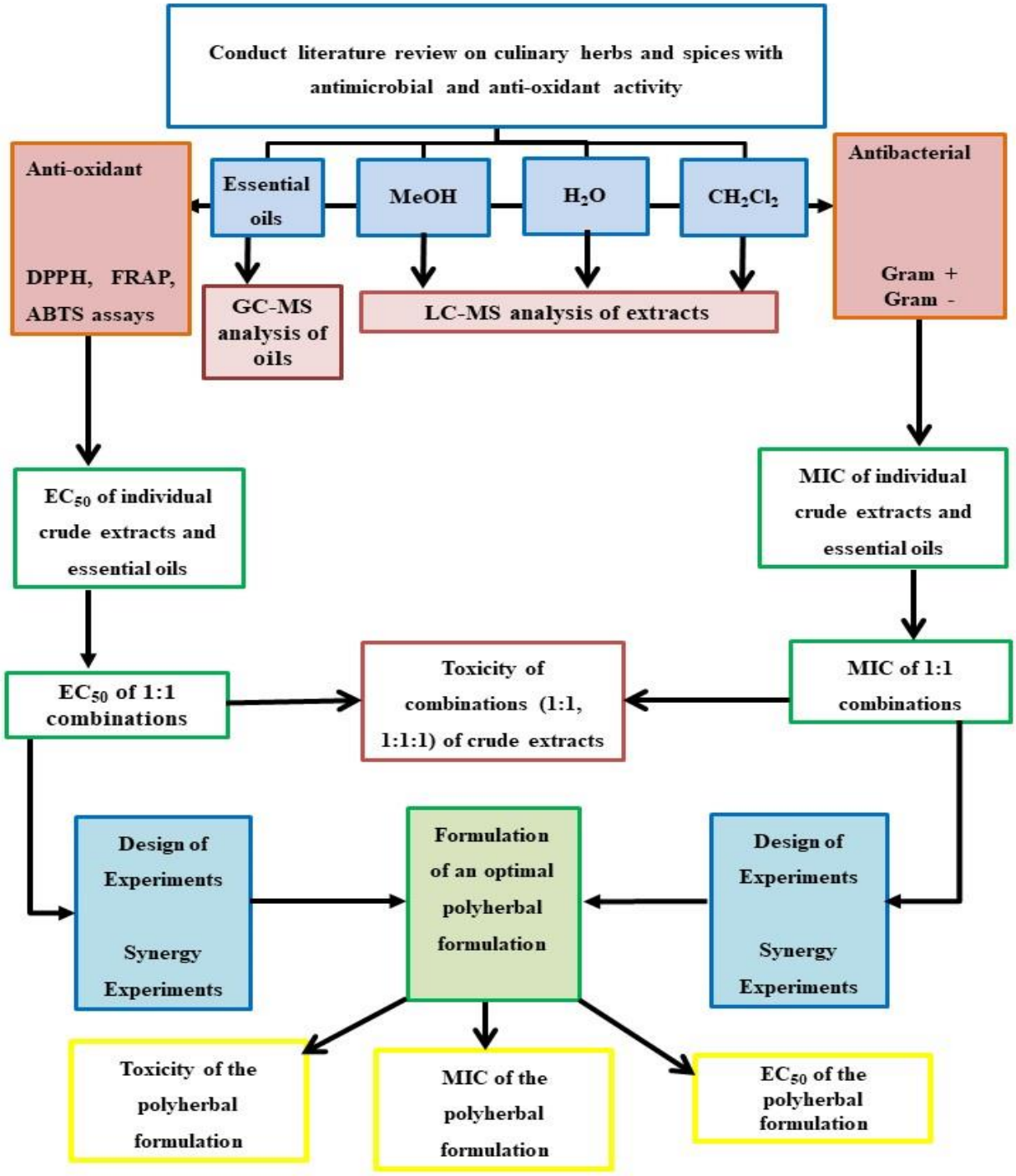

Figure 1.1 A schematic workflow of the study showing the research objectives

CHAPTER 2

Phytochemical profiling of crude extracts and essential oils of selected culinary herbs and spices

2.1 Introduction

Culinary herbs and spices contain volatile and non-volatile compounds. The volatile constituents are contained in the essential oils, which are odorous, concentrated hydrophobic liquids distilled from different plant parts such as leaves, seeds, stems, flowers, and peels. The essential oils contain variable mixtures of chemical compounds such as terpenoids (linalool, geraniol, borneol, menthol, thujanol, citronillol, α-terpineol), and a variety of low molecular weight aliphatic hydrocarbons like phenols (thymol, carvacrol, eugenol), as well as aromatic aldehydes such as cinnamaldehyde, cuminal, and phellandral (Embuscado, 2019). These chemical compounds may be present in the oil at higher concentrations (major constituents) or as minor constituents at trace levels (Bakkali *et al*., 2008).

The non-volatile metabolites are polyphenols such as flavonoids, phenolic acids, stilbenes, and ligands amongst others (Embuscado, 2019). The predominant classes of polyphenols found in herbs and spices are phenolic acids and flavonoids, mainly flavones and flavanols (Opara and Chohan, 2014). Phenolic compounds have gained much attention in the food, medicine, and pharmaceutical industries due to their antimicrobial and anti-oxidant potential, and associated health benefits. Flavonoids and phenolic acid content in herbs and spices are widely studied because of their important biological properties. Flavonoids are a group of plant secondary metabolites in which the molecular framework is characterized by variable phenolic structures, and are widely distributed in plants (Panche *et al*., 2016). The most common flavonoids in herbs and spices are flavonols, flavones, flavanones, anthocyanins, and isoflavonoids. Phenolic compounds are also plants' secondary metabolites, and mostly isolated phenolic acids in herbs and spices are hydroxybenzoic acid, and hydroxycinnamic acids.

In this study, GC-MS chromatography was used to identify the major compounds present in 15 neat essential oils of selected culinary herbs and spices, whereas UPLC-MS chromatography was used to produce a chemical fingerprint of the crude methanol, water, and dichloromethane

extracts. The chemical profiles of the compounds provided by GC-MS and UPLC-MS chromatograms were compared with the profiles of known anti-oxidant and antimicrobial components previously isolated from herbs and spices extracts.

2.2 Materials and methods

Spices and herbs were purchased from Warren Chemical Specialties (Pty) Ltd, South Africa with the appropriate certificate of analysis included (COA). Information regarding the purchased raw plant materials and essential oils, including the name of supplier and lot/ batch number is provided in Appendix E and F, respectively. The raw materials were kept in a cool and dry place before extraction. Extracts were prepared for each of the herbs and spice samples by macerating the powdered materials (20 g) in 200 mL of solvents with different polarities (water, methanol, and dichloromethane), followed by shaking in the dark for 24 hrs. at room temperature using a mechanical shaker. The mixtures were then filtered through a Whatman No.1 filter paper. The filtrates were evaporated to dryness under vacuum at 40°C using a vacuum evaporator (Labtech H50–500, Magna Analytical, South Africa). The 15 essential oils were purchased from Prana Monde (Belgium) with appropriate COA and were re-analyzed by GC-MS for confirmation of authenticity. The stock solutions of plant extracts and essential oils (1 mg/mL) were prepared with methanol for the chemical characterization study. The solutions were stored at 4°C until use. Ultra-Performance Liquid chromatography coupled with mass spectrometry (UPLC-MS) and Gas chromatography coupled with mass spectrometry (GC-MS) techniques were used for chemical characterization of the extracts and essential oils, respectively. It needs to be noted that it was not the objective to perform a comprehensive characterization of the commercially obtained samples. However, chromatographic profiles were generated to confirm the presence of marker compounds associated with each of the selected botanicals.

2.2.1 Gas chromatography coupled with mass spectroscopy characterization of neat essential oils

Gas chromatography-mass spectrometry (GC-MS) was used to analyse the chemical composition of the essential oils. The GC-MS, Agilent 6860N GC system coupled directly to a 5973 mass spectrometer and equipped with an HP-Innowax polyethylene glycol column (60

m×250 µm i.d. × 0.25 µm film thickness) was used for the analysis. A volume of 1 µL was injected into the GC with a 200:1 split ratio at 25.79 psi and an inlet temperature of 250°C. The oven temperature was initially maintained at 60°C, rising to 220°C at a rate of 4°C / min; after holding for 10 min, the temperature was raised to 240°C at a rate of 1°C / min. Helium was used as a carrier gas at a constant flow rate of 1.2 mL/min. The mass spectrometer was run in the electron impact mode of 70 eV and operated in a full scanning mode from *m/z*35 to 450. The percentage composition of the individual components was quantified by integration measurements using flame ionization detection (FID, 250°C). Compounds were identified using different libraries including Mass Finder®, Flavor®, and NIST® by comparing mass spectra and retention indices calculated using a series of straight-chain- *n*-alkanes (C_6–C_{20}) (Kamatou *et al.*, 2010).

2.2.2 Ultra-Performance Liquid chromatography coupled with mass spectroscopy profiling of crude extracts

The crude (methanol, water, and dichloromethane) extracts from selected herbs and spices were analysed using an Ultra-Performance Liquid chromatography-mass spectrometry (UPLC-MS) system with a PDA detector (Waters, Milford, MA, USA). Each of the extracts (1mg/mL) was injected in the mobile phase with an injection volume of 1.0 µL (full-loop injection). Separation of the compounds was achieved on an Acquity UPLC BEH C_{18} column (150 mm × 2.1 mm, i.d., 1.7 µm particle size, Waters) maintained in a column chamber at 40°C. The chromatographic conditions were optimized to obtain the best separation of the major peaks. The mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.3 mL/min for a total run of 18 min. The gradient elution was as follows: 90% A: 10% B, increased to 70% A: 30% B over 2 min, increased to 100% B over 13 min, hold for 1 min, and then returned to the initial ratio in 0.9 min, then equilibrating the system for 1.1 min.

The MS data were acquired in positive ionization mode. Nitrogen at 400°C was used as the desolvation gas at a flow rate of 600 L/hr. The cone voltage was set to 38 V and the capillary voltage to 3000 V. The accuracy and reproducibility of the results were ensured by using independent reference lock-mass ions via the LockSprayTM interface with an $[M + H]^+$ ion of 556.2771 Da. Data were collected from 100 to 1500 Da and processed using Marker Lynx (version 4.1, Waters Corporation, MA, USA) chromatographic software. Peaks were identified by comparing mass spectra and retention time (Rt) with those of commercially available

reference standards. Where standards were not available, tentative identification was done by comparing mass spectra in terms of *m/z* fragments with data reported in the literature (Ali *et al*., 2021).

2.3 Results and discussion

2.3.1 Profiling of neat essential oils of selected herbs and spices

The analysed essential oils varied in chemical composition. Some components for example β-caryophyllene and 1,8-cineole were common in several essential oils. The major constituents in the 15 essential oils analysed using GC-MS together with their respective percentage compositions are presented in Table 2.1. The GC-MS chromatograms of the essential oils are provided in Appendix C.

The GC-MS analysis of *A. graveolens* (celery) seed oil yielded 22 different compounds. The major compounds in the oil were identified as limonene (65.9%) and β-selinene (22.9%). The presence of major compounds in *A. graveolens* oil was also previously reported (Sowbhagya, 2014; Sarshar *et al*., 2018; Dąbrowska *et al*., 2020). Limonene, β-selinene, and sedanolide were reported to possess antimicrobial activity in some studies (Baananou *et al*., 2013; Ud Din *et al*., 2015).

The main constituent in *C. zeylanicum* bark oil was cinnamaldehyde (64.6%). Cinnamaldehyde and eugenol were previously identified as major compounds in *C. zeylanicum* bark oil (El-Baroty *et al*., 2010; Şimşek *et al*., 2013). The antimicrobial and anti-oxidant activities of *C. zeylanicum* oil were previously associated with the presence of cinnamaldehyde and eugenol (Singh *et al*., 2007; Al-Bayati *et al*., 2009; El-Baroty *et al*., 2010; Liang *et al*., 2019).

Linalool was identified as major compound in *C. sativum* seed oil (34.0%), and *O. basilicum* leaves oil (18.4%). Other studies also reported linalool as the major compound in *C. sativum* oil (Ebrahimi *et al*., 2010; Anwar *et al*., 2018; Shahwar *et al*., 2012) and *O. basilicum* oil (Govindarajan *et al*., 2013; Abd El-Azim *et al*., 2015; Abou *et al*., 2015). However, the concentrations of linalool in the oils were different to the current study. Linalool was also present in *C. zeylanicum* (5.7%) and *L. nobilis* (5.2%) essential oils.

The *C. citratus* leaf oil contains mainly citral (mixture of geranial and neral) at 44.1% and 30.7% respectively, which were comparable to respective values obtained in other studies (Bassolé *et al*., 2011; Mansour *et al*., 2015; Amini *et al*., 2016; Premathilake *et al*. 2018).

Table 2.1 Chemical composition of neat essential oils of selected spices and herbs

Essential oil	**Major compounds and % composition in brackets**
Apium graveolens	limonene (65.9), β-selinene (22.9)
Cinnamomum zeylanicum	cinnamaldehyde (64.6), cinnamyl acetate (7.9), eugenol (5.7), linalool (5.6), β-caryophyllene (5.5), 1,8-cineole (3.5)
Coriandrum sativum	linalool (34.0), decenol (14.02), decanal (5.9), decanol (5.3), 2-decen-1-ol (3.7), λ-terpinene (3.4)
Cymbopogon citratus	geranial (44.1), neral (30.7), geraniol (7.6), β- cartinene (4.3)
Laurus nobilis	1,8-cineole (42.9), terpinyl acetate (16.8), sabinene (6.6) linalool (5.2), α-pinene (3.8), β-pinene (3.8), terpinen-4-ol (3.4)
Melissa officinalis	geranial (31.4), neral (20.9), β-caryophyllene (7.3), citronellal (6.7), caryophyllene oxide (5.7), citronellol (3.1)
Mentha piperita	menthone (27.4), ρ-menthon-1-ol (27.3), menthofuran (6.3), 1,8-cineole (5.4), iso menthone (5.01), β-caryophyllene (4.1), pulegone (2.7), limonene (2.2)
Ocimum basilicum	estragole (74.5), linalool (18.4), bisabolene (2.1)
Origanum marjorana	terpinen-4-ol (29.8), cis-sabinene hydrate (17.8), λ- terpinene (13.5), α- terpinene (7.6), sabinene (6.3), cis-sabinene hydrate (4.6), α-terpineol (3.8), terpinolene (3.1)
Petroselinum crispum	myristicin (23.9), α-pinene (14.9), β-pinene (12.0), β-phellandrene (8.0), myrcene (5.1), elemicin (4.3), ρ- metha1,3- 8- triene (3.6), limonene (3.4), terpinolene (2.9)
Rosmarinus officinalis	1,8-cineole (43.01), camphor (16.03), α-pinene (12.5), β-pinene (5.6), β-caryophyllene (4.6), camphene (4.3), limonene (2.6)
Salvia officinalis	α-thujone (43.5), β-thujone (15.7),

Essential oil	Major compounds and % composition in brackets
	1,8-cineole (10.0), camphor (6.1), β-caryophyllene (5.4),
Syzygium aromaticum	eugenol(80.4), iso eugenol (13.8), β-caryophyllene (3.8)
Thymus vulgaris	thymol (68.1), terpinen-4-ol (7.5), myrcene (3.2), λ- terpinene (2.9), 1,8-cineole (2.3)
Zingiber officinalis	α-zingiberene (36.1), α-curcumene (28.7), camphene (5.7), zingiberone (4.6)

The major compounds in *C. citratus* oil studied by Louis *et al*., (2017) differed from this study, whereby citronellyl formate (42.8%), and isobornyl formate (33.5%) were identified as the main compounds in the oil. The presence of citral in the oil has been associated with the antibacterial and anti-oxidant activities of *C. citratus* oil (Soares *et al*., 2013; Godwin *et al*., 2014; Adukwu *et al*., 2016; Kapur *et al*., 2016; Sharma *et al*., 2021).

The most abundant *L. nobilis* leaf oil compounds were 1,8-cineole (42.9%) and α-terpinyl acetate (16.8%), which were comparable to respective values obtained in other studies (Zolfaghari *et al*., 2013; Said and Hussein, 2014; Kivrak *et al*., 2017; Fidan *et al*., 2019). However, Jemâa *et al*., (2012) identified 1,8-cineole (31.9%), sabinene (12.2%), and linalool (10.2%) as the main compounds in *L. nobilis* oil. The principal constituent, 1,8-cineole has been reported to have antimicrobial properties (Van Vuuren and Viljoen, 2007; Baser and Buchbauer, 2015; Caputo *et al*., 2017).

The major compounds identified in *M. officinalis* leaf oil were geranial (31.4%) and neral (20.9%). Other compounds present at lower concentrations (< 10.0%) were β-caryophyllene, citronellal, and citronellol. Congruent findings were reported by other researchers (Singh *et al*., 2011; Abdellatif and Hassani, 2015). However, there were some differences in other studies whereby α-citral (20.1%), β-caryophyllene (17.3%), and β-citral (13.6%) were identified as the major constituents of *M. officinalis* leaf oil (Schnizler *et al*., 2008). In their study, Taherpour *et al*. (2012), identified citral (37.2%), neral (23.9%), and citronellal (20.3%) as major constituents. Both geranial and neral have been associated with the antimicrobial and anti-oxidant activities of *M. officinalis* oil (Abdellatif and Hassani, 2015).

For *M. piperita* leaf oil, menthone (27.4%), and menthol (27.3%) were identified as major constituents followed by menthofuran (6.3%) 1,8-cineole (5.4%), iso-menthone or neo-

menthone (5.01%), and β-caryophyllene (4.1%). Similarly, these compounds were reported as major constituents by other researchers (Riachi and De Maria, 2015; Brahmi *et al*., 2017; Benzaid *et al*., 2019; Wu *et al*., 2019). However, Ramos *et al.* (2017) reported linalool (51.8%) as the main constituent of the oil. Menthol was found to be the most biologically active compound with its antimicrobial and anti-oxidant properties reported in numerous studies (Kumar *et al*., 2011; Kamatou *et al*., 2013; Sun *et al*., 2014; Wu *et al*., 2019a).

The GC-MS analysis of *O. basilicum* leaf oil yielded 28 compounds, and methyl eugenol (74.5%) and linalool (18.4%) were identified as major constituents in the oil. Govindarajan *et al*. (2013), also identified linalool (52.4%), and methyl eugenol (18.7%), as major constituents in *O. basilicum* oil. Methyl chavicol, methyl cinnamate, and linalool were reported as major constituents by other researchers (Stanojevic *et al*., 2017; Ahmed *et al*., 2019). Oliveira *et al*., (2009) reported linalool (72.1%), geraniol (12.9%), and 1,8-cineole (7.9%) as principal components in *O. basilicum* oil, whilst Joshi, (2014) reported methyl eugenol (39.3%), methyl chavicol (38.3%) as main constituents in the oil.

The major compounds in the essential oil of *O. marjorana* leaves were identified as terpinene-4-of (29.8%), cis-sabinene hydrate (17.8%), and γ-terpinene (13.5%). Hussain *et al*., (2011) identified terpinene-4-ol (20.9%), linalool (15.7%), linalyl-acetate (13.9%), limonene (13.4%), and α-terpineol (8.6%) as major compounds in *O. marjorana* leaf oil. However, Ramos *et al*., (2011) identified cis-sabinene hydrate (30.2%), terpinene-4-ol (28.8%), γ-terpinene (7.2%), α-terpineol (6.9%), trans-sabinene hydrate (4.4%), linalyl acetate (3.8%), and α-terpinene (3.6%) as main compounds present in *O. marjorana* leaf oil. Furthermore, Jiang *et al*. (2011), identified terpinene-4-ol (33.0%), caryophyllene oxide (11.9%), *p*-cymene (6.8%), α-terpineol (6.7%), and spathulenol (6.0%) as main compounds in the oil. Cis-sabinene hydrate has been claimed to be responsible for the antibacterial activity of *O. marjorana* oil (Ramos *et al*., 2011).

Myristicin and apiol are the major phytochemicals present in *P. crispum* leaf oil (Zhang *et al*., 2006). The main constituents identified in this study were myristicin (23.9%), α-pinene (14.9%), and β-pinene (12.0%). Compounds such as myrcene, limonene, β-phellandrene, terpinolene, *p*-menthol 1,3,8-triene, and elemicin were found at lower concentrations in the oil. The anti-oxidant activity of *P. crispum* oil has been associated with the presence of myristicin in the oil.

The main constituents in *R. officinalis* leaf oil were reported as 1,8-cineole (15.0-55.0%), camphor (5.0-21.0%), α-pinene (9.0-26.0%), borneol (1.5-5.0%), camphene (2.5-12.0%, β-pinene (2.0-9.0%), and limonene (1.5-5.0%) (Begum *et al*., 2013; Borges *et al*., 2019). These compounds may be present in different proportions in the oil due to factors such as the vegetative stage of the plant and bioclimatic conditions. The chemical composition of *R. officinalis* leaf oil in this study was dominated by 1,8-cineole (43.01%), camphor (16.03%), and α-pinene (12.5%), followed by β-pinene (5.6%), β-caryophyllene (4.6%), and limonene (2.6%). The presence of 1,8-cineole, α-pinene, and camphor have been related to the antibacterial and anti-oxidant activities of *R. officinalis* oil (Mekonnen *et al*., 2016; Bajalan *et al*., 2017; Borges *et al*., 2018; Pistelli *et al*., 2018).

Syzygium aromaticum bud oil is a mixture of different compounds, with eugenol, eugenyl acetate, β-caryophyllene, and α- humelen being the major compounds identified in the oil (Batiha *et al*., 2020). In this study, the predominant compounds identified in *S. aromaticum* bud oil were eugenol (80.4%), iso eugenol (13.8%), and β-caryophyllene (3.8%). Jirovetz *et al*., (2006) identified eugenol (76.8 %), β-caryophyllene (17.4 %), α-humulene (2.1 %), and eugenyl acetate (1.20 %) as the main components in *S. aromaticum* oil, whereas Alma *et al.* (2007), identified eugenol (87.0 %), eugenyl acetate (8.0 %) and β-caryophyllene (3.6 %) as major compounds in the *S. aromaticum* oil. Eugenol was also identified as major constituents in *S. aromaticum* oil by other researchers (Santin *et al.*, 2011; Kamatou *et al*., 2012; Nejad *et al*., 2017; Teles *et al*., 2021).

The major compounds in *S. officinalis* oil identified in this study were α-thujone (43.5%), β-thujone (15.7%), 1,8-cineole (10.0%), and camphor (6.1%). Similarly, Li *et al*. (2015), identified β-thujone (14.86%), 1,8-cineole (14.8%), and camphor (12.7%) as major compounds in *S. officinalis* oil. However, Khedher *et al*. (2017), and Craft *et al*. (2017), reported camphor (13.0%- 25.1%), α-thujone (18.8%- 27.0%), and 1,8-cineole (14.1-27.0%) as major compounds in *S. officinalis* oil. This variation in the chemical composition of the oil may be due to several factors such as the geographical location of the plant, time of harvest, postharvest processes, and method of extracting the oil from the plant to name a few. The presence of camphor, α-thujone, and 1,8-cineole in *S. officinalis* oil has been associated with the antibacterial activity of the oil (Delamare *et al*., 2007; Khedher *et al*., 2017; El-Jery *et al*., 2020).

The predominant compound in *T. vulgaris* oil was thymol (68.1%). Myrcene, 1,8-cineole, and γ-terpinene were also present at very low concentrations (2.3% –3.2%) in *T. vulgaris* oil. However, carvacrol, ρ-cymene, γ-terpinene, α-terpinene, and linalool were identified as major constituents in previous studies (Abdelli *et al*., 2017; Lemos *et al*., 2017; Aljabeili *et al*., 2018; Quynh *et al*., 2019). Thymol and carvacrol have been reported to have antibacterial and anti-oxidant activities (Ali *et al*., 2013; Lemos *et al*., 2017; Aljabeili *et al*., 2018; Gedikoğlu *et al*., 2019; Quynh *et al*., 2019).

The key constituents of *Z. officinalis* root oil have been identified as zingiberene, α-curcumene, β-sesquiphellandrene, β-bisabolene, and α-farnesene (Onyenekwe and Hashimoto, 1999; Jesudoss *et al*., 2017; Wang *et al*., 2020; Dillard, 2022). In this study, zingiberene (36.1%) and α-curcumene (28.7%) were identified as principal compounds of *Z. officinalis* root oil. Camphene and zingiberone were present, but at lower concentrations (5.7% and 4.6% respectively).

2.3.2 Profiling of crude extracts of selected culinary herbs and spices

A total of 45 bioactive compounds were identified in crude methanol, water and dichloromethane extracts of selected culinary herbs and spices. Table 2.2 provides a list of compounds identified by UPLC-MS along with their retention times. Full chromatograms of the crude extracts are provided in Appendix D. Some compounds such as apigenin, caffeic acid, proanthocyanidin, rosmarinic acid, and catechin were common in most of the analysed extracts.

Apigenin was identified in the crude extracts of *Apium graveolens, C. zeylanicum, C. sativum, C. citratus, O. basilicum,* and *O. marjorana*. The presence of apigenin in herbs and spices extracts has been reported by other researchers (Shah *et al*., 2004; Prakash *et al*., 2010; Patil *et al*., 2011; Mahboubi *et al*., 2014), and this compound has been associated with many biological activities of plant extracts, including antimicrobial, antibacterial and anti-oxidant activities. Caffeic acid was identified in *C. zeylanicum, C. sativum, C. citratus, L. nobilis, M. officinalis, M. piperita, O. basilicum, O. marjorana, P. crispum, S. officinalis,* and *T. vulgaris* crude extracts. Proanthocyanidins was identified in *C. zeylanicum, M. officinalis, O. marjorana, P. crispum, S. officinalis, S. aromaticum,* and *T. vulgaris* extracts.

Table 2.2 Chemical profiles of crude extracts of selected culinary herbs and spices

Proposed Compounds	**Retention time (min)**	**Molecular formula**	**$[M+H]^+$**	**Spice/ herb extracts (M,W,DCM)**
chlorogenic acid	3.35	$C_{16}H_{18}O_9$	355.0458	As
Epicatechin	2.27	$C_{15}H_{14}O_6$	291.1011	As
hesperitin-3´-O-glucuronide	7.88	$C_{22}H_{22}O_{12}$	479.2267	As
Catechin	6.75	$C_{15}H_{14}O_6$	291.1971	Apg, Cz, Cs, Cc, Ob, Om
Kaempferol	5.16	$C_{15}H_{10}O_6$	287.0548	Apg, So
Isorhamnetin	3.28	$C_{16}H_{12}O_7$	317.0660	Apg, Ln, So
Apigenin	5.02	$C_{15}H_9O_5$	271.0608	Apg, Mp, Ob, Om, Pc, Ro, Sa, Tv
Apiin	3.21	$C_{26}H_{28}O_{14}$	565.1604	Ang
Xanthotoxin	6.26	$C_{12}H_8O_4$	217.0488	Ang
cinnamic acid	2.67	$C_9H_8O_2$	149.0598	Cz
caffeic acid	6.49	$C_9H_8O_4$	181.1219	Cz, Cs, Cc, Ln, Mo, Mp, Ob, Om, Pc, So, Tv
Proanthocyanidins	14.77	$C_{31}H_{28}O_{12}$	593.2788	Cz, Mo, Om, Pc, So, Sa, Tv
vanillic acid	3.09	$C_8H_7O_4$	169.0758	Cs

ferulic acid	4.29	$C_{10}H_{10}O_4$	195.1382	Cc
rosmarinic acid	3.46	$C_{18}H_{16}O_8$	361.0924	Mo, Mp, Om, Ro, Sa, Tv
Diosmin	14.07	$C_{28}H_{32}O_{15}$	609.2755	Mo
Hesperidin	3.39	$C_{28}H_{34}O_{15}$	611.2048	Mp
Luteolin	4.33	$C_{15}H_9O_6$	287.0555	Mp, Sa
Rutin	2.83	$C_{27}H_{29}O_{16}$	611.1666	Ob
Tricin	4.67	$C_{17}H_{14}O_7$	331.0812	Om
luteolin-7-O-rutinoside	2.33	$C_{27}H_{30}O_{16}$	595.1698	Om
Carnosol	9.50	$C_{20}H_{26}O_4$	331.1911	Om, Ro, Sa
carnosic acid	4.61	$C_{20}H_{28}O_4$	333.0966	Om
*unidentified	11.29		496.3427	Om, Pc
Rosmadial	6.54	$C_{20}H_{24}O_5$	345.0974	Ro, Tv
Quercetin	4.34	$C_{15}H_{10}O_7$	303.0505	So
quercetin-3-O-hexoside	2.67	$C_{21}H_{19}O_{12}$	465.1050	Ro
isorhamnetin-3-hexoside	3.11	$C_{22}H_{22}O_{12}$	479.1212	Ro

luteolin-3-O-glucuronide	4.64	$C_{21}H_{18}O_{12}$	463.1259	Ro
Acacetin	7.27	$C_{16}H_{12}O_5$	285.0765	Ro, Tv
Epirosmanol	10.84	$C_{20}H_{12}O_5$	347.1865	Ro
Cirsimaritin	6.33	$C_{17}H_{14}O_6$	315.0873	Ro, Sa, Tv
hydroxybenzoic acid-O-hexoside	11.93	$C_{13}H_{16}O_8$	301.2164	Ro
methoxy carnosol	9.32	$C_{21}H_{27}O_5$	361.2026	Ro, Sa
Isobiflorin	2.17	$C_{16}H_{18}O_9$	355.1040	Sa
Biflorin	2.34	$C_{16}H_{18}O_9$	355.1022	Sa
Rhamnetin	3.49	$C_{16}H_{12}O_7$	317.0657	Sa
Medioresinol	8.43	$C_{21}H_{24}O_7$	389.1261	Tv
3´-O-methylcatechin	9.37	$C_{16}H_{16}O_6$	305.2120	Zo
4´-O-methyl epigallocatechin	10.49	$C_{16}H_{16}O_7$	321.2057	Zo
naringenin-7-O-glucoside	5.16	$C_{21}H_{22}O_{10}$	435.2034	Zo
Naringin	4.95	$C_{27}H_{32}O_{14}$	581.2430	Zo
Cirsilineol	9.37	$C_{18}H_{16}O_7$	345.2047	Zo

kaempferol 3-O-xylosyl-glucoside	4.94	$C_{26}H_{28}O_{15}$	581.2444	Zo
ρ-coumaric acid 4-O-glucoside	3.61	$C_{15}H_{18}O_{8}$	327.1596	Zo
ferulic acid 4-O-glucoside	4.94	$C_{16}H_{20}O_{9}$	357.1716	Zo

As: *A. sativum*; Apg: *Apium graveolens*; Ang: *Anethum graveolens*; Cz: *C. zeylanicum*; Cs: *C. sativum*; Cc: *C. citratus*; Ln: *L. nobilis*; Mo: *M. officinalis*; Mp: *M. piperita*; Ob: *O. basilicum*; Om: *O. marjorana*; Pc: *P. crispum*; Ro: *R. officinalis*; SO: *S. officinalis*; Sa: *S. aromaticum*; Tv: *T. vulgaris*; Zo: *Z. officinale.*

Both caffeic acid and proanthocyanidins were identified in spices and herbs extracts in some studies (Vallverdú-Queralt *et al*., 2014; Ali *et al*., 2021). These two compounds were reported to possess antimicrobial and anti-oxidant properties (Stojković *et al*., 2013; Vallverdú-Queralt *et al*., 2015; Rauf *et al*., 2019).

Rosmarinic acid was present in *M. officinalis*, *M. piperita*, *O. marjorana*, *R. officinalis*, *S. aromaticum*, and *T. vulgaris* extracts. This compound was identified in culinary herbs and spices extracts in some previous studies (Vallverdú-Queralt *et al*., 2014; Ali *et al*., 2021). Catechin was identified in *Apium graveolens*, *C. zeylanicum*, *C. sativum*, *C. citratus*, *O. basilicum*, and *O. marjorana* extracts. These compounds has a number of biological activities including anti-oxidant, anti-inflammatory, antibacterial, and antiviral (Embuscado, 2015; Nadeem *et al*., 2019).

Cirsimaritin was identified in *R. officinalis*, *S. aromaticum* and *T. vulgaris* extracts. The presence of this compound in these plant extracts was reported previously (Borras-Linare *et al*., 2014). Cirsimaritin is reported to possess antibacterial, antiviral, and anti-oxidant activities among others.

Other phenolic compounds identified were cinnamic acid in *C. zeylanicum*, ferulic acid in *C. citratus*, chlorogenic acid in *A. sativum* extracts. These compounds are known to possess antimicrobial and anti-oxidant properties (Park *et al*., 2011; Ruwizhi *et al*., 2020). It was observed that phenolic compounds such coumaric acid, syringic acid, vanillic acid were not identified in selected spices and herbs extracts as reported previously (Hossain *et al*., 2010).

Carnosol was present in *R. officinalis*, *S. aromaticum*, and *O. marjorana* extracts. Carnosic acid reported to be present in *R. officinalis* and *S. officinalis* extracts (Hossain *et al*., 2010; Birtić *et al*., 2015). However, this compound could only be identified in *O. marjorana* extracts. Both carnosol, carnosic acid were previously reported to possess anti-oxidant properties

(Vallverdú-Queralt *et al*., 2014; Birtić *et al*., 2015; Peixoto *et al*., 2021). Other compounds that were identified only in *O. marjorana* extracts are tricin and luteolin-7-O- rutinoside. Rosmanol was not identified from *R. officinalis* as reported previously (Peixoto *et al*., 2021). Another well-known flavonoid, kaempferol could not be detected in *R. officinalis* and *T. vulgaris* extracts as reported previously (Ali *et al*., 2021). Instead, kaempferol was identified only in *Apium graveolens* and *S. officinalis* extracts.

Quercetin, and rutin were identified only in *R. officinalis* and *O. basilicum* respectively. Quercetin and rutin were previously reported to have strong anti-oxidant potential (Kumar *et al*., 2017; Prasad *et al*., 2019; Chou *et al*., 2021). Other flavonoids identified in this study include acacetin, epicatechin, hesperitin-3´-O-glucuronide, diosmin, hesperidin, and luteolin. Phenolic diterpenes, rosmadial and epirosmanol were identified in *R. officinalis* and *T. vulgaris*. These compounds are reported to possess numerous biological activities, including antibacterial and anti-oxidant activities (Nieto *et al*., 2018).

Other compounds such as rhamnetin, biflorin, and isobiflorin were present in *S. aromaticum* extracts as reported in previous studies (Kim *et al*., 2001; Lee *et al*., 2016). Medioresinol was present in *T. vulgaris* extracts. Numerous phenolic compounds such as 3´-O-methylcatechin, 4´-O-methyl epigallocatechin, naringenin-7-O-glucoside, naringin, cirsilineol, kaempferol 3-O-xylosyl-glucoside, ρ-coumaric acid 4-O-glucoside, ferulic acid 4-O-glucoside were identified in *Z. officinalis* extracts. The presence of these compounds in *Z. officinalis* extracts have been associated with the anti-oxidant properties of this plant (Yang *et al*., 2020).

2.4 Summary

- The most frequently identified compounds in the analyzed essential oils were limonene, linalool, 1-8-cineole, β-caryophyllene, α-pinene, and β-pinene. The three compounds (β-

caryophyllene, α-pinene, and β-pinene) were, however, present at lower concentrations (≤ 10.0%) in essential oils of *C. zeylanicum*, *M. piperita*, *T. vulgaris*, and *S. officinalis*.

- Limonene (65.9%) and linalool (34.0%) were the principal compounds identified in *A. graveolens* leaf oil and *C. sativum* seed oil respectively.
- The key component in *R. officinalis* and *L. nobilis* leaf oil was 1,8-cineole at 43.01% and 42.9% respectively.
- Eugenol (80.4%), estragole (74.5%), cinnamaldehyde (65.9%), and thymol (68.1%) were the major constituents found at higher concentrations in *S. aromaticum* stem oil, *O. basilicum* leaf oil, *C. zeylanicum* bark oil, and *T. vulgaris* leaf oil respectively.
- Citral, a mixture of geranial (44.1% and 31.4%) and neral (30.7% and 20.9%) were identified as a major constituent in essential oils of *C. citratus* and *M. officinalis* leaves respectively.
- The major compounds in the essential oil of *O. marjorana* leaves were identified as terpinene-4-ol (29.8%), trans-sabinene hydrate (17.8%), and γ-terpinene (13.5%).
- The main constituents in *S. officinalis* oil were α- thujone (43.5%) and β-thujone (15.7%), 1, 8-cineole (10.0%), camphor (6.1%).
- Menthone (27.4%) and menthol (27.3%) were identified as major constituents in *M. piperita* oil.
- Myristicin (24.0%) was identified as the major compound in *P. crispum* leaf oil.
- The main constituents in *Z. officinalis* oil were identified as α-zingiberene (36.1%) and α-curcumene (28.7%), which were previously reported to possess antibacterial and anti-oxidant activities.
- A total of 45 polyphenolic compounds (phenolic acids, phenolic diterpenes, flavonoids, hydroxybenzoic acid derivative, and flourocoumarins) were identified from crude extracts.
- Compounds such as apigenin, caffeic acid, proanthocyanidins, catechin and rosmarinic acid were common in numerous spices/ herb extracts.
- Some compounds were present in only one spice even though they were identified previously in numerous spices or herbs extracts.

CHAPTER 3

Anti-oxidant interactions and optimization of synergy by Design of Experiments

3.1 Introduction

Culinary herbs and spices are widely investigated for their anti-oxidant properties as alternatives to synthetic counterparts, due to increasing concerns related to carcinogenicity and other adverse effects produced by chemical preservatives (Imhoff *et al.*, 2010; Taghvaei *et al*., 2015). However, many of these studies mainly focused on the anti-oxidant activity of individual extracts or oils (Kim *et al*., 2011; Devi *et al*., 2012; Asimi *et al*., 2013; Słowianek and Leszczyńska, 2016), but combination effects are less documented.

Spices and herbs are often added as blended mixtures in culinary preparations. Furthermore, many traditional healing modalities such as the Indian system of medicine (Ayurveda), Chinese and African traditional medicines often rely on combinations of highly complex herbal mixtures to achieve an enhanced effect. It could therefore be of great interest to investigate the possible anti-oxidant interactions occurring when spices and herbs are combined. This approach may increase their anti-oxidant efficacy at sufficiently low concentrations by taking advantage of their possible synergistic or additive interactions.

This study was designed to evaluate the anti-oxidant activity of crude extracts obtained from a selection of culinary herbs and spices as well as neat essential oils by comparatively evaluating the results of three anti-oxidant assays (DPPH, ABTS, and FRAP). Furthermore, the anti-oxidant interactions from various combinations of the most active crude extracts and neat essential oils were investigated, as well as optimizing the synergy potential by investigating the various combinations ratios using the DOE approach.

3.2 Materials and methods

Collection of spices and herbal material and essential oils as well as preparation of crude extracts were undertaken according to the procedures outlined in Chapter 2, Section 2.1. The working concentrations (500, 250, 125, 62.5, 31.25, 15.62, 7.81, and 3.91 µg/mL) were prepared from the stock solutions (1mg/mL) by diluting with the correct volume of methanol. Ascorbic acid was prepared in methanol, and used as a positive control. Ascorbic acid is a well-known naturally occurring and effective anti-oxidant present in spices and herbal plants, and therefore it was chosen as a positive control to comparatively evaluate the anti-oxidant activity of culinary herbs and spices.

The individual extracts and essential oils (Table 1.3) were screened for anti-oxidant activity using the DPPH, ABTS, and FRAP assays. The three assays were employed to provide broader information on the anti-oxidant activity of the tested extracts and essential oils. Measurements were obtained in triplicate for each sample in each assay. The half-maximal effective concentration (EC_{50}) values were calculated for the standard control and samples, representing the anti-oxidant capacity in the sample necessary for 50% of the maximal anti-oxidant effect. In all three assays, the EC_{50} values were determined using GraphPad Prism software, version 5 (GraphPad software, Inc. San Diego, California).

3.2.1 The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging effects of the extracts and essential oils were estimated according to the method of Brand-Williams *et al*. (1995), with some modifications. Briefly, a DPPH (Sigma-Aldrich) solution (0.1 Mm w/v) was prepared in methanol and 100 µL of the DPPH solution was mixed with 100 µL of the sample in each of the wells of a 96-well microtitre plate. For the DPPH and methanol controls, a volume of 200 µL of DPPH (96 µM in HPLC grade methanol (Merck, South Africa) and 200 µL of HPLC grade methanol, were added to the corresponding wells, respectively. Ascorbic acid (22.50 µg/mL) was used as a

positive control. The plates were then shaken at 960 rpm for 2 min and incubated in the dark at 27°C for 30 min. After incubation, the absorbance was read at a single wavelength of 517 nm (Wang *et al*., 1998) using a SpectraMax M2 Multimode Microplate Reader linked to a computer with SoftMax® Pro 6, Microplate Data Acquisition and Analysis software. Inhibition of the DPPH radical by the active samples was determined by calculating the DPPH percentage free radical scavenging activity according to Equation 3.1.

$$\% \text{ Decolorisation} = 100 \times \frac{(A_{control}) - (A_{test} + A_{methanol})}{A_{control}}$$

Where A = absorbance at 517 nm.

Acontrol = average absorbance of DPPH – average absorbance of methanol.

Amethanol = average absorbance obtained in the wells containing methanol and

Atest = average absorbance obtained in the wells containing DPPH and test sample.

Equation 3.1

3.2.2 The 2, 2´-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) cation radical scavenging assay

The spices and herbs were tested for their ABTS radical scavenging activity according to the method of Re *et al*. (1999), with some modifications. Two stock solutions of 7 mM ABTS (Sigma-Aldrich) in double-distilled water and 2.45 mM potassium persulphate (Merck, South Africa) were mixed. The mixture was incubated in the dark for 12-16 hrs. at room temperature and used as a working solution. The solution was adjusted with cold ethanol to obtain an absorbance of 0.700 (± 0.02) at 732 nm using a microplate reader (Spectra Max M2, Molecular Devices Inc., USA). A 100 µL of $ABTS^+$ solution was added to the microtiter plate wells containing 100 µL of crude extracts or essential oils. After 30 min of incubation, the percentage decolorization of $ABTS^+$ at 734 nm was calculated for each concentration relative to the blank according to Equation 3.2 Ascorbic acid (22.5 µg/ mL) was used as a positive control.

$$\% \text{ Decolorisation} = 100 \times \frac{(A_{control}) - (A_{test} + A_{methanol})}{A_{control}}$$

Where A = absorbance at 734 nm.

Acontrol = average absorbance of $ABST^{+}$ – average absorbance of methanol.

Amethanol = average absorbance obtained in the wells containing methanol and

Atest = average absorbance obtained in the wells containing $ABST^{+}$ and test sample.

Equation 3.2

3.2.3 Ferric iron-reducing anti-oxidant power (FRAP) assay

The ferric iron-reducing anti-oxidant (FRAP) assay is based on the ability of the anti-oxidant to reduce Fe^{3+} to Fe^{2+} in the presence of 2,4,6-Tris(2-pyridyl)-1,3,5-triazine (TPTZ), forming an intense blue Fe^{2+}-TPTZ (Benzie and Strain, 1996). The FRAP reagent was freshly prepared before each experiment by mixing 300 mM acetate buffer (pH 3.6), 20 mM ferric chloride in distilled water, and 10 mM TPTZ (Sigma-Aldrich, in 40 mM HCl) in a proportion of 10:1:1. The FRAP solution (100 µL) was mixed with 100 µL of the test samples and standard solution in each of the wells of a 96-well microtitre plate. Following the same procedure, a blank test-containing methanol instead of the extract was used as a negative control whilst ascorbic acid at 22.5 µg/mL served as the positive control. The reaction mixtures were then incubated in the dark for 30 min and the reduction of Fe^{3+}-TPTZ complex to a colored Fe^{2+}-TPTZ complex by the extracts and essential oils was monitored by measuring the absorbance after 4 min of incubation, at 593 nm using a microplate reader (SpectraMax M2, Molecular Devices Inc., USA). The FRAP value of each sample was calculated using Equation 3.3 and expressed as µg/mL ascorbic acid.

$$\text{FRAP value of sample } (\mu M) = A_{sample} \times \frac{\text{FRAP value of sample } (\mu M)}{A_{standard}}$$

Where A = absorbance at 593 nm.

Equation 3.3

3.2.4 Statistical analysis

A one-way analysis of variance (ANOVA) was done on each plant extract, and a post Tukey's multiple comparison test was conducted to conclude on the differences between the different extracts of the same plant. The data from the three anti-oxidant assays (DPPH, ABTS and FRAP) were correlated by calculating the Pearson's correlation coefficient (r) using Microsoft Excel.

3.2.5 Sum of fractional inhibitory concentration (ΣFIC) index

The sum of the fractional inhibitory concentration index (ΣFICI) was used to measure interactions from different 1:1 combination of herbs or spices extracts and essential oils with promising anti-oxidant effects when tested using DPPH, FRAP and ABTS assays. Fifty µL of sample A and B for the combinations were plated out in each of the wells marked accordingly followed by the addition of an anti-oxidant reagent (100 µL), to final concentrations of 3.1 to 500 µg/mL. The ΣFICI for each of the combinations were calculated using Equation 3.4.

$$\Sigma FICI = FIC\,(i) + FIC\,(ii)$$

$$FIC(i) = \frac{EC_{50}\text{ (a) in combination with (b)}}{EC_{50}\text{ (a) independently}}$$

$$FIC(ii) = \frac{EC_{50}\text{ (b) in combination with (a)}}{EC_{50}\text{ (b) independently}}$$

Equation 3.4

Where (a) is the EC_{50}, of one spice extract or essential oil in the combination and (b) is the EC_{50} of the other extract or essential oil. The ΣFICI for each combination were interpreted as synergy where the ΣFICI less than or equal to 0.5, additive effects are greater than 0.5 but less than or equal to 1.0. For indifference, the ΣFICI must be greater than 1.0 but less than or equal

to 4.0 and for antagonism an ΣFICI is greater than 4.0 (Van Vuuren and Viljoen, 2011). For all anti-oxidant assays positive and negative controls were included, with a known anti-oxidant, ascorbic acid (22.5 µg/mL) used as a positive control. Methanol, which was used as a diluent to dissolve the test samples, was used as a negative control.

3.2.6 Experimental design using the DOE model to determine effective anti-oxidant combinations

The DoE model was prepared and evaluated using MODDE 9.1® software (Umetrics AB. Umea. Sweden). The models were fitted with partial least square (PLS) and were adjusted by removing non-significant terms. To determine which input parameters resulted in the desired outcomes. Screening experiments were carried out using fractional factorial design. Both the independent and dependent variables were fitted to a linear model. This required 12 experimental runs with three centre points. All the experiments were completely randomized by the software to reduce bias and experimental errors. After the experimental runs were completed, the results were analysed using MODDE 9.1® software, and the data set gave close to normal distribution hence it did not require logarithmic transformation.

A mixture design worksheet for the chosen extracts was produced and the modelling of responses was generated to confirm the best model fit. A prediction contour plot was generated which showed the average prediction (point estimate prediction) for every possible combination of the tested extracts. Predictions with desired EC_{50} values were selected for the model validation. These model predictions were then verified by carrying out additional laboratory experiments and then compared to the predicted values.

3.3 Results and discussion

3.3.1 Anti-oxidant activity of individual crude extracts and neat essential oils

The data on the comparative analysis of the anti-oxidant activities of crude extracts and essential oils is presented in Table 3.1. The total anti-oxidant activity measured by DPPH assay ranged from EC_{50} values of 5.48 to 497.10 µg/mL for methanol extracts, 7.66 to 1340.00 µg/mL for the water extracts, 17.85 to 807.80 µg/mL for dichloromethane extracts and 2.55 to 861.50 µg/mL for the essential oils. The anti-oxidant properties of a spice or herb differed depending on the solvent used for the extraction. Based on the results in Table 3.1, *C. zeylanicum* methanol and water extracts demonstrated noteworthy DPPH radical scavenging activity with EC_{50} values of 5.48 and 7.66 µg/mL, respectively. Furthermore, the recorded activity was better than that of the positive control, ascorbic acid (EC_{50} value of 10.25 µg/mL).

Gupta *et al*. (2013), and Ramadan *et al*. (2017) in their studies also reported high DPPH radical scavenging activity for *C. zeylanicum* methanol extracts though the values were expressed as % DPPH inhibition. Kim *et al*. (2011), reported *C. zeylanicum* water extract to possess higher DPPH radical scavenging activity compared to other spice extracts. However, the EC_{50} value was higher (0.25 mg/mL) compared to that obtained in this study. The least active extract was *C. sativum* water extract (1340.00 µg/mL).

With regards to the essential oils, noteworthy activity was displayed by *S. aromaticum* oil (EC_{50} value of 2.55 µg/mL), followed by *M. piperita* and *C. zeylanicum* oils with EC_{50} value of 6.88 and 7.17 µg/mL, respectively. Other oils displayed moderate to low activity, with *T. vulgaris* essential oil displaying the least anti-oxidant activity with EC_{50} value of 861.50 µg/mL. The anti-oxidant trend for the ABTS assay was different from the DPPH assay, with the total anti-oxidant activity ranging from EC_{50} values of 6.06 to 69.19 µg/mL for methanol extracts, 5.79 to 145.90 µg/mL for water extracts, 3.09 to 258.40 µg/mL for dichloromethane extracts and 5.81 to 1397.00 µg/mL for the essential oils.

The *S. aromaticum* and *Z. officinalis* dichloromethane extracts displayed notable activities with EC_{50} value of 3.09 and 4.15 µg/mL, respectively. The activity was higher than that of ascorbic acid (EC_{50} value of 4.88 µg/mL). This was followed by *C. zeylanicum* (EC_{50} value of 5.79 µg/mL), *O. marjorana* (EC_{50} value of 6.57 µg/mL) and *M. officinalis* (EC_{50} value of 5.95 µg/mL) water extracts. Methanol extracts of *C. zeylanicum*, *M. officinalis*, *M. piperita* and *R. officinalis* also demonstrated promising ABTS radical scavenging effects with EC_{50} values of 6.06, 6.94, 6.42 and 7.18 µg/mL, respectively. As noted in the DPPH assay, *S. aromaticum* oil exhibited notable radical scavenging effect in the FRAP assay with EC_{50} value of 5.96 µg/mL.

The FRAP assay demonstrated a higher variation compared to DPPH and ABTS assays, with EC_{50} for the methanol extracts varying from 33.45 to 11498.00 µg/mL and 42.96 to 1143.00 µg/mL for the water extracts. The values for the dichloromethane extracts ranged from 36.26 to 81083.00 µg/mL, while essential oils ranged from 5.96 to 794.80 µg/mL. The order of active methanol extracts according to the FRAP assay, in comparison to ascorbic acid was *R. officinalis* > *C. zeylanicum* > *M. officinalis* > *T. vulgaris* > *M. piperita* > *S. officinalis* > *S. aromaticum* > *C. citratus* > ascorbic acid.

The *C. zeylanicum* and *R. officinalis* water and dichloromethane extracts and *S. aromaticum* dichloromethane extract, showed promising FRAP reducing activity (EC_{50} values of 36.44, 33.45 µg/mL for the water extract and 36.26, 58.92, 40.95 µg/mL for the dichloromethane extracts, respectively). Other extracts had moderate, low or no activity with reference to ascorbic acid. In their study, Chan *et al*. (2012), reported notable FRAP reducing ability of *C. zeylanicum* water extracts. However, the EC_{50} value was higher (0.24 mg/mL) compared to the value obtained in the current study.

Table 3.1 Anti-oxidant activity of 17 culinary herbs and spice crude extracts and essential oils expressed as EC_{50} (µg/mL)

Spice/herb	**Type of extract**	**DPPH EC_{50} (µg/mL)**	**ABTS EC_{50} (µg/mL)**	**FRAP (µg/mL)**
Allium sativum	Methanol	356.90 ±0.41	47.46 ±0.11	11498.00 ±4.04
	Water	176.60 ±0.76**	142.60 ±0.15	188.50 ±0.32*
	Dichloromethane	57.98 ±0.17	19.10 ±0.16	373.00 ±0.15*
	Essential oil	165.80± 0.20	159.60 ±0.02	146.40 ±0.03
Anethum graveolens	Methanol	96.47 ±0.04*	14.29 ±0.08	454.30 ±0.17*
	Water	144.50 ±0.22*	26.82 ±0.09	192.30 ±0.99
	Dichloromethane	271.90 ±1.59***	129.30 ±0.10	81083.00 ± 00*
	Essential oil	217.60 ±0.13	194.90 ±0.54	158.90 ±0.12
Apium graveolens	Methanol	114.20 ±0.11	21.47 ±0.04	784.50 ±0.17
	Water	294.80 ±0.18	87.98 ±0.05	1143.00 ±0.40
	Dichloromethane	235.80 ±0.99	55.23 ±0.13	305.90 ±0.05
	Essential oil	547.30 ±0.31	42.86 ±0.12	**69.68** ±0.11
Cinnamomum zeylanicum	Methanol	**5.48** ±0.44***	**6.06** ±0.32*	**36.44** ±0.06*
	Water	**7.66** ±0.22***	**5.95** ±0.43*	**42.96** ±0.04**
	Dichloromethane	287.90 ±0.55***	21.09 ±0.05*	**36.26** ±0.10**
	Essential oil	**7.17** ±0.17	11.42 ±0.10	**25.18** ±0.03
Coriandrum sativum	Methanol	92.41 ±0.11**	22.40 ±0.02	432.70 ±0.30*
	Water	1340.00 ±3.26*	111.50 ±0.09	440.00 ±0.24*
	Dichloromethane	232.80 ±0.53*	15.96 ±0.08	742.70 ±0.65

Spice/herb	Type of extract	DPPH EC_{50} (µg/mL)	ABTS EC_{50} (µg/mL)	FRAP (µg/mL)
	Essential oil	385.00 ±1.21	1397 ±3.13	159.70 ±0.13
Cymbopogon citratus	Methanol	245.10 ±0.14*	35.92 ±0.03	**61.99** ±0.20*
	Water	72.90 ±0.07*	29.99 ±0.04	**79.13** ±0.19
	Dichloromethane	807.80 ±1.68***	45.43 ±0.09	615.30 ±0.23*
	Essential oil	601.60 ±0.53	253.20 ±0.34	794.80 ±0.28
Laurus nobilis	Methanol	27.58 ±0.04	**8.77** ±0.03**	120.70 ±0.06
	Water	22.53 ±0.24***	17.58 ±0.05*	161.40 ±0.07***
	Dichloromethane	148.40 ±0.26***	47.57 ±0.07**	356.30 ±0.11***
	Essential oil	152.40 ±0.63	49.48 ±0.06	684.90 ±1.03
Melissa officinalis	Methanol	17.27 ±0.05***	**6.94** ±0.40**	**39.49** ±0.05***
	Water	117.10 ±0.08***	**6.57** ±0.25**	326.20 ±0.35***
	Dichloromethane	321.00 ±0.26***	68.81 ±0.13**	669.80 ±0.40
	Essential oil	62.38 ±0.63	73.65 ±0.07	472.50 ±0.20
Mentha piperita	Methanol	17.67 ±0.049***	**6.42** ±0.85	**53.40** ±0.04**
	Water	53.90 ±0.082**	19.96 ±0.03	234.10 ±0.11**
	Dichloromethane	142.60 ±0.23***	125.50 ±0.12	807.60 ±0.87***
	Essential oil	**6.88** ±0.13	34.08 ±0.13	257.40 ±0.88
Ocimum basilicum	Methanol	497.10 ±0.63	19.2 ±0.03	1090.00 ±0.55
	Water	125.70 ±0.05	53.54 ±0.08	429.50 ±1.64
	Dichloromethane	172.30 ±0.07	29.48 ±0.05	323.30 ±0.15

Spice/herb	Type of extract	DPPH EC_{50} (µg/mL)	ABTS EC_{50} (µg/mL)	FRAP (µg/mL)
	Essential oil	309.60 ±0.37	756.0 ±3.36	1092.00 ±1.61
Origanum marjorana	Methanol	35.25 ±0.11	**8.18** ±0.03	100.70 ±0.06
	Water	32.02 ±0.21	**5.79** ±0.40	136.80 ±0.03
	Dichloromethane	56.41 ±0.33	12.30 ±0.20	158.50 ±0.04
	Essential oil	524.00 ±3.42	162.00 ±0.12	374.80 ±0.21
Petroselinum crispum	Methanol	365.70 ±1.09	69.19 ±0.07**	**77.35** ±0.21*
	Water	81.83 ±0.12*	15.70 ±0.41**	136.40 ±0.12*
	Dichloromethane	98.47 ±0.16*	258.40 ±0.41	**81.42** ±0.47
	Essential oil	15.51 ±0.15	126.30 ±0.10	769.60 ±0.52
Rosmarinus officinalis	Methanol	**11.64** ±0.05*	**7.18** ±0.30	**33.45** ±0.13*
	Water	36.15 ±0.12*	10.56 ±0.21	**45.86** ±0.11*
	Dichloromethane	17.85 ±0.03	16.78 ±0.15	**58.92** ±0.17
	Essential oil	444.30 ±0.58	195.90 ±0.24	484.10 ±0.58
Salvia officinalis	Methanol	40.16 ±0.03	16.36 ±0.03	**58.31** ±0.03
	Water	39.03 ±0.03	17.18 ±0.06	223.50 ±0.01
	Dichloromethane	35.20 ±0.05	**8.55** ±0.12	94.77 ±0.02
	Essential oil	59.07 ±0.07	149.10 ±0.08	171.40 ±0.04
Syzygium aromaticum	Methanol	**11.07** ±0.05	10.21 ±0.04	**58.47** ±0.15
	Water	18.91 ±0.07	**8.43** ±0.17	102.10 ±0.26
	Dichloromethane	28.96 ±0.05	**3.09** ±1.89	**40.95** ±0.19

Spice/herb	Type of extract	DPPH EC_{50} (µg/mL)	ABTS EC_{50} (µg/mL)	FRAP (µg/mL)
	Essential oil	**2.55** ±0.40	**5.81** ±1.35	**5.96** ±0.71
Thymus vulgaris	Methanol	32.73 ±0.04	**7.27** ±0.17	**45.10** ±0.05*
	Water	135.80 ±0.57	27.48 ±0.05	249.80 ±0.09*
	Dichloromethane	74.30 ±0.05	16.01 ±0.04	233.20 ±0.35
	Essential oil	861.50 ±5.05	147.90 ±0.09	537.80 ±0.85
Zingiber officinalis	Methanol	22.18 ±0.05***	**6.85** ±0.25	220.00 ±0.02*
	Water	893.60 ±0.11***	145.90 ±0.33	569.50 ±0.34*
	Dichloromethane	30.41 ±0.05***	**4.15** ±0.57	133.60 ±0.11*
	Essential oil	129.40 ±0.26	275 ±0.82	363.90 ±0.24
	Positive control	10.25	4.88	90.59
	Negative control	> 500	> 500	> 500

Values represent means ± standard deviations for experiments performed in triplicate. Values in bold indicate noteworthy anti-oxidant activity in comparison with the positive control, ascorbic acid. The lower the EC_{50} value, the higher the anti-oxidant activity. Extracts from same spice/ herb with significant differences are shown with * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$).

The results on the anti-oxidant activity of spices or herbal extracts and essential oils demonstrated moderate to weak correlations among the three assays used in the study, except for the high correlation (r = 0.71) between DPPH and ABTS assays for the water extracts (Table 3.2). A weaker correlation existed between the DPPH and FRAP for the water (r = 0.42) and methanol (r = 0.37) extracts. This is contrary to the findings by Ulewicz-Magulska and Wesolowski (2019), who reported a stronger relationship between the two assays (r = 0.94). Meanwhile very weak correlations existed for the dichloromethane extracts between DPPH and ABTS assays (r = 0.10), and between DPPH and FRAP assays (r = 0.13). Furthermore, weak correlation existed between the ABTS and FRAP assays for essential oils.

Table 3.2 Pearson's correlation between anti-oxidant activity of solvent extracts by different anti-oxidant assays

Type of extract	**DPPH/FRAP (r)**	**DPPH/ABTS (r)**	**FRAP/ABTS (r)**
Methanol	0.37	0.005	0.20
Water	0.42	0.71	0.48
Dichloromethane	0.13	0.10	0.31
Essential oil	0.23	0.22	0.16

3.3.2 Anti-oxidant interactive studies

Extracts (12) and essential oils (two) demonstrating promising activity when compared to the positive control were combined (1:1 v/v) to evaluate the interactive anti-oxidant activity using DPPH, ABTS and FRAP assays. Data on the comparative analysis of the anti-oxidant interactions from the different combinations of the extracts and essential oils are presented in Table 3.3. The results show that 3.8% of the combinations were synergistic in the DPPH assay, 7.7% in the FRAP and ABTS assays, whilst 50% of the extracts were additive using the DPPH and FRAP assays, and 19.2% with ABTS assay. Other combinations were indifferent with ABTS (69.2%) and 38.4% with DPPH and FRAP assays. Antagonism was shown for 11.5% of the combinations with DPPH assay and 3.8% for the FRAP assay. None of the combinations were antagonistic following the ABTS assay.

Combining *T. vulgaris* and *Z. officinalis* methanol extracts yielded synergy (ΣFICI of 0.19) in the ABTS assay, whilst the combination was indifferent using the DPPH and FRAP assays.

Synergy was also observed when combining *M. piperita* and *T. vulgaris* methanol extracts using the FRAP (ΣFICI of 0.15) and DPPH assays (ΣFICI of 0.32), meanwhile these combinations were indifferent in the ABTS assay. Other combinations that were synergistic include *R. officinalis* with *S. aromaticum* methanol extracts at ΣFICI of 0.47 following the FRAP method. However, this combination was additive in DPPH and ABTS assays.

This clearly demonstrates that variability exists between methods studied and including different assays, as in this study, provides a better overall assessment of efficacy. Similar observations were made when comparing the assays, whereby *M. officinalis* combined with *S. aromaticum* methanol extracts, and *M. piperita* with *R. officinalis* methanol extracts, displayed an additive effect. Meanwhile, combinations between *R. officinalis* with either *C. zeylanicum* or *Z. officinalis* methanol extracts were indifferent in the three assays. The combination of *M. piperita* methanol extract with either *C. zeylanicum* or *S. aromaticum* extracts were antagonistic using the DPPH method.

Mansour *et al*. (2017), reported synergistic anti-oxidant effects from a combination of *C. zeylanicum* and *S. aromaticum* methanol extracts, with the DPPH assay. Ramadan *et al*. (2017), also reported synergy from combining *C. zeylanicum* with *S. aromaticum* water extracts using the DPPH assay, meanwhile an additive effect was observed in the current study. The differences with their study and the results obtained in this study is that they used the combination index (CI) to evaluate the anti-oxidant interactions meanwhile in the current study the ΣFICI was calculated. Furthermore, in their study, synergy was interpreted as any value < 1, whilst in this study more stringent criteria (ΣFICI ≤0.5 interpreted as synergy) was used.

Purkait *et al*. (2020), reported synergy from combining *C. zeylanicum* and *S. aromaticum* oils using the DPPH assay. However, the combination was indifferent in this study. Synergy was also observed when *R. officinalis* and *Z. officinalis* dichloromethane extracts were combined (ΣFICI of 0.22), when ABTS assay was used. However, the combination was additive in DPPH and FRAP assays. Overall, the results from the interactive study therefore demonstrated that selected crude extracts from culinary herbs and spices can be combined to achieve synergistic or additive anti-oxidant effects, and thus enhance their efficacy.

Table 3.3 Anti-oxidant effect from the combination (1:1 v/v) of active extracts and essential oils with the DPPH, ABTS, and FRAP assays

Extracts / essential oils	**DPPH assay**		**ABTS assay**		**FRAP assay**	
	ΣFICI	**Interpretation**	**ΣFICI**	**Interpretation**	**ΣFICI**	**Interpretation**
C. zeylanicum + *M. officinalis* methanol	0.99	ADD	1.55	I	0.87	ADD
C. zeylanicum + *M. piperita* methanol	30.13	A	1.70	I	0.87	ADD
C. zeylanicum + *R. officinalis* methanol	2.46	I	1.72	I	2.42	I
C. zeylanicum + *S. aromaticum* methanol	2.15	I	1.09	I	0.93	ADD
C. zeylanicum + *T. vulgaris* methanol	0.95	ADD	2.29	I	1.14	I
C. zeylanicum + *Z. officinalis* methanol	1.94	I	1.28	I	0.62	ADD
M. officinalis + *M. piperita* methanol	0.87	ADD	1.19	I	2.36	I
M. officinalis + *R. officinalis* methanol	1.36	I	1.12	I	3.46	I

Extracts / essential oils	DPPH assay		ABTS assay		FRAP assay	
	ΣFICI	**Interpretation**	**ΣFICI**	**Interpretation**	**ΣFICI**	**Interpretation**
M. officinalis + *S. aromaticum* methanol	0.92	ADD	0.83	ADD	0.55	ADD
M. officinalis + *T. vulgaris* methanol	0.72	ADD	1.12	I	0.79	ADD
M. officinalis + *Z. officinalis* methanol	1.39	I	1.65	I	0.83	ADD
M . piperita + *R. officinalis* methanol	0.51	ADD	0.55	ADD	0.90	ADD
M. piperita + *S. aromaticum* methanol	16.93	A	1.04	I	0.93	ADD
M. piperita + *T. vulgaris* methanol	0.32	S	1.03	I	0.15	S
M. piperita + *Z. officinalis* methanol	0.54	ADD	1.82	I	0.67	ADD
R. officinalis .+ *S. aromaticum* methanol	0.86	ADD	1.00	ADD	0.47	S
R. officinalis + *T. vulgaris* methanol	0.90	ADD	1.16	I	1.19	I

Extracts / essential oils	DPPH assay		ABTS assay		FRAP assay	
	ΣFICI	**Interpretation**	**ΣFICI**	**Interpretation**	**ΣFICI**	**Interpretation**
R. officinalis + *Z. officinalis* methanol	1.07	I	1.08	I	1.03	I
S. aromaticum + *T. vulgaris* methanol	1.04	I	0.96	ADD	1.15	I
S. aromaticum + *Z. officinalis* methanol	2.07	I	1.03	I	0.51	ADD
T. vulgaris + *Z. officinalis* methanol	1.22	I	0.19	S	1.28	I
C. zeylanicum + *S. aromaticum* water	0.78	ADD	1.48	I	1.49	I
R. officinalis + *S. aromaticum* dichloromethane	0.79	ADD	0.84	ADD	1.17	I
R. officinalis + *Z. officinalis* dichloromethane	0.51	ADD	0.22	S	0.65	ADD

Extracts / essential oils	DPPH assay		ABTS assay		FRAP assay	
	ΣFICI	**Interpretation**	**ΣFICI**	**Interpretation**	**ΣFICI**	**Interpretation**
S. aromaticum + *Z. officinalis* dichloromethane	1.66	I	2.80	I	0.80	ADD
C. zeylanicum + *S. aromaticum* essential oil	18.29	A	4.33	A	21.51	A

ΣFICI: Sum of Fractional inhibitory concentration index; ADD: additive; S: synergy; I: indifferent; A: antagonism

3.3.3 Design of Experiments (DOE) data analysis and model verification

Three extracts (*M. piperita*, *T. vulgaris* and *Z. officinalis*) were selected for DOE studies based on a synergistic outcome from the combination/interaction studies. This was done in order to determine the optimum ratio at which a formulation of the three extracts can be combined to obtain the highest anti-oxidant effect. Twelve experimental runs with the combinations indicated in Table 3.4 were obtained from the design and the EC_{50} values obtained for each combination using the DPPH, ABTS and FRAP assays were imported into MODDE® software. The data in Table 3.4 was modelled and the replication plot, histogram, summary of fit, coefficient plot, residual N- plot, observed versus predicted plot and the response contour plots were obtained and used to assess the suitability of the PLS model for predictions.

3.3.3.1 Summary of fit plot for the anti-oxidant combination

The linear model generated was fitted against the data and the response is shown in the summary of the fit plot in Figure 3.1, which provides the information on the strength and robustness of the model.

The R2 value (0.91) signified a low variation in the response and a strong fit between the data and the model, meanwhile, the Q2 value of 0.71 (ideally > 0.5) demonstrated a high predictive power of the model. Furthermore, the model demonstrated a strong validity of 0.44, which is greater than 0.25 as required for good models. The model reproducibility of 0.97 is far greater than the requisite value of 0.50, indicating good model design and low pure error (Figure 3.1).

Table 3.4 The worksheet used to fit the model for anti-oxidant combinations

Exp No	Exp Name	Run Order	*M. piperita*	*Z. officinalis*	*T. vulgaris*	EC_{50} (µg/mL)
1	N1	3	1	0	0	53.40
2	N2	2	0	1	0	220.00
3	N3	8	0	0	1	45.10
4	N4	5	0.67	0.17	0.17	62.48
5	N5	9	0.17	0.67	0.17	146.10
6	N6	6	0.17	0.17	0.67	92.00
7	N7	1	0	0.50	0.50	111.90
8	N8	11	0.50	0	0.50	7.25
9	N9	4	0.50	0.50	0	68.71
10	N10	10	0.33	0.33	0.33	94.50
	N11	7	0.33	0.33	0.33	96.10
12	N12	12	0.33	0.33	0.33	85.25

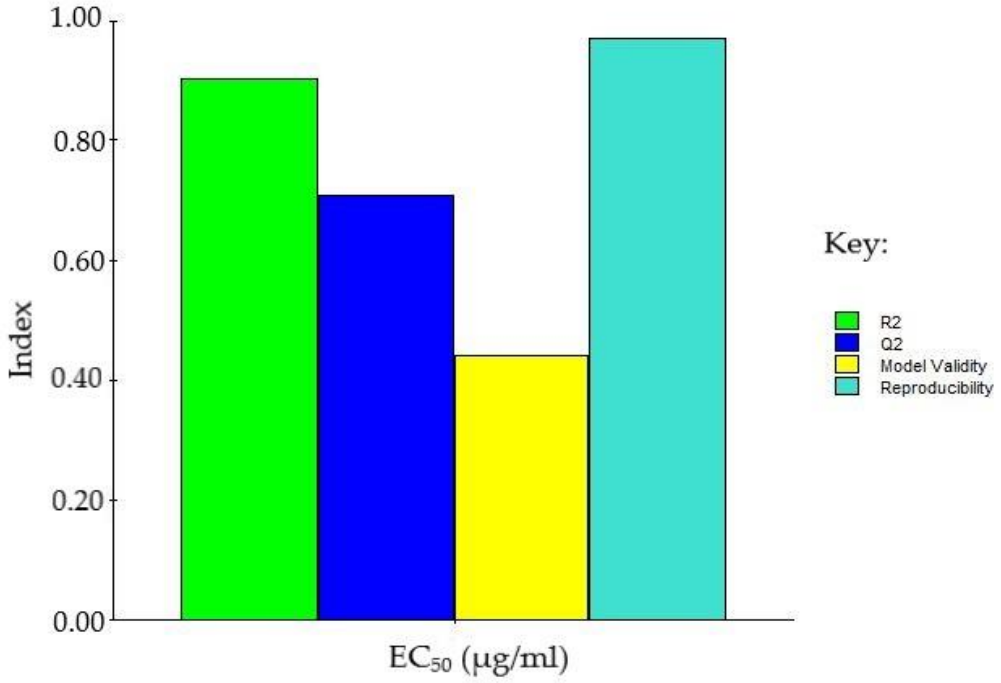

Figure 3.1 Summary of the fit plot for EC_{50} of methanol extract mixtures. R2: the model fit; Q2: an estimate of the future prediction precision; Model validity: a test of diverse model problems; Reproducibility: the variation of the replicates compared to overall variability

3.3.3.2 Coefficient plot for the anti-oxidant combination

The centre-scaled coefficients of each term in the model were used to estimate the significance of the factors to the desired response (EC_{50}). It was determined that *M. piperita*, *Z. officinalis* and *T. vulgaris* methanol extracts and the interactions between *M. piperita* and *Z. officinalis* were important factors to the model outcome, whereas interactions between *T. vulgaris* with either *M. piperita* or with *Z. officinalis* were found to be non-significant factors (Figure 3.2). Typically, large regression coefficients represent factors with a large contribution to the model response, such as *M. piperita* extract. While regressions with a positive number denote a positive contribution towards the response such as for the *Z. officinalis* extract and a negative number denotes a negative response.

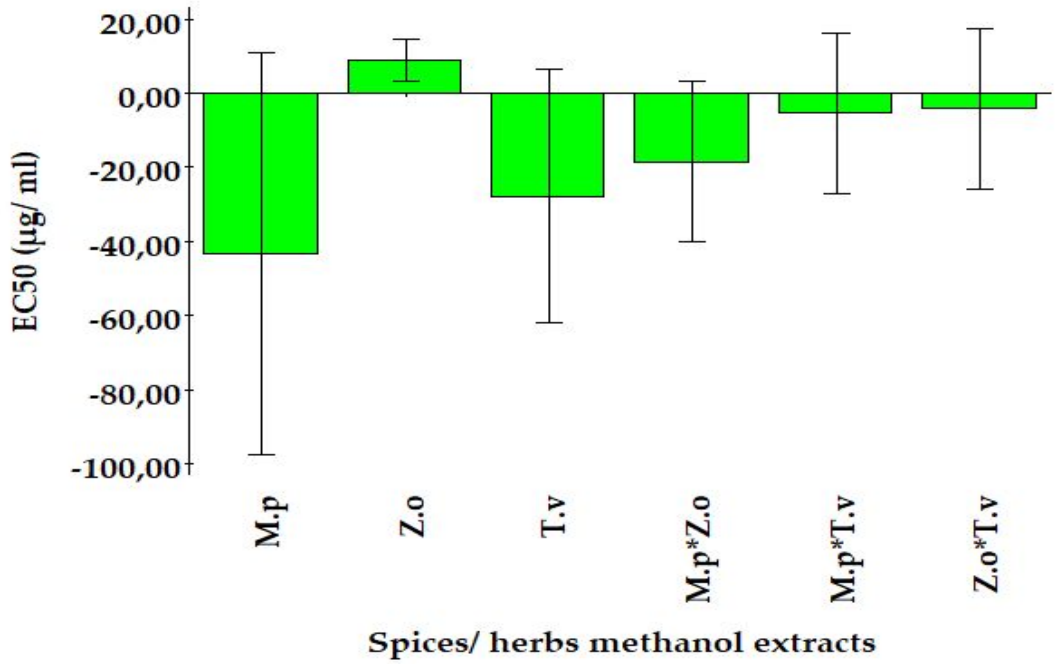

Figure 3.2 A coefficient plot showing the effect of the three factors; *M. piperita* (MP), *Z. officinalis* (Z.o), and *T. vulgaris* (T.v) on the anti-oxidant effect of the mixture (*)

3.3.3.3 Response contour plot for the anti-oxidant combination

The response contour plot allows for the identification of ratios of the combinations that demonstrate the best and worst overall anti-oxidant effect (Figure 3.3). The model generated predicted various ratios at which spice/herb extracts can be combined to produce an optimal anti-oxidant effect. Extract combinations in the red region, would produce high EC_{50} values thus low anti-oxidant activity. These combinations generally comprise of higher proportions of *Z. officinalis* and *T. vulgaris*, and low *M. piperita*. On the opposite end of the colour spectrum, combinations in the blue region were predicted to produce lower EC_{50} values and hence higher anti-oxidant activity. In the blue region predicted EC_{50} values are given with ratio of combinations with high *M. piperita*, low *T. vulgaris* and a wider range of *Z. officinalis* content.

The optimizer function was then used to determine the best ratio at which the extracts should be combined to obtain optimum synergistic anti-oxidant effects. Five combinations in the blue region of the response contour plot were selected as displayed in Table 3.5, to validate the model predictions. The DPPH, ABTS and FRAP methods were used for the model design.

However, the FRAP method was chosen as it produced the best model predictions compared to DPPH and ABTS methods.

In this study, a statistically significant model was produced, which provided the best combination ratios of the extracts, using limited time and resources. The optimizer showed the optimal anti-oxidant activity achieved with a mixture of *M. piperita* (55%), *T. vulgaris* (44%) and *Z. officinalis* (1 %) at EC_{50} value of 39.59 µg/mL using FRAP method. The results from the validation experiments confirmed the reliability and fitness of the model because the correlation coefficient (r= 0.76), calculated using Microsoft Excel shows strong correlation (Schober *et al*., 2018) between the experimental and the predicted EC_{50} values for all the combinations (Table 3.5).

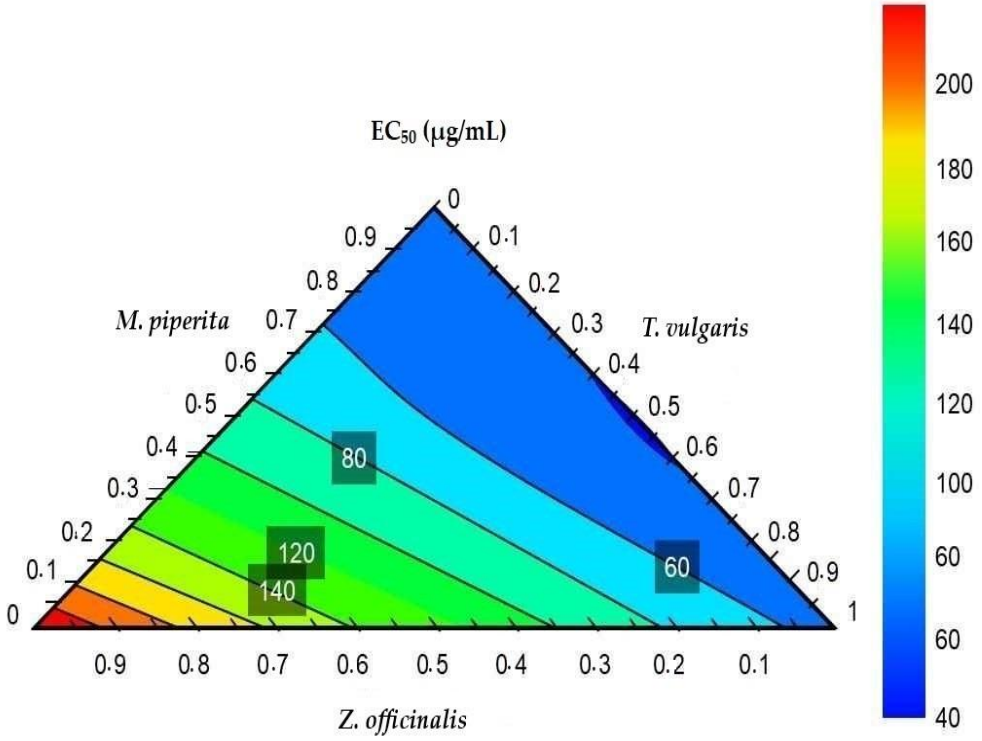

Figure 3.3 Response contour plot showing the extract combinations and predicted responses (EC_{50} values). The dark blue regions gives an indication of different ratios wheredby the three extracts should be combined to generate desired EC_{50} values. Combinations in other regions (e.g. far red region) will give higher EC_{50} values, which are undesirable.

Table 3.5 DOE Model validation for experimental runs using FRAP assay

M. piperita	***Z. officinalis***	***T. vulgaris***	**Predicted**	**Experimental validation**
0.55	0.01	0.44	39.59	47.61
0.82	0.15	0.02	52.99	57.26
0.71	0.26	0.03	59.34	59.60
0.38	0.08	0.54	46.89	40.87
0.48	0.10	0.42	47.46	47.56

3.4 Summary

- The results of this study demonstrated the anti-oxidant variability between crude extracts and essential oils from culinary herbs and spices using different *in vitro* methods (DPPH, ABTS and FRAP assays). Generally, the methanol extract demonstrated the better anti-oxidant activity compared to the other extracts.
- The *C. zeylanicum* methanol and water extracts were found to be more effective DPPH radical scavengers compared to the standard anti-oxidant, ascorbic acid.
- The most superior ABTS radical scavengers were *S. aromaticum* and *Z. officinalis* dichloromethane extracts, with activity greater than the activity of ascorbic acid.
- The most effective extracts in reducing ferric iron were *R. officinalis, C. zeylanicum, M. officinalis, T. vulgaris, M. piperita* and *S. aromaticum* methanol extracts.
- *Cinnamomum zeylanicum, M. piperita* and *S. aromaticum* essential oils were found to be effective anti-oxidants.
- The results from the different assays could not be correlated, except for the relationship between DPPH and FRAP assays for the water extract, which showed a high positive correlation.
- The 1:1 combination displayed synergistic, additive, indifferent and antagonistic effects. Synergism was shown by combining *T. vulgaris* with *Z. officinalis* (methanol extract) using the ABTS assay (ΣFIC index of 0.19), *R. officinalis* with *S. aromaticum* (methanol extracts) using the FRAP assay (ΣFIC index of 0.47), and from combining *T. vulgaris* and *M. piperita* methanol extracts using the DPPH and FRAP assays (ΣFIC index of 0.32 and 0.15

respectively). Synergy was also observed when *R. officinalis* and *Z. officinalis* dichloromethane extracts were combined using the ABTS assay (ΣFIC index of 0.22).

- Using the DOE, a model that could easily predict the ratios at which the spice extracts can be combined to achieve the highest anti-oxidant effect was produced. The optimal anti-oxidant activity (EC_{50} value of 39.59 µg/mL) was achieved with a mixture of *M. piperita* (55%), *T. vulgaris* (44%) and *Z. officinalis* (1 %).

CHAPTER 4

A Combinational Approach to Testing Antimicrobial Efficacy of Some Common Herbs and Spices

4.1 Introduction

There are numerous studies reporting on the antibacterial activities of spice and herb extracts, as well as essential oils, towards different types of micro-organisms, including food-borne pathogens (Sethi *et al*., 2013; Witkowska *et al*., 2013; Mith *et al*., 2014; Pandey *et al*., 2014; Hassanen *et al*., 2015; Sah *et al*., 2020; Karagözlü *et al*., 2021; Yadav *et al*., 2022). Spices and herbs such as *Allium sativum* (garlic), *Cinnamomum zeylanicum* (cinnamon), *Syzygium aromaticum* (clove), *Thymus vulgaris* (thyme), and *Salvia officinalis* (sage) to name a few, were reported to possess antibacterial activity against a range of Gram-positive and Gram-negative food-borne and spoilage bacteria (Pavithra *et al*., 2014; Liu *et al*., 2017). Although the individual effects of these plant products are widely documented, fewer studies have reported on their combinatory antibacterial activity (Das *et al*., 2012; Bag and Chattopadhyay, 2015; García-Díez *et al*., 2017; Maharjan *et al*., 2019).

Spices and herbs are commonly used in combination for their medicinal as well as flavour enhancing effects. Traditional knowledge points to synergistic interactions in polyherbal formulations which enhances the biological effects compared to single-herb extracts. This also allows sufficiently lower concentrations of extracts to be used, which has the potential to minimise adverse effects. It is therefore important to channel more research effort towards polyherbal formulations, in order to document as much as possible scientific evidence which supports their popular use.

To determine the herb combinations which produce the best antimicrobial activity is a huge challenge in a laboratory setting. Practically, it is time consuming and wasteful to combine different extracts randomly, and run many experiments with the hope of a favourable outcome. Furthermore, when experiments are based on trial and error, without proper experimental design, there is a possibility of low reproducibility, reliability, and versatility (Das *et al*., 2016). The use of design of experiments (DOE) makes it possible to find the optimal combination

ratios through predictive statistical models, making it possible to obtain the best antimicrobial efficacy. The DOE models aim to produce optimized mixtures, with a limited number of experimental runs, thus saving time and resources. In the present study, the broth microdilution method was used to determine the minimum inhibitory concentrations (MICs) of individual crude extracts and essential oils of selected culinary herbs and spices against a selection of Gram-positive and Gram-negative bacteria. Optimization of the synergy potential of various herb combinations was subsequently performed using the DOE approach.

4.2 Materials and methods

Collection of spices and herbal material and essential oils as well as preparation of crude extracts were undertaken according to the procedures outlined in Chapter 2, Section 2.1.

4.2.1 Micro-organisms and growth conditions

Six pathogens associated with food borne illnesses and gastrointestinal tract infections were used in the study to evaluate the antimicrobial effects of the selected herbs and spices. The strains included *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Enterobacter faecalis* ATCC 29212, *Shigella sonnei* ATCC 9290, *Escherichia coli* ATCC 8739 and *Salmonella typhimurium* ATCC 1403 (Davies Diagnostics, South Africa). Stock cultures were kept at -20°C, and then subcultured onto/into Tryptone Soya agar and broth (TSA/TSB), and incubated at 37°C for 24 hrs.

4.2.2 Minimum inhibitory concentration (MIC)

Broth micro-dilution assays were performed as described by Eloff (1998) and the NCCLS (2003) to determine the MIC of extracts and oils against the test organisms. Although the herb and spice selection has been well studied in the past, base-line MIC values were obtained for all samples prior to combination studies, as variability may exist between present samples in the study and that found in the literature. Extracts and oils were diluted to a concentration of 32 mg/mL using acetone as a diluent. A volume of 100 µL of sterile broth was plated out in duplicate in each of the wells of a 96- well microtiter plate. Extracts and oils were added as a

volume of 100 µL, followed by serial dilutions to yield concentrations of 8, 4, 2, 1, 0.5, 0.25, 0.12, 0.06 mg/mL.

Firstly, test cultures were diluted using sterile Tryptone Soya broth at a 1: 100 dilution in order to achieve an approximate concentration of 1 x 10^6 colony forming units (CFU)/ mL. Test cultures were then added as a 0.5 McFarland turbidity standard to all the wells of their respective microtiter plates, at a volume of 100 µL. The plates were sealed with a sterile adhesive sealing film to prevent loss of volatile components and then incubated at 37ºC for 24 hrs. After incubation, 40 µL of *p*-iodonitrotetrazolium chloride (INT) at concentration of 0.40 mg/mL was added to each well. Viable micro-organisms interact with INT to create a colour change from clear to a red/ purple in colour. The lowest dilution with no colour change was considered the MIC for that extract or oil.

For all antimicrobial assays positive and negative controls were included, with ciprofloxacin (0.01 mg/mL) used as a positive control. Acetone (32 mg/mL), which was used as a diluent to dissolve the test samples, was used as a negative control. Assays were done in triplicate on consecutive occasions and the mean values noted.

4.2.3 Sum of fractional inhibitory concentration index (ΣFICI)

The sum of the fractional inhibitory concentration index (ΣFICI) was used to measure interactions from different 1:1 combination of herbs, spices extracts, and essential oils with promising antibacterial effects based on the results from MIC study. Samples of A and B (50 µL) for the combinations were plated out in each of the wells, which were marked accordingly, and the same procedure for determining the MIC was followed. The ΣFICI for each of the combinations were calculated using Equation 4.1.

$$(\Sigma FICI) = FIC\,(i) + FIC\,(ii)$$

$$FIC(i) = \frac{\text{MIC (a) in combination with (b)}}{\text{MIC (a) independently}}$$

$$FIC(ii) = \frac{\text{MIC (b) in combination with (a)}}{\text{MIC (b) independently}}$$

Equation 4.1

Where (a) is the MIC, of one spice extract or essential oil in the combination and (b) is the MIC of the other extract or essential oil. The ΣFICI for each combination were interpreted as synergy where the ΣFICI less than or equal to 0.5. Additive effects were considered for values greater than 0.5 but less than or equal to 1.0. For indifference, the ΣFICI must be greater than 1.0 but less than or equal to 4.0 and for antagonism an ΣFICI was considered for values greater than 4.0 (Van Vuuren and Viljoen, 2011).

4.2.4 Experimental design using the DOE model to determine effective antibacterial combinations

The DoE model was prepared and evaluated using MODDE 9.1® software as mentioned in Chapter 3, Section 3.2.6. A prediction contour plot was generated which showed the average prediction (point estimate prediction) for every possible combination of the tested extracts. Predictions with lowest MIC values (0.17–0.38 mg/mL) were selected for the model validation. These model predictions were then verified by carrying out additional broth micro-dilution assays as described in Section 4.2.2. The assay was done to determine the MIC values for the experimental validation, which were then compared to the predicted MIC values.

4.3 Results and discussion

4.3.1 Antibacterial activity of individual extracts and essential oils

The antibacterial activity results of crude extracts and neat essential oils are presented in Table 4.1. For the extracts MIC values of 0.10–0.62 mg/mL were regarded as moderate antibacterial activity, whereas weaker activity were considered for values > 0.62 mg/mL (Kuete *et al*., 2010).

All the spice/herb extracts exhibited moderate, weak or no antimicrobial activity against the tested bacteria. In comparison to other extracts, *S. officinalis* methanol and dichloromethane extracts were found to be more effective in inhibiting the growth of *B. cereus* and *S. sonnei*,

respectively at MIC value of 0.25 mg/mL, followed by *R. officinalis* dichloromethane extract at MIC value of 0.33 mg/mL against *S. sonnei*. Generalić *et al*. (2012), made similar observation on the activity of *S. officinalis* extract against *B. cereus* (MIC value of 0.23 mg/mL). Although in their study ethanol was used as a solvent for extraction. It was observed that *C. zeylanicum* and *S. aromaticum* methanol extracts inhibited *E. faecalis* at MIC value of 0.42 mg/mL. Both *A. sativum* and *S. aromaticum* dichloromethane extracts inhibited the growth of *E. faecalis* at MIC value of 0.46 mg/mL. In addition, *S. aromaticum* methanol and dichloromethane extracts showed moderate activity against *S. aureus* (MIC value of 0.62 mg/mL). It was further noted that *R. officinalis* and *S. aromaticum* methanol extracts could inhibit the growth of *E. coli* at MIC value of 0.50 mg/mL, whilst *R. officinalis* methanol extract also inhibited *B. cereus* at the same concentration.

It was observed that *C. zeylanicum, O. marjorana*, *R. officinalis*, and *S. aromaticum* dichloromethane inhibited *B. cereus*, *S. aureus,* and *S. sonnei,* respectively all at MIC value of 0.50 mg/mL. The methanol and dichloromethane extracts of *S. aromaticum* inhibited *S. aureus* and *B. cereus* at an MIC value of 0.62 mg/mL. The inhibitory effect of *S. aromaticum* methanol extract on *S. aureus* was also reported by Betoni *et al*. (2006), and Pandey and Singh (2011). The MIC value of 0.36 mg/mL was more or less congruent to the values obtained in these studies. It was also noted that *C. zeylanicum* dichloromethane extracts inhibited *E. coil* and *S. typhimurium* at MIC value of 0.67 mg/mL, whilst *S. aromaticum* methanol extract inhibited *B. cereus* at the same concentration. Generally, the water extracts displayed weaker or no antibacterial activity. Similar observations were made from previous studies (Weerakoddy *et al*., 2010; Witkowska *et al*., 2012).

For the essential oils, MIC values of 0.10–0.50 mg/mL were regarded as strong antibacterial activity, whereas the oils with MIC values ranging from 0.50–1.00 mg/mL showed moderate antibacterial activity (Freires *et al*., 2015) Therefore, the essential oils of *C. zeylanicum, C. citratus, L. nobilis, M. officinalis, R. officinalis, S. aromaticum*, and *Z. officinale* demonstrated strong antibacterial activity against *B. cereus* (MIC values ranging from 0.25–0.50 mg/mL), whereas *A. sativum* had stronger antibacterial effect on *E. faecalis*. The highest inhibition was shown by *C. zeylanicum* and *C. citratus* essential oils against *B. cereus* (MIC values of 0.25 mg/mL). The results are congruent with previous studies (Unlu *et al*., 2010; Nanasombat *et al*., 2011).

Table 4.1 Antibacterial activity of 17 culinary herbs and spice crude extracts and essential oils expressed as MICs (mg/mL)

Spice/herb	Type of extract	*B. cereus* ATCC 11778	*E. coli* ATCC 8739	*E. faecalis* ATCC 29212	*S. aureus* ATCC 25923	*S. sonnei* ATCC 9220	*S. typhimurium* ATCC 1403
Allium sativum	Methanol	6.67	5.33	4.00	>8.00	3.00	>8.00
	Water	4.00	6.67	4.00	>8.00	4.00	3.00
	Dichloromethane	2.00	4.00	**0.46**	3.33	3.33	1.27
	Essential oil	2.00	4.00	1.00	4.00	4.00	2.00
Anethum graveolens	Methanol	2.00	>8.00	4.00	4.00	2.67	2.67
	Water	4.67	4.00	6.00	4.00	4.00	8.00
	Dichloromethane	2.00	>8.00	2.67	3.33	2.67	2.67
	Essential oil	2.00	2.67	2.00	4.00	2.00	2.00
Apium graveolens	Methanol	2.67	2.67	2.67	4.00	4.00	2.00
	Water	2.67	6.00	2.67	>8.00	4.00	4.00
	Dichloromethane	5.33	8.00	5.33	2.67	2.00	2.00
	Essential oil	0.67	2.00	4.00	2.67	4.00	2.00
Cinnamomum zeylanicum	Methanol	2.67	4.00	**0.42**	2.67	4.00	>8.00
	Water	2.67	4.00	2.67	>8.00	4.00	2.00
	Dichloromethane	**0.50**	0.67	1.00	1.00	1.67	0.67
	Essential oil	**0.25**	1.00	0.67	1.00	1.00	1.00
Coriandrum sativum	Methanol	6.67	>8.00	8.00	8.00	>8.00	2.00
	Water	2.67	4.00	2.67	8.00	>8.00	2.67
	Dichloromethane	2.00	2.67	2.67	2.00	2.67	2.00
	Essential oil	0.67	1.00	0.83	2.00	2.00	2.00

Spice/herb	Type of extract	*B. cereus* ATCC 11778	*E. coli* ATCC 8739	*E. faecalis* ATCC 29212	*S. aureus* ATCC 25923	*S. sonnei* ATCC 9220	*S. typhimurium* ATCC 1403
	Methanol	4.00	>8.00	>8.00	5.33	>8.00	2.67
Cymbopogon	Water	5.33	5.33	2.00	8.00	>8.00	8.00
citratus	Dichloromethane	1.33	2.00	>8.00	1.00	2.00	2.67
	Essential oil	**0.25**	1.00	1.00	>8.00	2.00	2.00
	Methanol	>8.00	5.33	4.00	8.00	8.00	>8.00
Laurus	Water	5.33	8.00	2.67	5.33	2.67	2.67
nobilis	Dichloromethane	1.33	5.33	2.67	2.67	2.67	2.00
	Essential oil	**0.50**	2.00	2.67	4.00	2.67	4.00
	Methanol	2.00	4.00	2.67	5.33	>8.00	2.67
Melissa	Water	1.67	2.67	1.67	2.67	2.67	2.67
officinalis	Dichloromethane	2.00	8.00	2.00	6.67	4.00	8.00
	Essential oil	**0.50**	2.00	1.33	>8.00	2.00	2.00
	Methanol	4.00	2.00	2.00	8.00	2.33	>8.00
Mentha	Water	2.00	4.00	6.67	>8.00	4.00	2.67
piperita	Dichloromethane	2.67	8.00	2.00	2.67	2.67	2.67
	Essential oil	1.00	2.00	4.00	2.00	4.00	2.00
	Methanol	2.67	4.00	>8.00	6.00	2.67	2.00
Ocimum	Water	2.67	6.00	6.67	>8.00	4.00	4.00
basilicum	Dichloromethane	2.00	8.00	6.00	2.67	2.00	2.00
	Essential oil	1.17	2.00	>8.00	4.00	4.00	2.00
Origanum	Methanol	2.00	8.00	1.33	4.00	8.00	2.67
marjorana	Water	2.67	5.33	2.67	4.00	8.00	2.67

Spice/herb	Type of extract	*B. cereus* ATCC 11778	*E. coli* ATCC 8739	*E. faecalis* ATCC 29212	*S. aureus* ATCC 25923	*S. sonnei* ATCC 9220	*S. typhimurium* ATCC 1403
	Dichloromethane	6.00	6.67	**0.50**	2.67	2.00	4.00
	Essential oil	0.67	1.33	4.00	4.00	3.33	>8.00
	Methanol	2.00	4.00	2.00	4.00	5.33	>8.00
Petroselinum	Water	4.00	5.33	4.00	4.00	4.00	>8.00
crispum	Dichloromethane	2.00	8.00	2.67	2.67	4.00	2.00
	Essential oil	0.83	2.00	3.33	4.00	4.00	2.00
	Methanol	**0.50**	**0.50**	0.80	1.33	1.00	2.00
Rosmarinus	Water	2.00	4.00	>8.00	4.00	>8.00	5.33
officinalis	Dichloromethane	2.67	1.33	1.00	**0.50**	**0.33**	4.00
	Essential oil	**0.50**	2.00	3.33	8.00	>8.00	2.00
	Methanol	**0.25**	**0.42**	0.80	2.00	2.67	2.00
Salvia	Water	6.00	4.00	4.00	2.00	>8.00	2.00
officinalis	Dichloromethane	1.00	2.67	1.00	**0.50**	**0.25**	1.67
	Essential oil	1.00	2.00	2.00	4.00	3.33	2.00
	Methanol	0.67	**0.50**	**0.42**	**0.62**	6.67	2.00
Syzygium	Water	5.33	1.00	1.67	2.00	2.00	2.67
aromaticum	Dichloromethane	2.67	1.33	**0.46**	**0.62**	2.00	2.00
	Essential oil	**0.42**	1.00	1.00	2.00	2.00	1.00
	Methanol	2.00	>8.00	2.00	2.67	2.67	2.67
Thymus	Water	5.33	5.33	>8.00	4.00	>8.00	2.67
vulgaris	Dichloromethane	2.00	8.00	1.67	2.00	2.00	2.67
	Essential oil	1.00	1.00	3.33	2.00	4.00	2.00

Spice/herb	**Type of extract**	***B. cereus* ATCC 11778**	***E. coli* ATCC 8739**	***E. faecalis* ATCC 29212**	***S. aureus* ATCC 25923**	***S. sonnei* ATCC 9220**	***S. typhimurium* ATCC 1403**
	Methanol	2.00	4.00	2.00	2.00	2.67	4.00
Zingiber	Water	2.67	4.00	2.00	8.00	4.00	2.00
officinalis	Dichloromethane	1.33	2.00	1.33	2.67	2.00	1.67
	Essential oil	**0.42**	1.00	1.00	4.00	2.00	1.00
Positive control	Ciprofloxacin (µg/mL)	0.62	0.31	0.62	0.16	0.62	0.62
Negative control	Acetone in water (mg/mL)	>8	>8	>8	>8	>8	>8

Values represent mean MICs for experiments performed in triplicates. Values in bold indicate moderate antimicrobial activity for the crude extracts (MIC values of 0.10–0.62 mg/mL), and strong antimicrobial activity for the essential oils (MIC values of 0.10–0.50 mg/mL).

Other essential oils demonstrated moderate to weak activity. *Apium graveolens*, *C. sativum*, and *O. marjorana* essential oils inhibited *B. cereus* at 0.67 mg/mL, whereas *M. piperita* oil, *P. crispum* oil, *S. officinalis* oil, and *T. vulgaris* oil inhibited the bacteria at MIC values of 0.83 and 1.00 mg/mL. Linde *et al*. (2016), reported MIC value of 1mg/mL for *P. crispum* oil against *B. cereus*, which relates to the findings in this study. Chaibi *et al*. (1997), reported on the antibacterial activity of *R. officinalis* oil against *B. cereus* with MIC value of 0.23 mg/mL. Their results are congruent with the results in this study.

Tomar *et al*. (2020), reported the activity of *L. nobilis* oil against *B. cereus* at MIC value of 0.75 mg/mL. Their findings are consistent with the current study. The inhibitory effect of *M. officinalis* and *O. marjorana* essential oils was also reported by other researchers (Hussain *et al*., 2011; Hajlaoui *et al*., 2016). The MIC values of 0.08 and 0.20 mg/mL differed from the values obtained in this study. Furthermore, *A. sativum* oil, *C. citratus* oil, *C. sativum* oil, *S. aromaticum* oil, *T. vulgaris* oil, and *Z. officinale* oil showed moderate activity against *E. coli* and *E. faecalis* (MIC values ranging from 0.83–1.00 mg/mL).

4.3.2 Antibacterial interactive studies

All the extracts displaying moderate activity (MIC values of 0.10–0.62 mg/mL), and the essential oils demonstrating strong activity (MIC values of 0.10–0.50 mg/mL) were subsequently selected for the interactive studies. Furthermore, extracts with MIC values closest to moderate activity (0.67 mg/mL) were also selected for the interactive study. A total of 14 extracts combinations (1:1), and 21 essential oils combinations (1:1) were evaluated for antimicrobial interaction using the broth microdilution assay. The results of the antimicrobial interactions are presented in Table 4.2. Four possible effects or outcomes are reported from the combination studies as determined by the sum of fractional inhibitory concentration index (ΣFICI). The combinations were classified as either synergistic (ΣFICI is ≤ 0.5), antagonistic (ΣFICI is > 4.0), additive (ΣFICI is > 0.5 but ≤1.0) or indifferent (ΣFICI is > 0.5 but ≤1.0) (Van Vuuren and Viljoen, 2011).

The data from interactive study has shown antibacterial enhancement (synergistic and additive effects) Out of the 14 extract combinations tested, two displayed synergistic interactions (ΣFICI index of 0.25 and 0.31), and two combinations were additive (ΣFICI value of 0.88 and 1.00). The remaining combinations were either indifferent (eight) or antagonistic (two).

Synergy was observed from combination of *R. officinalis* and *S. aromaticum* methanol extracts (ΣFICI value of 0.25), and from combination of *R. officinalis* and *S. officinalis* methanol extracts (ΣFICI value of 0.31) all against *B. cereus*.

The combination of *R. officinalis* and *S. aromaticum* methanol extracts had an additive effect against *E. coli* (ΣFICI value of 1.00), whilst the combination of *R. officinalis* and *S. officinalis* dichloromethane extracts was additive against *S. sonnei* (ΣFICI value of 0.88). Witkowska *et al*. (2013), reported an additive effect from combining alcoholic extracts of *Origanum vulgare* (oregano) with *R. officinalis* and *S. officinalis* against *S. aureus*. In the present study, none of the evaluated essential oil combinations exhibited synergy or additive effects. However, some studies reported additive activity when *S. aromaticum* and *R. officinalis* oils were combined against *E. coli* and *S. aureus* (Fu *et al*., 2007).

Bag and Chattopadhyay, (2015), reported synergy from combining *C. sativum* and *C. cyminum* essential oils against *B. cereus, E. coli,* and *S. aureus*, with (ΣFIC values of 0.25 and 0.50 respectively). Bassolé and colleagues also reported synergism from combining *M. piperita* and *O. basilicum* essential oils against *S. aureus, E. faecalis, L. monocytogenes, E. coli*, and *Shigella dysenteriae* with ΣFIC values of 0.36, 0.37, 0.27, 0.29, and 0.35, respectively, whilst the combination was additive against *S. typhimurium* at ΣFIC value of 0.69 (Bassolé *et al*., 2010). Combining *O. vulgare* with either *O. marjorana*, *R. officinalis*, *T. vulgaris* or *O. basilicum* oil yielded additive effects against *B. cereus* and *E. coli* (Gutierrez *et al*., 2008 and 2009; Lv *et al*., 2011).

Kon and Rai (2012), evaluated the antibacterial activity of *T. vulgaris* oil in combination with *C. zeylanicum*, *M. piperita*, and *M. officinalis* oils against *E. coli* and *S. aureus*. Synergistic interactions were observed when *T. vulgaris* and *C. zeylanicum* oils were combined and tested against *S. aureus* (ΣFIC value of 0.26), and when *T. vulgaris* and *M. officinalis* oils were combined and tested against *E. coli* (ΣFIC value of 0.34). Additive effects were observed when *T. vulgaris* and *M. piperita* oils were combined and tested against *E. coli* and *S. aureus* with ΣFIC values of 0.55. Overall, the results from the interactive study demonstrated that selected crude extracts from culinary herbs and spices can be combined to achieve synergistic or additive antibacterial effects.

Table 4.2 Antibacterial interactions from 1:1 combination of active extracts and essential oils against selected pathogens

Name of pathogen	Extracts / essential oils 1:1 combination	ΣFICI	Interpretation
B. cereus ATCC 11778	*R. officinalis* + *S. aromaticum* (methanol)	0.25	S
	R. officinalis + *S. officinalis* (methanol)	0.31	S
	S. aromaticum + *S. officinalis* (methanol)	1.31	I
E. coli ATCC 8739	*R. officinalis* + *S. aromaticum* (methanol)	1.00	ADD
	R. officinalis + *S. officinalis* (methanol)	1.83	I
	S. aromaticum + *S. officinalis* (methanol)	2.19	I
E. faecalis ATCC 29212	*C. zeylanicum* + *S. aromaticum* (methanol)	2.50	I
	A. sativum + *O. marjorana* (dichloromethane)	4.00	A
	A. sativum + *S. aromaticum* (dichloromethane)	2.00	I
	O. marjorana + *S. aromaticum* (dichloromethane)	4.00	A
S. aureus ATCC 25923	*R. officinalis* + *S. officinalis* (dichloromethane)	3.49	I
	R. officinalis + *S. aromaticum (*dichloromethane)	1.81	I

Name of pathogen	Extracts / essential oils 1:1 combination	ΣFICI	Interpretation
	S. officinalis + *S. aromaticum* (dichloromethane)	2.40	I
S. sonnei ATCC 9290	*R. officinalis* + *S. officinalis* (dichloromethane)	0.88	ADD
B. cereus ATCC 11778	*C. citratus* + *R. officinalis* essential oils	6.00	A
	C. citratus + *M. officinalis* essential oils	6.00	A
	C. citratus + *L. nobilis* essential oils	6.00	A
	C. citratus + *Z. officinalis* essential oils	6.38	A
	C. citratus + *C. zeylanicum* essential oils	4.00	I
	C citratus + *S. aromaticum* essential oils	6.38	A
	R. officinalis + *M. officinalis* essential oils	5.33	A
	R. officinalis + *L. nobilis* essential oils	4.00	I
	R. officinalis + *Z. officinalis* essential oils	8.76	A
	R. officinalis + *C. zeylanicum* essential oils	6.00	A
	R. officinalis +	4.38	A

Name of pathogen	Extracts / essential oils 1:1 combination	ΣFICI	Interpretation
	S. aromaticum essential oils		
	M. officinalis + *L. nobilis* essential oils	4.00	I
	M. officinalis + *Z. officinalis* essential oils	4.38	A
	M. officinalis + *C. zeylanicum* essential oils	3.00	I
	M. officinalis + *S. aromaticum* essential oils	3.98	I
	L. nobilis + *Z. officinalis* essential oils	6.43	A
	L. nobilis + *C. zeylanicum* essential oils	3.00	I
	L. nobilis + *S. aromaticum* essential oils	4.38	A
	Z. officinalis + *C. zeylanicum* essential oils	4.25	A
	Z. officinalis + *S. aromaticum* essential oils	7.94	A
	C. zeylanicum + *S. aromaticum* essential oils	3.19	I

ΣFIC: Sum of Fractional inhibitory concentration index; S: synergy; ADD: additive; I: Indifferent; A: antagonism

4.3.3 Design of Experiments (DOE) data analysis and model verification

The *R. officinalis*, *S. aromaticum*, and *S. officinalis* methanol extracts were selected for DOE studies based on a synergistic outcome from the combination studies. The DOE was performed in order to determine the optimum ratio at which a combination of the three extracts could be prepared to obtain the best antibacterial effect. Table 4.3 indicates the DOE output of twelve experimental runs with the different combinations tested as determined by the PLS model. The MIC values were determined using the broth microdilution assay and the results indicate noteworthy antimicrobial activity (≤ 1 mg/mL) for all the 12 combinations.

Table 4.3 The worksheet used to fit the model for the antimicrobial combination

Exp No	**Exp Name**	**Run Order**	***R. officinalis***	***S. aromaticum***	***S. officinalis***	**MIC (mg/mL)**
1	N1	2	1	0	0	0.50
2	N2	8	0	1	0	1.00
3	N3	1	0	0	1	0.25
4	N4	6	0.67	0.17	0.17	0.37
5	N5	11	0.17	0.67	0.17	0.37
6	N6	7	0.17	0.17	0.67	0.25
7	N7	10	0	0.50	0.50	0.75
8	N8	4	0.50	0	0.50	0.12
9	N9	9	0.50	0.50	0	0.25
10	N10	3	0.33	0.33	0.33	0.50
11	N11	5	0.33	0.33	0.33	0.50
12	N12	12	0.33	0.33	0.33	0.25

4.3.3.1 Summary of fit plot for the antimicrobial combination

The DOE generated a linear model which was fitted against the data, and the response thereof is shown in the summary of the fit plot (Figure 4.1), which provides information on the strength and the robustness of the model. The R^2 value of 0.72 signified a high variation in the response (MIC), and a strong fit between the data and the model. The Q^2 value of 0.18 demonstrated a significant predictive power of the model (Q^2 > 0.10 for significant model and > 0.50 for a good model) (Bravo *et al*., 2013; Bhatia *et al*., 2016). The model yielded a strong score for validity (0.84), which is far exceeding the required value of >0.25 for good models (Bravo *et al*., 2013). The value obtained for model reproducibility of 0.52 exceeded the requisite value of 0.5, indicating good experimental control and low pure error (Figure 4.1).

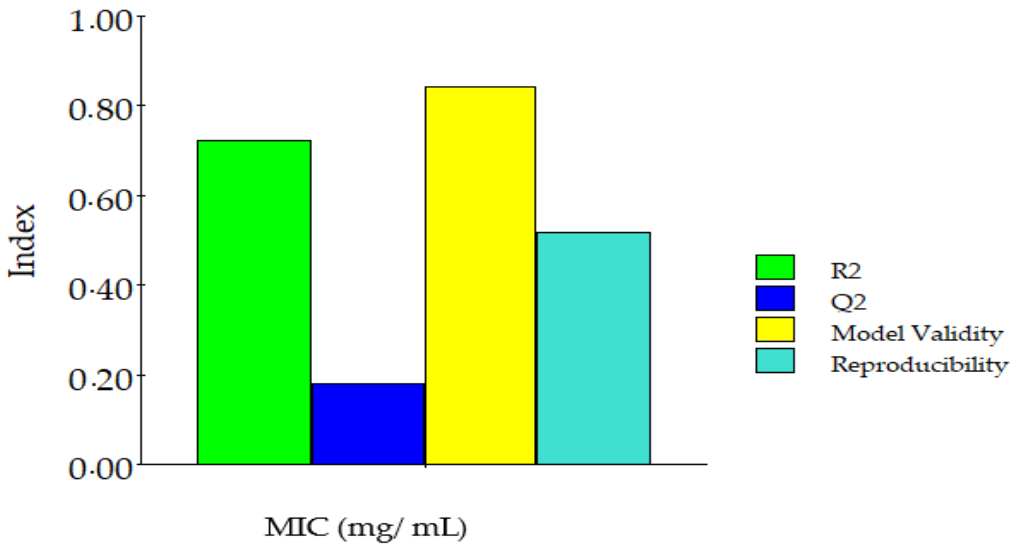

Figure 4.1 Summary of fit plot for MIC values of methanol extract mixtures

4.3.3.2 Coefficient plot for the antimicrobial combination

An assessment of the effect of *R. officinalis* (R.o), *S. officinalis* (S.o) and *S. aromaticum* (S.a) on the antimicrobial activity (MIC) was also done on the coefficient plot which showed that varying the proportions of the three extract had minimal impact on the antimicrobial effect. (Figure 4.2).

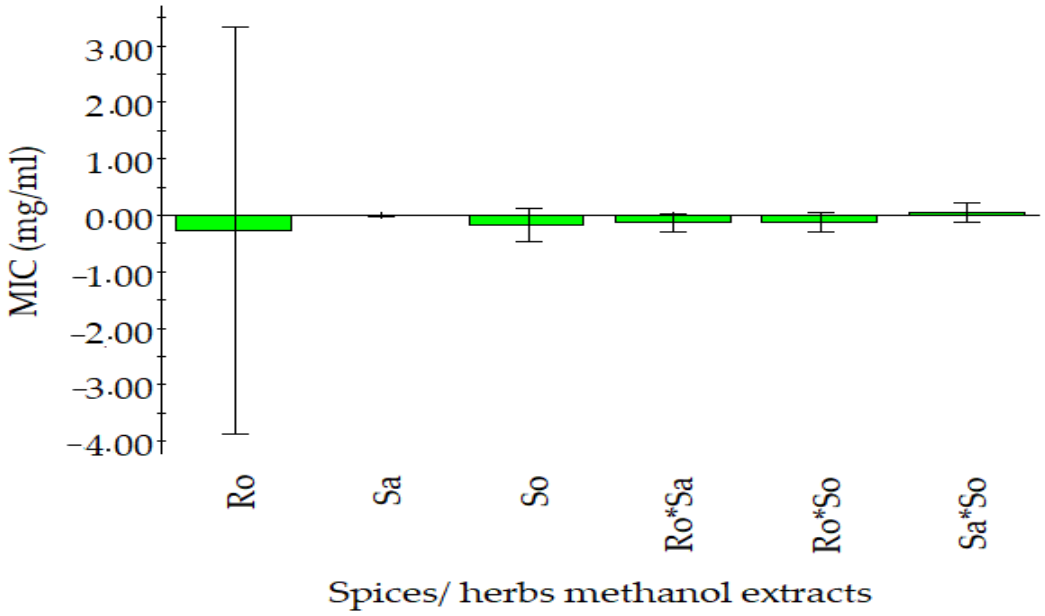

Figure 4.2 A coefficient plot showing the effect of the three factors *R. officinalis* (R.o), *S. officinalis* (S.o), and *S. aromaticum* (S.a) on the antimicrobial activity of the mixture (*)

4.3.3.3 Response contour plot for the antimicrobial combination

The response contour plot which provides a better indication of the extract combinations and predicted responses was then generated (Figure 4.3). According to the key, the lowest MIC values (< 0.3 mg/mL) which translates to the best antimicrobial activity, were predicted for combinations with proportions corresponding to the co-ordinates within the blue region. The predicted MIC values in the blue region were therefore based on the combinations with high *R. officinalis*, low *S. aromaticum*, and a wider range of *S. officinalis* content. The optimizer function assisted in determining the best ratio at which the extracts should be combined for optimum synergistic antibacterial effects. Five combinations in the blue region of the response contour plot were selected as displayed in Table 4.4, to validate the model predictions. The highest antibacterial activity (MIC value of 0.19 mg/mL) was achieved with a mixture of *R. officinalis* (59.5%), *S. officinalis* (40.0%) and *S. aromaticum* (0.5%) methanol extracts. The results from the validation experiments confirmed the reliability and fitness of the model because the correlation coefficient (r= 0.73), calculated using Microsoft Excel shows a strong correlation (Schober *et al*., 2018). between the experimental and the predicted MIC values for all the combinations (Table 4.4).

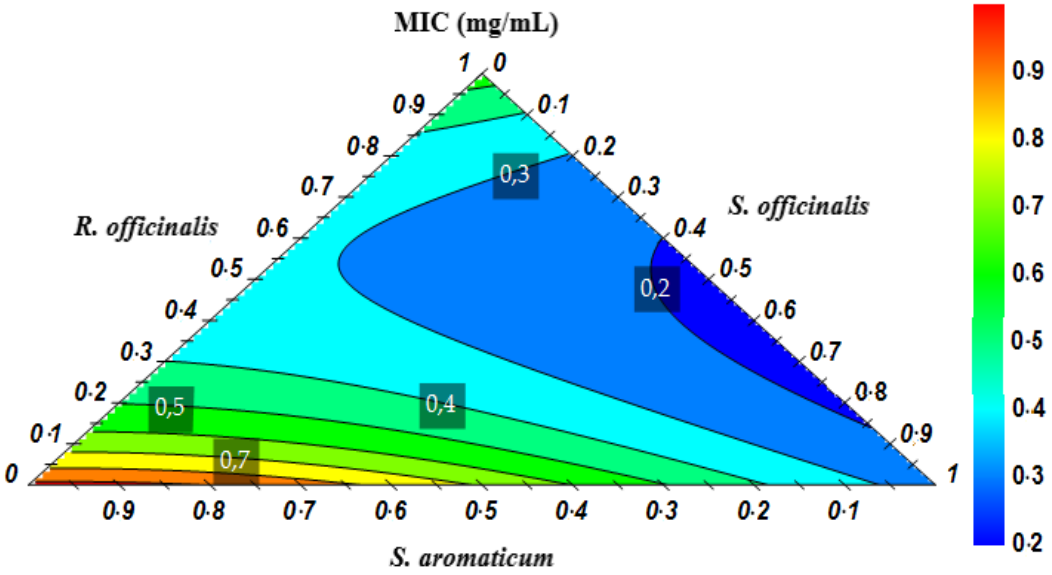

Figure 4.3 Response contour plot showing the extract combinations and predicted responses (MIC values)

Table 4.4 DOE *Model validation for experimental runs using the MIC assay against* B. cereus

R. officinalis	***S. aromaticum***	***S. officinalis***	**Predicted**	**Experimental validation**
0.78	0.01	0.21	0.30	0.38
0.56	0.00	0.44	0.19	0.19
0.59	0.01	0.40	0.17	0.19
0.23	0.23	0.54	0.29	0.25
0.04	0.03	0.92	0.25	0.38

4.4 Summary

- The results from the antimicrobial study demonstrated moderate, weaker, and no antimicrobial activities for the selected spices or herbs crude extracts and essential oils.

- For the methanol extracts, the highest inhibition (MIC value of 0.25 mg/mL) was demonstrated by *S. officinalis* methanol extract against *B. cereus* ATCC 11778.

- The highest inhibition among the dichloromethane extracts were from *R. officinalis* and *S. aromaticum* extracts (MIC values of 0.25 and 0.33 mg/mL, respectively) against *S. sonnei* ATCC 9220.

- Essential oils of *C. zeylanicum*, *C. citratus*, *L. nobilis*, *M. officinalis*, *R. officinalis*, *S. aromaticum*, and *Z. officinale* exhibited strong antibacterial activity against *B. cereus*, with MIC values ≤0.5 mg/mL.

- Synergy interaction was achieved by combining (1:1) *R. officinalis* methanol extract with either *S. officinalis* or *S. aromaticum* methanol extracts against *B. cereus*(ΣFIC index of 0.25 and 0.31, respectively).

- Using the DOE, a model was produced that could easily predict the ratio at which the extracts could be combined to achieve the highest antibacterial efficacy. This was achieved with a mixture of *R. officinalis* (59.5%), *S. officinalis* (40%) and *S. aromaticum* (0.5%) methanol extracts.

- The results from predicted MIC values were validated successfully, and a strong correlation (r= 0.83) existed between the predicted MIC values and the experimental MIC values.

CHAPTER 5

Formulation and evaluation of an anti-oxidant and antibacterial polyherbal capsule

5.1 Introduction

Herbal formulations have been used since ancient times to treat various ailments. They were used either in the form of a single herb (monoherbal) or combinations of herbs (polyherbal). There is some scientific evidence that the polyherbal formulations are more efficacious compared to monoherbal formulations due to the synergism among the constituents (Parasuraman *et al*., 2014; Choudhari *et al*., 2020; Rahim *et al*., 2020).

A polyherbal product can be formulated into various pharmaceutical dosage forms such as Tablets or capsules, gels and liquids etc. Capsules are oral dosage forms made from gelatine or other suitable material which is filled with medicines to produce a unit dosage. The gelatine shell may be hard or soft. The advantage of gelatine capsules as a dosage form is that they are easy to swallow, tasteless, non-toxic, and gelatine is readily soluble in biological fluids at body temperature (Aulton and Taylor, 2013).

Therefore, in this study, a hard gelatine capsule containing methanol extracts of *R. officinalis*, *S. aromaticum*, and *S. officinalis* was formulated. The three extracts were used for the formulation because they demonstrated promising anti-oxidant and antibacterial activities. The three extracts were combined based on the most effective combination ratio as predicted by the DOE software. The developed formulation was evaluated for toxicity using the brine shrimp lethality assay. Furthermore, the formulation was evaluated for anti-oxidant and antibacterial activity.

5.2 Materials and methods

5.2.1 Preparation of crude herbal extracts

The preparation of methanol extracts was done according to the method explained previously in Chapter 2, Section 2.1.

5.2.2 Development of the polyherbal formulation

5.2.2.1 Selection of excipients

In addition to the active ingredients, excipients like diluents (filler), glidants and lubricants, are required for the formulation of capsule. The choice of excipients was made keeping in mind the current FDA regulations. Diluents or fillers are added to bulk up a dosage form when the active ingredient is not present in sufficient quantity for filling up capsule. Common capsule fillers include microcrystalline cellulose, lactose, and mannitol (Aulton and Taylor, 2013). Dry herbal extracts are invariably hygroscopic, hence in this study a high porosity excipient such as microcrystalline cellulose was selected as capsule filler.

Furthermore, microcrystalline cellulose has lubricating effect. Lubricants are important in that they reduce the friction between the surfaces of manufacturing equipment and organic solids during the filling process. In addition, they prevent adherence of capsule materials to the capsule filling machine (Aulton and Taylor, 2013). A glidant is added to capsules to improve the flow of powder materials by reducing the friction between particles. The most commonly used and effective glidants are colloidal silicon dioxide, talc and starch (Aulton and Taylor, 2013). In this study, colloidal silicon dioxide was selected because it is considered to be biologically inert and silicon dioxide is generally regarded as safe by the FDA.

5.2.2.2 Preparation and filling of polyherbal capsules

A polyherbal mixture containing three methanol extracts of *R. officinalis, S. officinalis,* and *S. aromaticum* based on the DOE model predictions was prepared (Table 5.1). The three methanol extracts were completely dried, grounded, and sieved using a 590 microns sieve to produce a

fine powder. The extracts were then mixed with microcrystalline cellulose and colloidal silicon dioxide by trituration until the mixture was homogenous. The powder was then collected and used to fill 150 hard gelatine capsules (Size 0) to an average net content weight of 500 mg. A hand operated capsule filling machine (ZUMA, Milano Italy) was used in this study for encapsulation of the capsules.

Table 5.1 Final batch composition of 500 mg capsule

Actives and Excipients	Reasons for inclusion in the formulation	Quantity (g) per capsules	Quantity (g) per 150 capsules
R. officinalis methanol extract	Active ingredient	0.13	19.06
S. officinalis methanol extract	Active ingredient	0.27	40.60
S. aromaticum methanol extract	Active ingredient	0.002	0.35
Microcrystalline Cellulose (Ph 101)	Capsule filler	0.07	10.19
Colloidal silicon Dioxide	Glidant	0.002	0.35
Total		0.47	70.54

5.2.3 Standardization of polyherbal capsule

The developed polyherbal capsules was standardized for its description, mass variation, length and diameter, total mass, and disintegration rate. Standardizations were carried out according to United States Pharmacopoeia (USP), 2021 methods. The following quality control parameters were evaluated.

5.2.3.1 Description

The general appearance, visual identity, and overall “elegance” of a capsule are essential for consumer acceptance. Thus, the shape, colour, and odour for each capsule was noted.

5.2.3.2 Mass variation test

The test for uniformity of mass for the capsules was carried out. Twenty capsules out of a batch of 150, were selected randomly and accurately weighed individually on an analytical balance. The content of each capsule was then completely removed, and the weight of each individual emptied shell was recorded. The net mass of the capsule content was calculated by subtracting the shell mass from the respective total mass. The average mass and the standard deviation were calculated. The USP limit for mass variation in case of capsule weighing more than 300.00 mg is 7.5% and for capsules weighing less than 300.00 mg is 10%. In addition to the mass variation test, other physical parameters such as the diameter and the length of each of the 20 capsules were measured.

5.2.3.3 Disintegration test

Complete disintegration is defined as that state in which any residue of the unit, except fragments of insoluble coating or capsule shell, remaining on the screen of the test apparatus or adhering to the lower surface of the disc, if used, is a soft mass having no palpably firm core (USP, 2021). The test is used as a quality assurance measure to demonstrate that the disintegration performance of hard gelatine capsules is comparable and reliable. Six out of a batch of 150 capsules were randomly selected to determine the disintegration time of the capsules. The test was carried out using disintegration test apparatus (Erweka ZT120 supplier). One capsule was introduced into each of the six test tubes of the basket, and a disc was added to each tube. The assembly was suspended in 900 mL distilled water in a 1000 mL beaker. The volume of water was such that the wire mesh was at its highest point in at least 25 mm above the surface of the water, and at its lowest point at least 25 mm below the surface of the water. The apparatus was operated and maintained at 37ºC ± 2ºC throughout the experiment. The time taken for each of the capsule to disintegrate with the disks were recorded as shown in Table 5.3.

5.2.4 Toxicity study using brine shrimp lethality assay

Brine shrimp lethality assay (BSLA) was used to screen for toxicity of the polyherbal formulation as well as of *R. officinalis*, *S. officinalis*, and *S. aromaticum* methanol extracts independently and in combination. The bioassay was done as described by Meyer *et al*. (1982), and McLaughlin *et al*. (1998), using freshly hatched *Artemia franciscana* (brine shrimp).

5.2.4.1 Hatching of brine shrimp larvae

Artificial sea water was prepared by dissolving 16g of Tropic ® Marine sea salt in 500 mL of deionised water, which mimics the natural environment for growth and development of brine shrimp. Dried brine shrimp eggs (Ocean Nutrition ^{TM}) (0.5 g) were then added to the artificial sea water. To ensure a high hatch rate, the sea water was aerated with rotary pump (Kiho) and kept in constant motion to keep the eggs well dispersed at all times. The eggs were incubated under bright luminescent light (220-240 V) at 25°C for 18-24 hrs.

5.2.4.2 Preparation of test samples

All samples were tested for toxicity at a concentration of 1 mg/mL or lower. If concentrations above 1 mg/mL are necessary to exhibit a toxic effect against the brine shrimp, the sample is not considered toxic in nature (Bussmann *et al*., 2011). Each test sample (plant extracts and polyherbal formulation) was prepared at a concentration of 2 mg/mL, considering the dilution factor of two when plated out. The test samples were dissolved in distilled, deionised water. An organic solvent such as DMSO was used to dissolve samples that were insoluble in water. The concentration of the solvent used was always kept below 1% to ensure that the solvent itself would not cause any toxic effects on brine shrimp. Samples to be tested in combination were also prepared in the same manner, except to a concentration of 4 mg/mL, considering a dilution factor of four when plating out.

5.2.4.3 Method of brine shrimp lethality bioassay

After the incubation period, the artificial sea water containing the brine shrimp was placed in a shallow container, which was tilted at an angle for approximately 30 minutes. This was done to attract the brine shrimps towards the light, thus allowing for a large amount of brine shrimp

to be collected. After 30 minutes, 400 µL of artificial sea water containing on average 40-60 live brine shrimps was added to each of the 48 wells in a microtiter plate. Test samples (400 µL) were then added to the wells. The tests were done in triplicates. The viability of the brine shrimps were confirmed before adding the test samples.

For the testing of individual extracts and the polyherbal formulation, 400 µL (at 2 mg/mL) of samples were added to each of the wells and marked accordingly. For screening of the 1:1 combination, 200 µL of each of the extract used in the combination at different concentrations were added to each of the wells, whereas for the triple combination (1:1:1), 133.3 µL of each sample was added to the wells marked accordingly. The plates were then incubated for 24 hrs. at room temperature. A highly toxic compound, potassium dichromate (Sigma-Aldrich) at a concentration of 1.6 mg/mL was used as a positive control. Artificial sea water was prepared to a working concentration of (32 g/L), and used as negative, toxin-free control. The solvent control was 10% DMSO. All the tests were done in triplicates.

The plates were observed under a light microscope (Olympus) (magnification 40x), and any motionless brine shrimps during 10 seconds of observation were regarded as dead (Carballo *et al*., 2002). The number of dead brine shrimps were counted at time 0 (i.e., immediately after adding the test samples). The dead shrimps at time 0 were excluded from the total mortality calculation. Dead brine shrimp were again counted after 24 hrs. of exposure to the test samples. After the dead brine-shrimp count at 24 hrs., a lethal dose of 50 µL of glacial acetic acid (Saarchem,100% v/v) was added to each well, and after 30 minutes, a final count was undertaken to calculate the percentage mortality (Cock and Kalt, 2010). The percentage mortality of the brine shrimp after 24 hrs. was then calculated for each of the individual extracts and combinations using Equation 5.1.

$$\% \text{ Mortality} = \frac{\text{dead shrimp at 24 hrs. (before acetic acid) (time 0)}}{\text{dead shrimp (after acetic acid)}}$$

Equation 5.1

Extracts that induced percentage mortality greater than 50% were considered biologically toxic (Bussmann *et al*., 2011; Hübsch *et al*., 2014) and were further diluted with 2% v/v DMSO to

concentrations of 1, 0.5, 0.25, 0.125, 0.063 mg/mL to be tested again. The median lethal concentration of the test extracts and of the formulation necessary to have a lethal effect on 50% of the brine-shrimp (LC_{50}) was determined using the probit method. The LC_{50} values ≤249 µg/mL was considered highly toxic, 250–499 µg/mL was considered moderately toxic, 500–999 µg/mL was considered weak or low in toxicity, and ≥1000 µg/mL was considered non-toxic (Bussmann *et al*., 2011).

5.2.5 *In vitro* screening for anti-oxidant activity

The anti-oxidant activity of the polyherbal formulation was measured using DPPH, ABTS and FRAP assays. The methods for the anti-oxidant assays were described under Chapter 3, Section 3.2.

5.2.6 *In vitro* screening for antibacterial activity

The antibacterial activity of the polyherbal formulation was measured against Gram-positive bacteria (*S. aureus*, *B. cereus* and *E. faecalis*) and Gram-negative bacteria (*E. coli*, *S. sonnei*, and *S. typhumirium*). The MIC of the formulation was determined using the broth dilution method as described under Chapter 4, Section 4.2.2.

5.3 Results and discussion

5.3.1 Description

The shape, colour of the formulated capsules are shown in Figure 5.1. Furthermore, the mass variation, length, diameter and disintegration time for the capsule are shown in Table 5.2.

5.3.2 Mass, length and diameter variation of polyherbal capsule

The capsule mass is a critical parameter that can have an impact on the dimensions, machinability and disintegration behaviour of the capsules (Stegemann *et al*., 2014). The average weight of the empty gelatine capsule was 90.00 mg, which fall under the standard weight (99.00 mg) of size 0 hard gelatine capsule (https://www.capsulemachines.in/capsule-

size-guide.htmL). Each capsule contained 0.13 g of *R. officinalis*, 0.27 g of *S. officinalis*, and 0.002 g of *S. aromaticum* methanol extracts (Table 5.1).

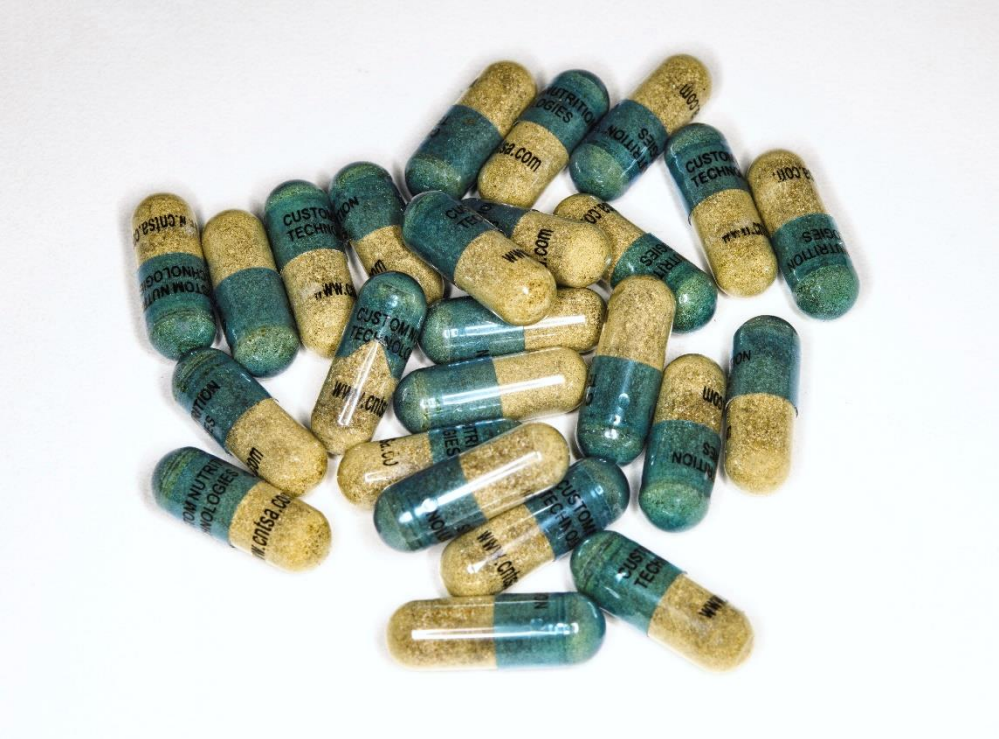

Figure 5.1 Polyherbal hard gelatine capsules containing mixture of *R. officinalis* (130 mg), *S. aromaticum* (0.002 mg), and *S. officinalis* (0.27 mg) methanol extracts

The overall mass of ingredients in the formulation was 0.47 g (Table 5.1), and total mass of the filled capsule was 0.52 g as indicated in Table 5.2. It was observed that all the 20 capsules analysed were under the upper and lower average limit of mass variation, whereby 95% of the capsule had a limit variation of 7.5% and 100% had a variation limit of 10%. Furthermore, the average diameter and length of the capsules was 7.22 and 20.93 mm respectively (Table 5.2), and these were well within the acceptable limits for size 0 capsule.
(http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances)

5.3.3 Capsule disintegration test

The disintegration test was conducted on six randomly selected polyherbal capsules out of a batch of 150 capsules (4%) with and without the discs. The disintegration time of hard gelatine

capsules is set to no more than 900 s (15 min) (USP, 2021). Based on the results in Table 5.2, the mean disintegration time of polyherbal hard gelatine capsules was 4.30 ± 0.17 min without the disk, and 7.70 ± 0.33 min with the disk. The results falls within the specifications (USP, 2021).

Table 5.2 Characterization of polyherbal capsule

Parameters	**Observations**
Average mass of capsule content: *R. officinalis* methanol extract *S. officinalis* methanol extract *S. aromaticum* methanol extract	 130.00 mg 270.00 mg 2.00 mg
Average mass of filled capsules	520.00 mg ± 6.70
Average diameter	7.22 mm
Average length	20.93 mm
Mass variation	95% of capsules had variation limit of 7.5% 100% of capsules had variation limit of 10%
Disintegration time (min)	with the disc 4.30 ± 0.17 without the disc 7.70 ± 0.33

5.3.4 Brine shrimp lethality bioassay

Plant extracts were diluted with 2.0% DMSO and tested at different concentrations (1000.00, 500.00, 250.00, 125.00, and 63.00 µg/mL). The extent of lethality at 24 hrs was directly proportional to the concentration of the extracts in the formulation. Maximum mortality was observed at a concentration of 1000.00 µg/mL, and least mortality at a concentration of 63.00 µg/mL. However, *R. officinalis* methanol extract alone and in 1:1 combination with *S. officinalis* methanol extract exhibited mortality below 50.0% even at higher concentrations (1000.00 to 500.00 µg/mL). Whereas the polyherbal formulation showed 72.1% and 50.3% mortality at 1000.00 and 500.00 µg/mL respectively. The lowest percentage mortality (9.1%) was shown by the formulation at a concentration of 63.00 µg/mL (Table 5.3). Followed by *R. officinalis* and *S. officinalis* (1:1) combination (17.5%) and *R. officinalis* (19.4%) whereas the

1:1:1 combination (*R. officinalis*: *S. officinalis*: *S. aromaticum* methanol extracts) had mortality of 19.9%. Furthermore, the formulation and *S. aromaticum* methanol extract would kill less than 50% of brine shrimps even at concentrations of 250.00 and 125.00 µg/mL. No mortality was observed in the negative control.

Table 5.3 Mortality (%) of brine shrimp at different concentrations of test samples at 24 hrs

Concentration (µg/mL)	Mortality (%) of test samples									
	Ro	**Sa**	**So**	**Ro:Sa (1:1)**	**Ro:So (1:1)**	**So:Sa (1:1)**	**Ro:Sa:So (1:1:1)**	**PHF**	**Positive control**	**Negative control**
1000	69	71.3	88.1	86.5	38.02	59.5	89.01	77.2	100	0
500	30.4	56.4	79.7	63.8	35.4	55.3	53.6	50.3	100	0
250	28.03	38.7	54.7	52.7	28.7	53.9	53.0	33.9	100	0
125	22.2	24.2	49.2	39.3	18.2	35.1	32.6	28.7	100	0
63	19.4	20.6	25.8	35.1	17.5	31.1	19.9	9.1	100	0

Ro: *Rosmarinus officinalis*; Sa: *Syzygium aromaticum*; So: *Salvia officinalis*; PHF: Polyherbal formulation; Positive control: Potassium dichromate; Negative control: artificial sea salt water

Table 5.4 The LC_{50} values of test samples at 24 hrs

Spice/ herb extract	LC_{50} (µg/mL) at 24 hrs.
R. officinalis methanol	703.64
S. officinalis methanol	159.04
S. aromaticum methanol	386.99
R. officinalis + *S. officinalis* methanol	163.37
R. officinalis + *S. aromaticum* methanol	178.57
S. aromaticum + *S. officinalis* methanol	331.56
Ro: Sa: So (1:1:1)	240.96
Polyherbal formulation	395.94
Positive control: Potassium dichromate (1.6 mg/ mL)	100% mortality
Negative control: Tropic ® marine (artificial sea salt water)	0

5.3.5 Anti-oxidant activity of the polyherbal formulation

The anti-oxidant activity of the polyherbal formulation is shown in Table 5.5. Ascorbic acid was used as a reference standard. With the DPPH assay, the EC_{50} value for the formulation (13.34 µg/mL) was comparable with that of ascorbic acid (10.25 µg/mL). Furthermore, the polyherbal formulation demonstrated notable activity (EC_{50} value of 88.10 µg/mL) when compared to ascorbic acid (90.95 µg/mL) using FRAP assay, whilst its ABTS radical scavenging effect was lower than that of ascorbic acid (EC_{50} value of 11.63 µg/mL).

Table 5.5 Anti-oxidant activity of the polyherbal formulation

Anti-oxidant assay	Sample	Average EC_{50} (µg/mL)
DPPH	PHF	13.34±0.10
	Ascorbic acid	10.25
ABTS	PHF	11.63±0.04
	Ascorbic acid	4.88
FRAP	PHF	88.10±0.02
	Ascorbic acid	90.95

Values represent means ± standard deviations for the experiments performed in triplicates. The lower the EC_{50} value, the higher the anti-oxidant activity. PHF: polyherbal formulation.

5.3.6 Antibacterial activity of the polyherbal formulation

The MIC results for the polyherbal formulation and ciprofloxacin are displayed in Table 5.6. The polyherbal formulation demonstrated moderate antibacterial activity against *B. cereus* (MIC value of 0.12 mg/mL), and weaker activity against other test pathogens with MIC values > 0.62 mg/mL (Kuete *et al*., 2010).

Table 5.6 Antibacterial activity of the polyherbal formulation

Micro-organism	Average MIC (mg/mL)
B. cereus ATCC 11778	0.12
E. faecalis ATCC 29212	2.00
S. aureus ATCC 25926	2.00
E. coli ATCC 8739	1.00
S. typhimurium ATCC 1403	2.00
S. sonnei ATCC 9220	1.00
Positive control: ciprofloxacin	≤ 0.62 µg/mL
Negative control: acetone	> 8 mg/mL

Values represent mean MIC values of the experiments done in triplicates.

5.4 Summary

- The individual weight, length, height, and diameter of each capsule was within the limits as specified in the USP.
- The mean disintegration time of 4.30 ± 0.17 min for the polyherbal capsules was within the USP specified limits.
- The polyherbal combination within the capsule dose demonstrated moderate antibacterial activity (MIC = 0.12 mg/mL) against *B. cereus*.
- The polyherbal formulation exhibited notable DPPH and ABTS radical scavenging activity with EC_{50} values of 13.34 and 11.63 µg/mL respectively. For the FRAP assay, the activity was comparable to that of ascorbic acid.
- The polyherbal formulation demonstrated dose dependent mortalities, with maximum mortalities occurring at concentrations of 1000 µg/mL. Less than 50% mortalities occurred at concentrations below 500 µg/mL.
- At 24 hrs., the formulation was less toxic at LC_{50} value of 395.94 µg/mL compared to when the three extracts were combined at equal ratios of 1:1, and 1:1:1.

CHAPTER 6

General conclusions and recommendations

6.1 Introduction

The study aimed to formulate an optimal polyherbal capsule with antimicrobial and anti-oxidant potential. Various crude extracts and neat essential oils from some commonly used culinary herbs and spices were screened for antibacterial and anti-oxidant activities. The most active extracts were combined at 1:1 ratio to evaluate their antimicrobial and anti-oxidant interactions. The design of experiments (DOE) approach was used to predict the most effective combination ratio of herbal extracts required to produce an optimal polyherbal combination to be incorporated into a capsule.

The aim of the study was achieved and the findings in the study are discussed as follows;

6.1.1 Chemical characterization of neat essential oils and crude extracts

Most of the compounds identified from neat essential oils of selected spices and herbs were also reported in previous studies as major constituents in the oils. However, some variations in terms of the quantity of major constituents existed when compared to reports from previous studies. The most frequently identified major compounds were limonene, linalool, and 1-8-cineole. The identified compounds in the essential oils were previously reported to possess antimicrobial and anti-oxidant activities.

The crude extracts were characterized by the presence of phenolic acids, phenolic diterpenes, flavonoids, flourocoumarins, and hydroxybenzoic acid derivative. Apigenin, caffeic acid, proanthocyanidins, catechin and rosmarinic acid were identified in several crude extracts of selected herbs and spices. These compounds were reported to exhibit a host of biological activities, including anti-oxidant and antimicrobial activities.

6.1.2 Anti-oxidant activities and optimization of synergy interactions

The results of the anti-oxidant study demonstrated anti-oxidant variability between crude extracts and essential oils of selected culinary herbs and spices when using different *in vitro* methods (DPPH, ABTS, FRAP assays). The methanol extracts demonstrated better radical scavenging activity compared to the water and dichloromethane extracts, based on DPPH and FRAP assays. The *C. zeylanicum* methanol and water extracts were found to be more effective radical scavengers compared to the standard anti-oxidant, ascorbic acid. The most superior ABTS radical scavengers were *S. aromaticum* and *Z. officinalis* dichloromethane extracts, which had greater activity than ascorbic acid. The most effective extracts in reducing ferric iron were *R. officinalis*, *C. zeylanicum*, *M. officinalis*, *T. vulgaris*, *M. piperita*, *S. aromaticum*, and *S. officinalis* methanol extracts. It was also observed that *C. zeylanicum* and *S. aromaticum* essential oils were effective anti-oxidants.

The 1:1 combination of crude extracts displayed synergistic, additive, indifferent, and antagonistic effects. Strong synergism was shown by combining *T. vulgaris* and *Z. officinalis* methanol extracts using ABTS assay, *T. vulgaris* and *M. piperita* methanol extracts using FRAP assays, and from combining *R. officinalis* and *Z. officinalis* dichloromethane extracts using ABTS assay.

Using the DOE, a model that could easily predict the ratios at which the extracts can be combined to achieve the highest anti-oxidant effect was produced, which was shown by a mixture of *M. piperita* (55%), *T. vulgaris* (44%) and *Z. officinalis* (1 %) methanol extracts. The use of crude extracts in various combinations resulted in an optimum anti-oxidant effect, even at lower concentrations compared to single extracts due to synergistic interactions. This approach creates an innovative opportunity for the future development of anti-oxidant supplements for optimum health.

6.1.3 Antibacterial activity and optimization of synergy interactions

The results from antimicrobial study demonstrated moderate to low antimicrobial activities for the selected spices or herbs crude extracts and neat essential oils. The highest inhibition (MIC value of 0.25 mg/mL) was shown by *S. officinalis* methanol and dichloromethane extracts against *B. cereus* and *S. sonnei* respectively, and by *R. officinalis* dichloromethane extract

against *S. sonnei*. Methanol extracts of *C. zeylanicum, S. aromaticum, S. officinalis,* and *R. officinalis* also demonstrated moderate antibacterial activity against *E. faecalis*, *E. coli*, and *B. cereus* respectively, with MIC values of 0.42–0.50 mg/mL. For the dichloromethane extracts, moderate activity was shown by *S aromaticum, O. marjorana, C. zeylanicum*, *R. officinalis*, and *S. officinalis* against *E. faecalis*, *B. cereus*, *S. sonnei*, and *S. aureus* respectively. Essential oils of *C. zeylanicum*, *C. citratus*, *L. nobilis*, *M. officinalis*, *R. officinalis*, *S. aromaticum*, and *Z. officinalis* were found to be more active against *B. cereus* than other essential oils used in this study.

The 1:1 combination yielded synergistic, additive, indifferent, and antagonistic antibacterial activities. Synergy was observed from combining *R. officinalis* methanol extract with either *S. officinalis* or *S. aromaticum* methanol extracts against *B. cereus*.

Using the DOE, a model was produced that could easily predict the ratio at which the extracts could be combined to achieve the highest antibacterial efficacy, and this was achieved with a mixture of *R. officinalis* (59.5%), *S. officinalis* (40%), and *S. aromaticum* (0.5%) methanol extracts. It was evident that use of spice extracts in combination increases the antibacterial activity due to synergistic interactions.

6.1.4 Formulation and evaluation of a polyherbal capsule

A polyherbal capsule was formulated from a mixture of methanol extracts of *R. officinalis* (59.5%,), *S. officinalis* (40.0%), and *S. aromaticum* (0.5%). The formulated capsule was within the pharmaceutical limits as specified in the United States Pharmacopoeia.

Furthermore, the polyherbal formulation demonstrated dose related toxicity on brine shrimp, and was least toxic at concentrations below 500 µg/mL. The anti-oxidant activity of the polyherbal formulation was comparable to the activity of ascorbic acid. In addition, the formulation demonstrated moderate activity against *B. cereus* with MIC value of 0.12 mg/mL.

Based on the results from the *in vitro* study, it is possible that through synergistic interactions, different combinations of herbs or spice extracts can result in enhanced antibacterial and anti-oxidant efficacy even at sufficiently lower concentrations, and thus minimising the adverse effects produced by each individual herb or spice. The transition of *in vitro* studies to *in vivo*

experiments, and to human clinical trials is required to have a better understanding of antimicrobial and anti-oxidant effects of combinations of spice and herb extracts *in vivo*, and whether these can be considered as an alternative to control contamination and spoilage of food by micro-organisms or as supplements for optimum health.

Instead of combining extracts randomly, the DOE study provides an interesting and promising cost-effective approach whereby spices and herbs extracts can be combined for optimum biological effect.

The study also provides valuable preliminary data on the possible toxicity of the polyherbal formulation. Further *in vivo* and long-term toxicity studies are required to complete the safety profile of the polyherbal formulation.

Further studies on stability and the gastrointestinal absorption of the polyherbal formulation are required.

6.2. Study limitations and recommendations

Though the three assays employed in the study were technically simple, each had its own limitations. For example, the DPPH assay required that the analysis be done in the dark because absorbance of DPPH tends to decrease when exposed to light. For the FRAP assay is based on aqueous solutions, hence the method is limited to hydrophilic substances, and therefore may not be suitable for plant essential oils since they are hydrophilic. The generation of the $ABTS^{*+}$ radical took 12–16 hours, thus making the reaction time slower compared to DPPH assay. Furthermore, the $ABTS^{*+}$ radicals are not biomolecules, and are only generated through chemical reactions. This makes it difficult to relate the *in vitro* test results to biological systems. In addition, *In vitro* anti-oxidant assays are carried out at pH values far from the physiological pH, and therefore cannot give meaningful information on the estimation of anti-oxidant effects *in vivo*.

Further studies on the characterization of anti-oxidant active phenolic compounds present in herbs and spices can be conducted through bioassay guided fractionation and biochemometric approaches".

Since the stability of phenolic compounds is greatly affected by heat, light, and pH during formulation steps,various formulation approaches such as encapsulation may therefore be employed in future to protect these compounds from degradation.

6.3. Final conclusion

The results provide evidence that the anti-oxidant and antibacterial activities of culinary herbs and spices crude extracts were improved when in combination. The DOE approach created an innovative opportunity for the future development of most effective natural anti-oxidant and antimicrobial agents for the pharmaceutical and food industries. The work further demonstrates the importance of producing a product-prototype as ingredients in a formulation may exhibit different properties and activities when tested *in vitro* compared to what they can do *in vivo*.

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APPENDIX A

Abstract of paper published in Molecules Journal

molecules

MDPI

Article

Optimization of Antioxidant Synergy in a Polyherbal Combination by Experimental Design

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Abstract: Culinary herbs and spices are known to be good sources of natural antioxidants. Although the antioxidant effects of individual culinary herbs and spices are widely reported, little is known about their effects when used in combination. The current study was therefore undertaken to compare the antioxidant effects of crude extracts and essential oils of some common culinary herbs and spices in various combinations. The antioxidant interactions of 1:1 combinations of the most active individual extracts and essential oils were investigated as well as the optimization of various ratios using the design of experiments (DoE) approach. The 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) assays were used to determine the antioxidant activity, and MODDE 9.1® software (Umetrics AB, Umea, Sweden) was used to determine the DoE. The results revealed synergism for the following combinations: *Mentha piperita* with *Thymus vulgaris* methanol extract (ΣFIC = 0.32 and ΣFIC = 0.15 using the DPPH and FRAP assays, respectively); *Rosmarinus officinalis* with *Syzygium aromaticum* methanol extract (ΣFIC = 0.47 using the FRAP assay); *T. vulgaris* with *Zingiber officinalis* methanol extracts (ΣFIC = 0.19 using the ABTS assay); and *R. officinalis* with *Z. officinalis* dichloromethane extract (ΣFIC = 0.22 using the ABTS assay). The DoE produced a statistically significant (R2 = 0.905 and Q2 = 0.710) model that was able to predict extract combinations with high antioxidant activities, as validated experimentally. The antioxidant activities of the crude extracts from a selection of culinary herbs and spices were improved when in combination, hence creating an innovative opportunity for the future development of supplements for optimum health.

Keywords: culinary herbs; spices; essential oils; combination; antioxidant; design of experiments; MODDE; 2,2-diphenyl-1-picrylhydrazyl; ferric reducing antioxidant power; 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)

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Citation: Mapeka, T.M.; Sandasi, M.; Viljoen, A.M.; van Vuuren, S.F. Optimization of Antioxidant Synergy in a Polyherbal Combination by Experimental Design. *Molecules* **2022**, *27*, 4196. https://doi.org/10.3390/molecules27134196

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1. Introduction

Culinary herbs and spices are an important part of human nutrition, and they have been used for centuries not only as flavoring agents but as food preservatives and for health benefits. Each spice or herb contains many bioactive compounds such as flavonoids, phenolics, sulfur-containing compounds, tannins, alkaloids, vitamins, and essential oils. Some of these compounds are responsible for their reported antioxidant activities [1,2]. Antioxidants are free radical scavengers and thus inhibit lipid peroxidation and other free-radical-mediated processes, protecting the human body from several diseases attributed to the reactions of free radicals [3–6]. Furthermore, antioxidants are added to food to prevent deterioration through oxidative processes [7,8]. Natural antioxidants, such as those present in culinary herbs and spices, have been investigated as alternatives to synthetic counterparts due to the concerns of potential carcinogenicity and other adverse effects [9,10]. In recent

APPENDIX B

Abstract of paper submitted to *Antibiotics* Journal

A Combinational Approach to Testing Antimicrobial Efficacy of Some Common Herbs and Spices

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ABSTRACT

The antimicrobial activity of crude extracts and essential oils of culinary herbs and spices is widely reported. Although herbs and spices are commonly used in combination there are few studies reporting on their combination effects as antimicrobial agents. The current study was designed to investigate the antibacterial effects of crude extracts and essential oils of some common culinary herbs and spices in combination, exploring synergy as an approach to produce combinations with enhanced antibacterial action. The broth microdilution method was used to determine the minimum inhibitory concentrations (MIC) of the crude extracts and essential oils towards six common food-borne disease pathogens namely; *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 8739, *Enterococcus faecalis* ATCC 29212,

Staphylococcus aureus ATCC 25923, *Shigella sonnei* ATCC 9220, and *Salmonella typhimurium* ATCC 1403. The antimicrobial activity was first determined for individual extracts and oils, followed by 1:1 combinations of the most active samples and finally a Design of Experiments (MODDE 9.1® software) approach was used to identify extract combinations with optimum antimicrobial synergistic effects towards the pathogens. The results revealed synergism for the following combinations: *R. officinalis* and *S. aromaticum* methanol extracts (ΣFIC index value of 0.25), and for *R. officinalis* and *S. officinalis* methanol extract (ΣFIC index value of 0.31) against *B. cereus*. The DOE optimization allowed for the identification of the most effective spice/ herb extracts mixture required to inhibit the test bacteria. The highest inhibition (MIC value of 0.17 mg/mL) was achieved with a mixture of *R. officinalis* (59.5%), *S. officinalis* (40%), and *S. aromaticum* (0.5 %) methanol extracts. The results from predicted MIC values were validated and a strong correlation (*r* value of 0.73) existed between the predicted and the experimental MIC values.

APPENDIX C

GC-MS Chromatograms of neat essential oils of selected culinary herbs and spices

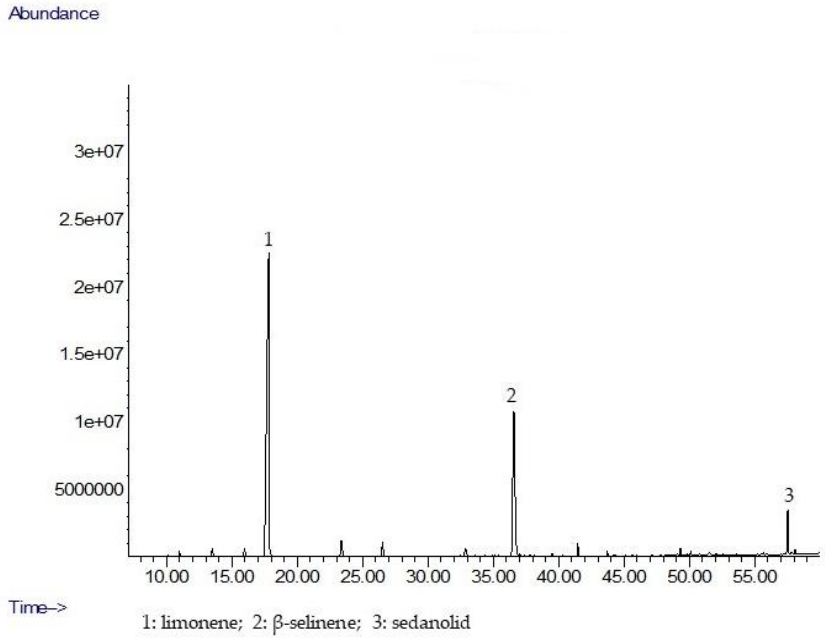


Figure 1: Total ion chromatogram of *Apium graveolens* oil

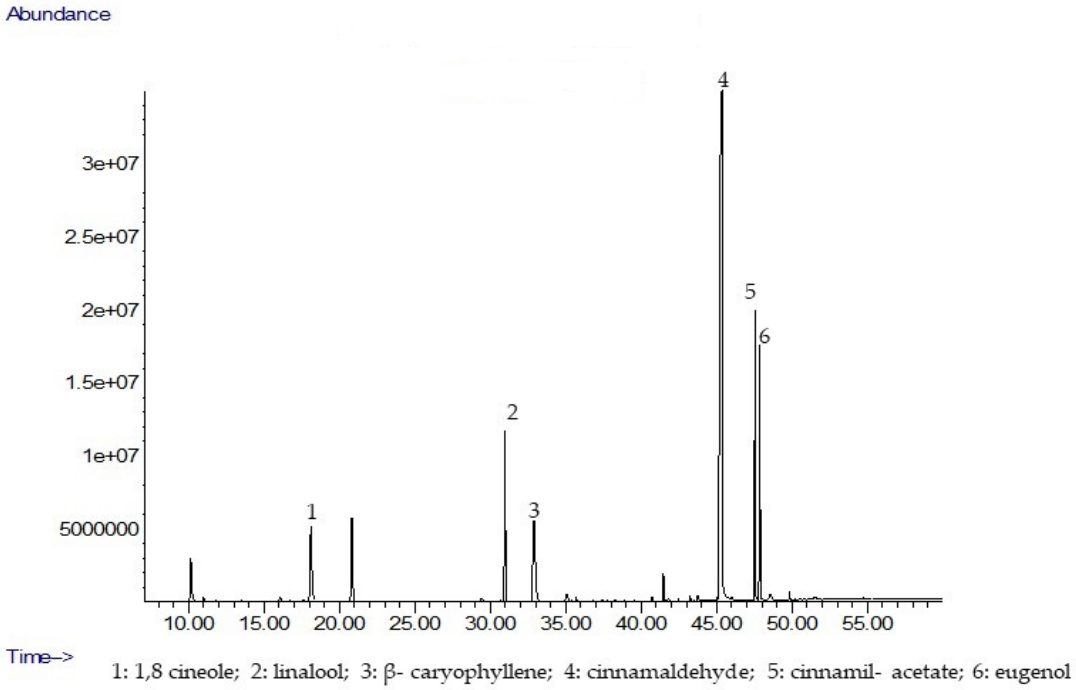


Figure 2: Total ion chromatogram of *Cinnamomum zeylanicum* oil

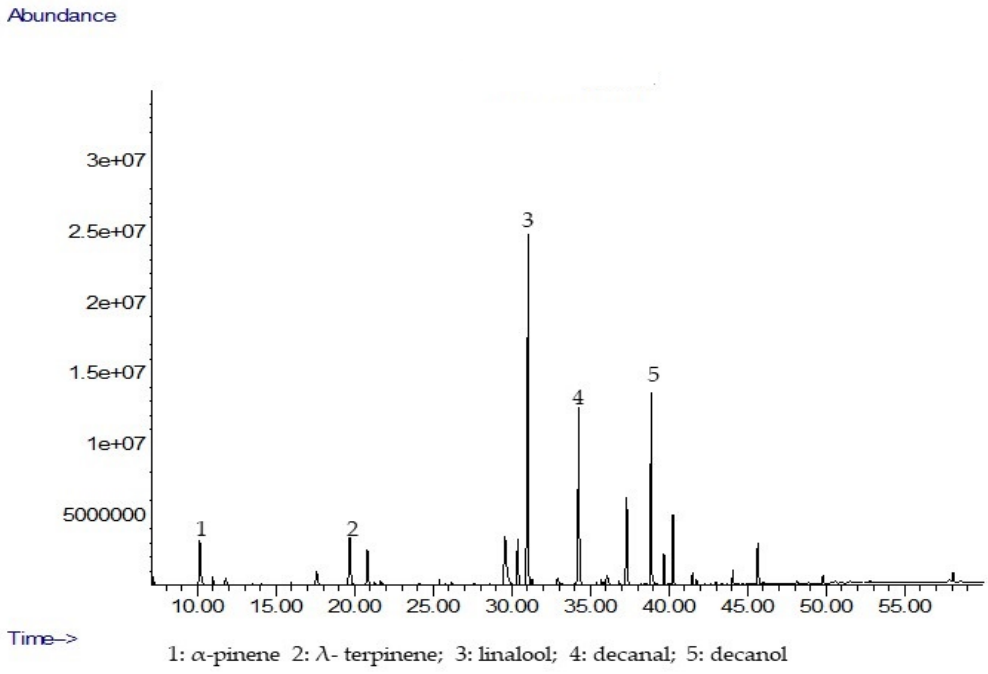


Figure 3: Total ion chromatogram of *Coriandrum sativum* oil

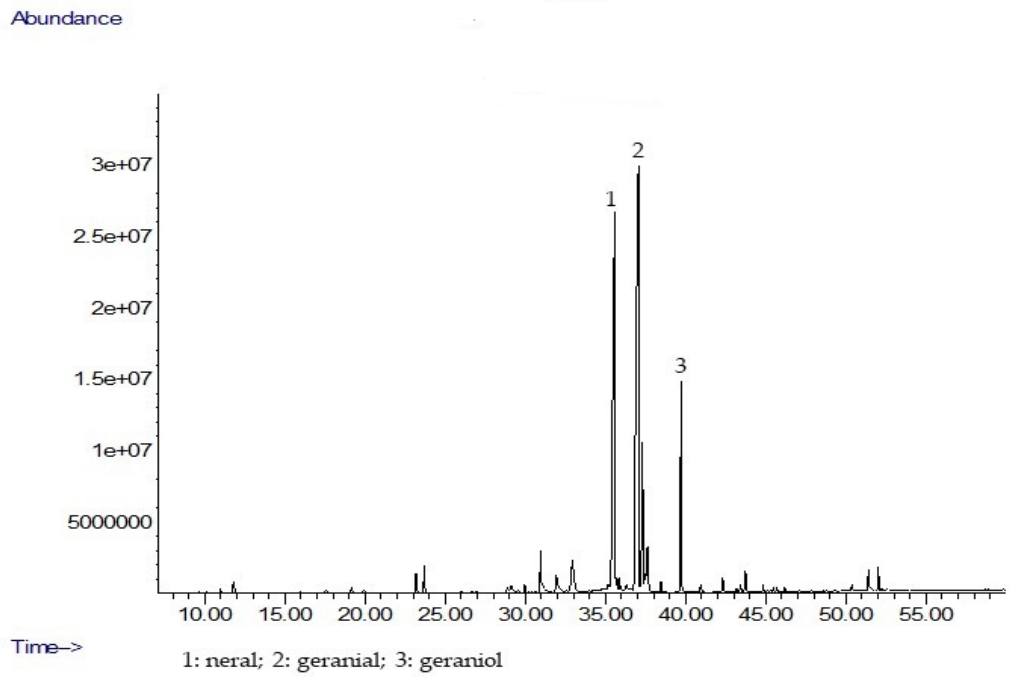


Figure 4: Total ion chromatogram of *Cymbopogon citratus* (lemon grass) oil

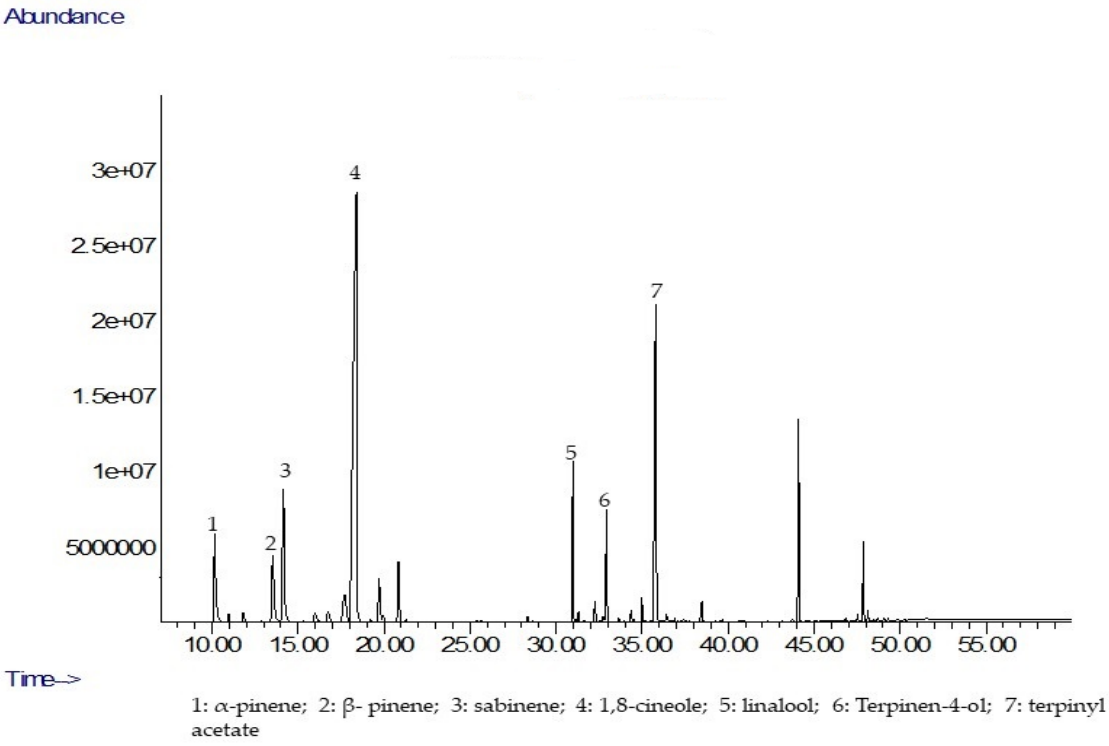


Figure 5: Total ion chromatogram of *Laurus nobilis* oil

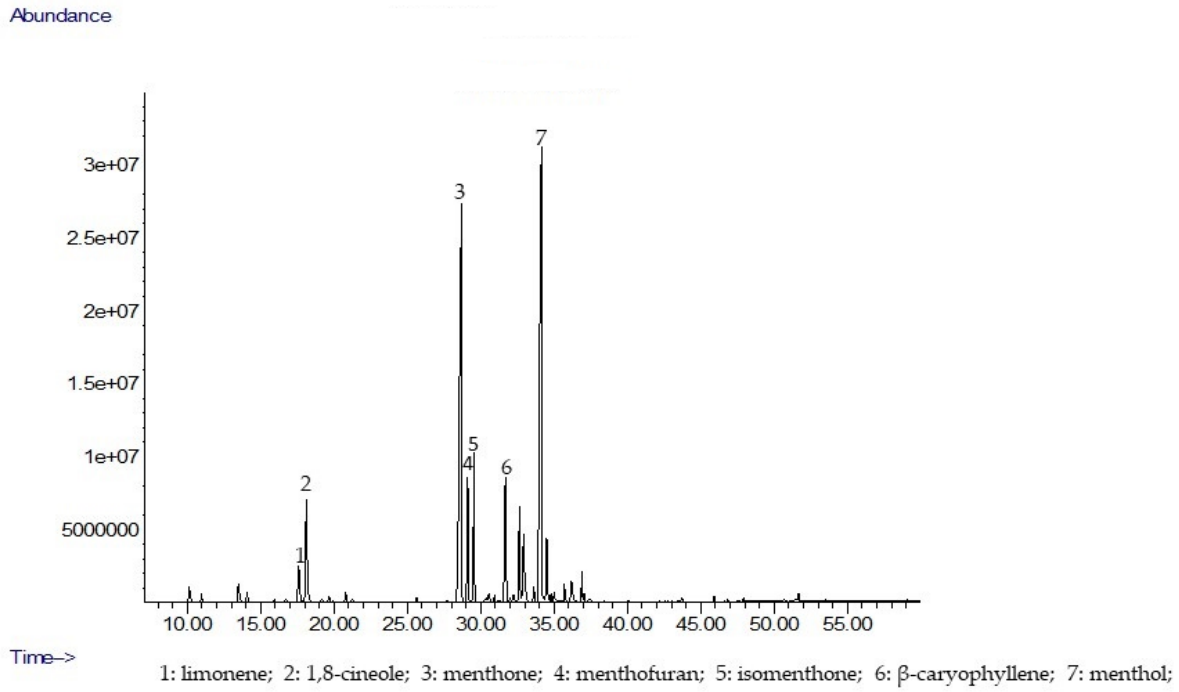


Figure 6: Total ion chromatogram of *Mentha piperita* oil

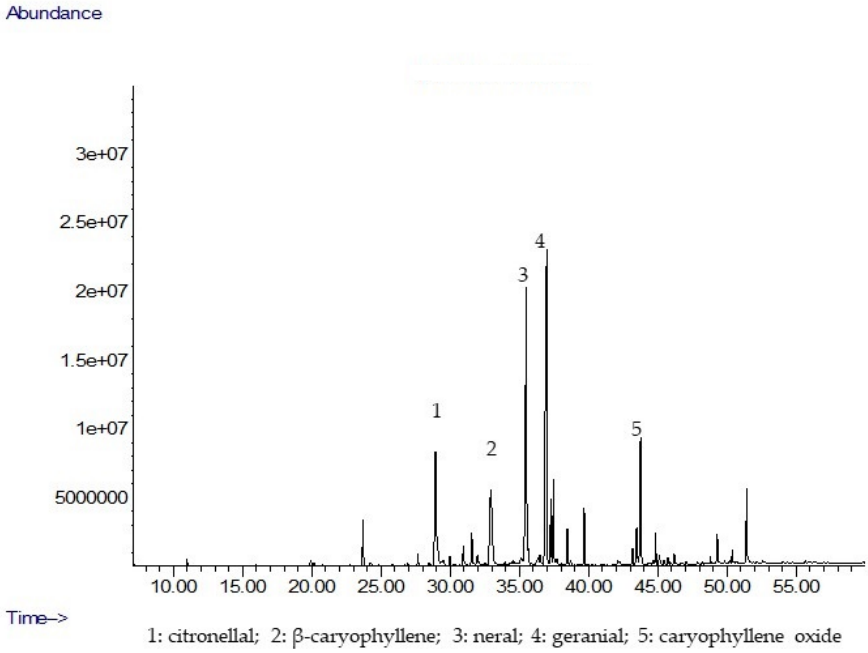


Figure 7: Total ion chromatogram of *Melissa officinalis* oil

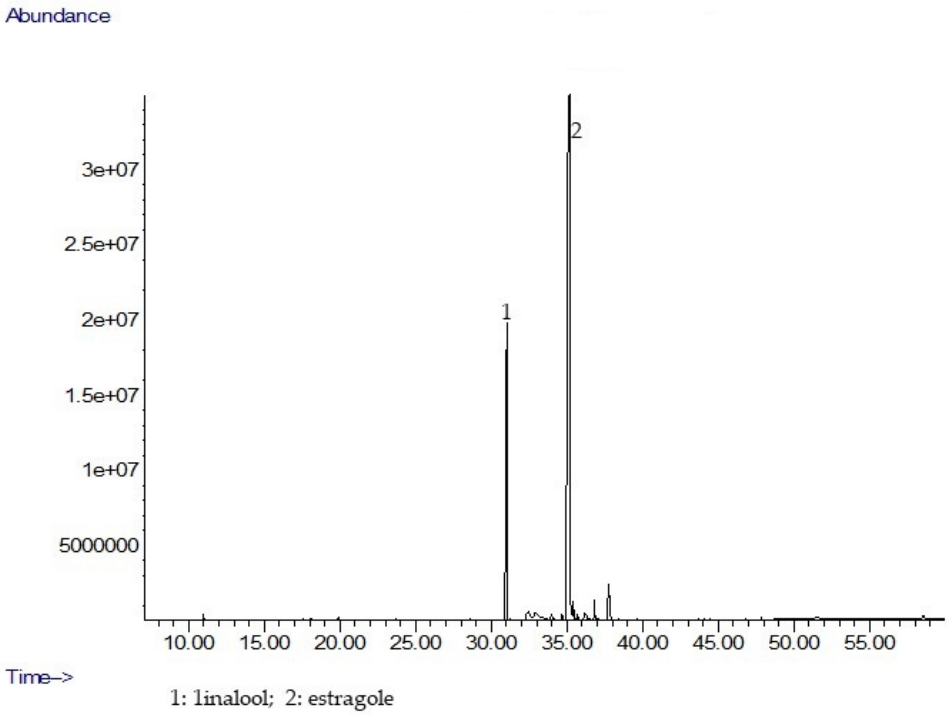


Figure 8: Total ion chromatogram of *Ocimum basilicum* oil

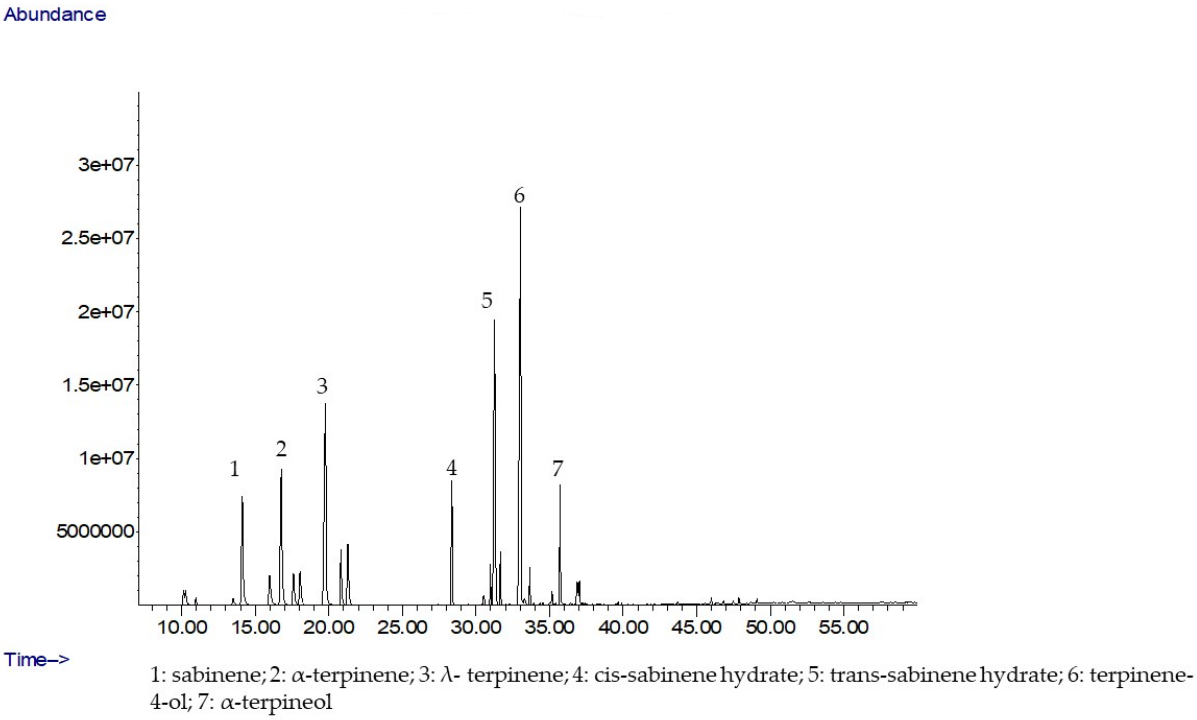
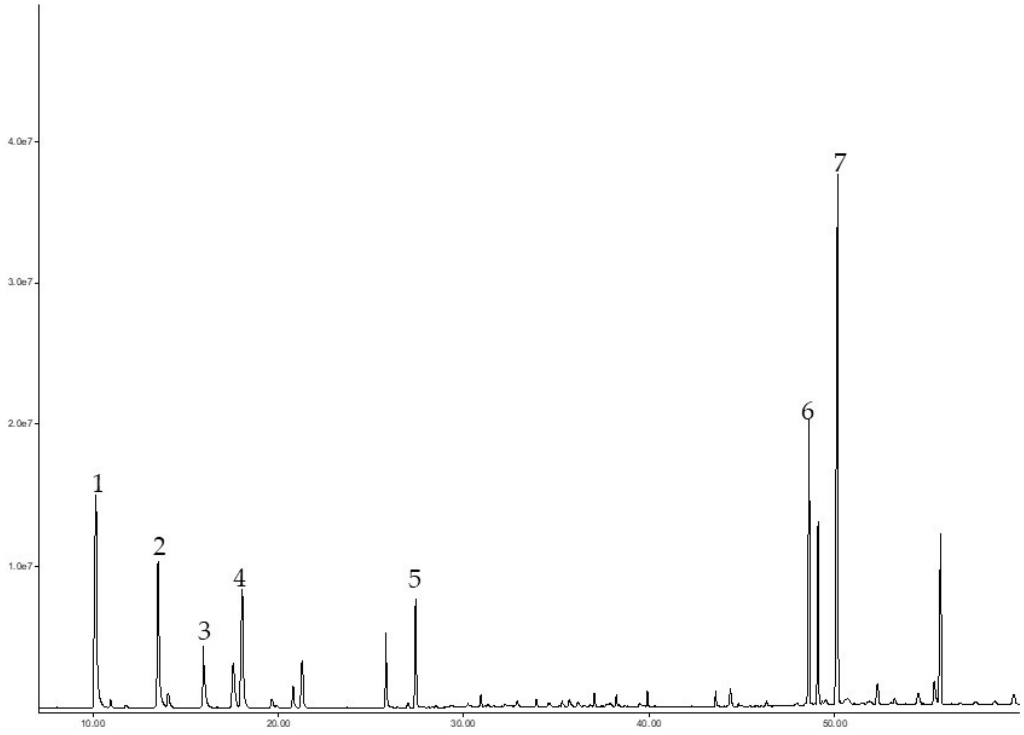
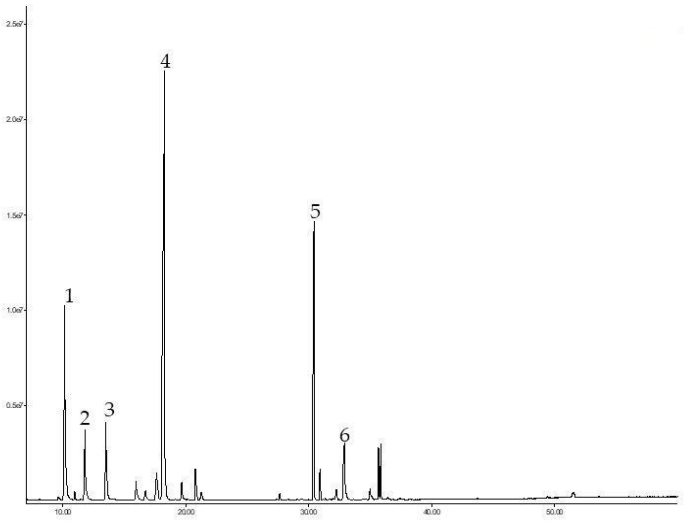


Figure 9: Total ion chromatogram of *Origanum marjorana* oil



1: α-pinene; 2: β-pinene; 3: myrcene; 4: limonene; 5: ρ-menthal 1,3,8-triene; 6: elimicin; 7: myristicin

Figure 10: Total ion chromatogram of *P. crispum* oil



1: α-pinene; 2: camphene; 3: β-pinene; 4: 1,8-cineole; 5: camphor; 6: β-caryophyllene

Figure 11: Total ion chromatogram of *R. officinalis* oil

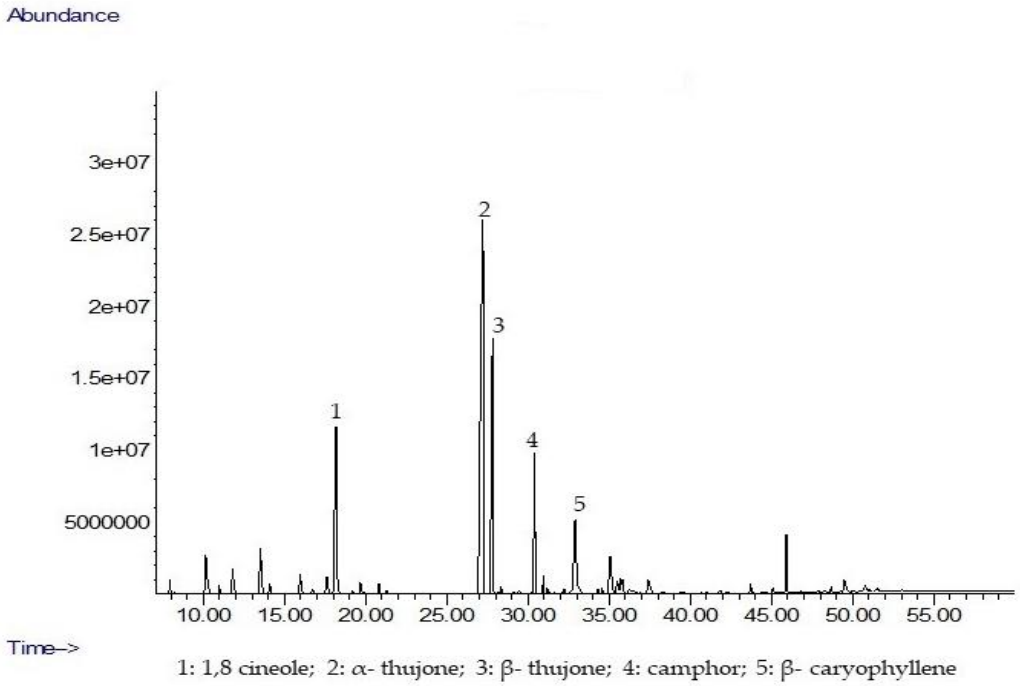


Figure 12: Total ion chromatogram of *Salvia officinalis* oil

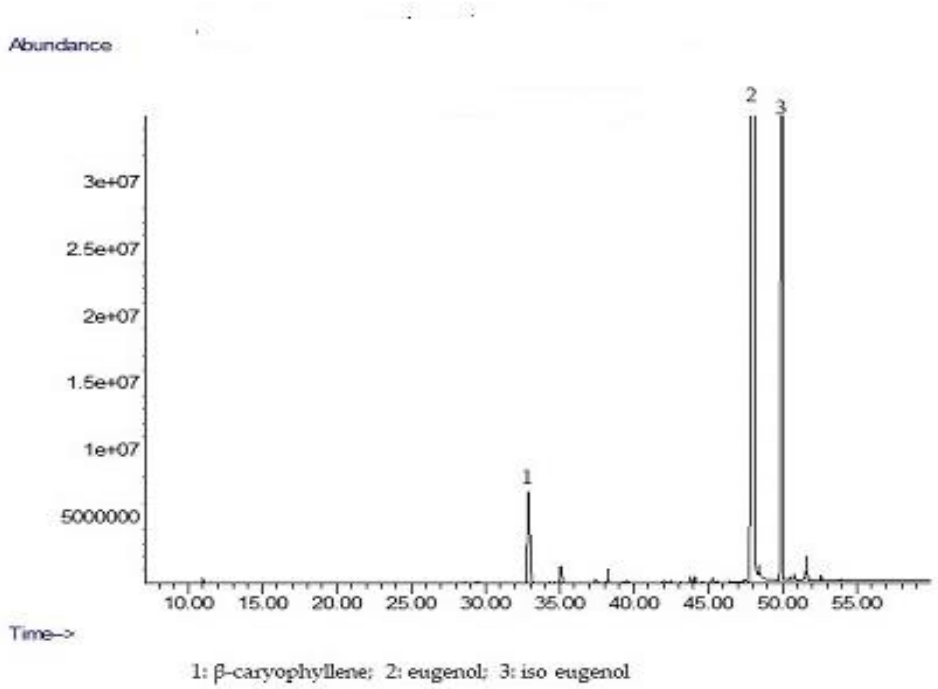


Figure 13: Total ion chromatogram of *Syzygium aromaticum* oil

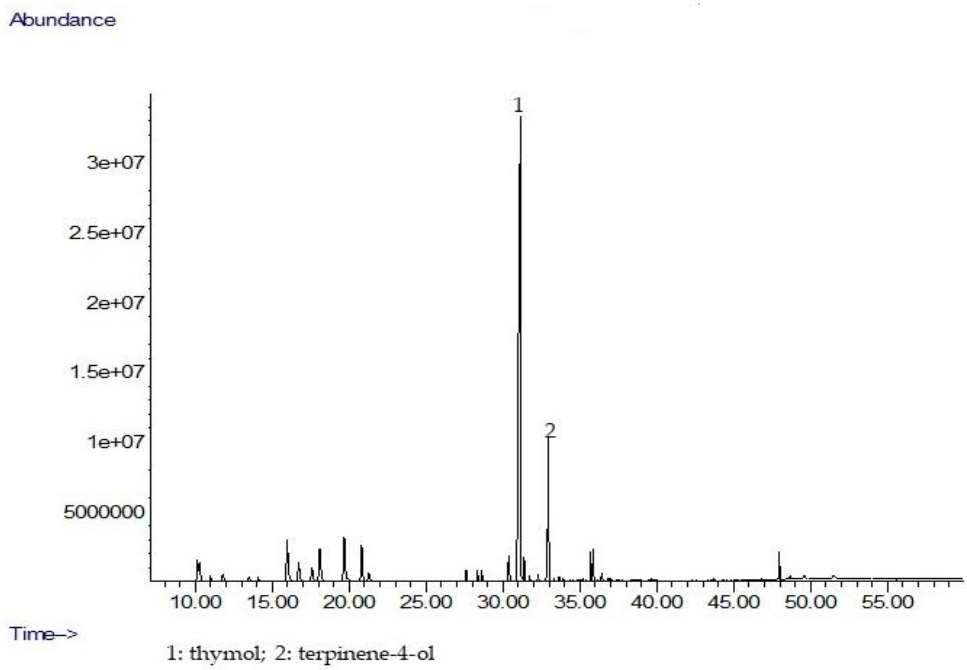


Figure 14: Total ion chromatogram of *Thymus vulgaris* oil

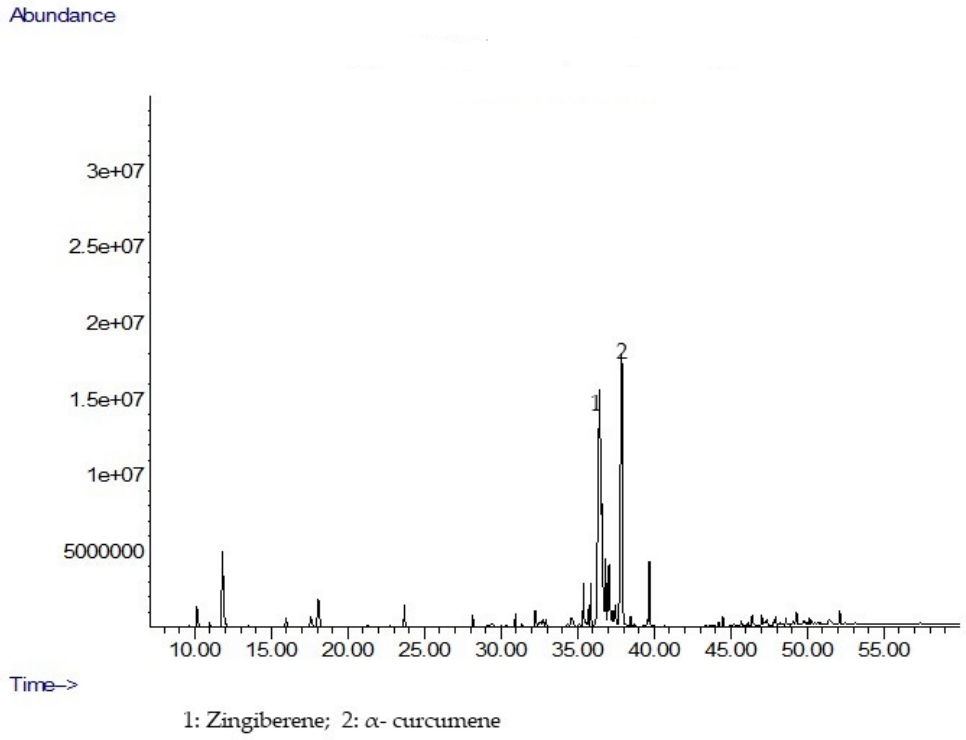
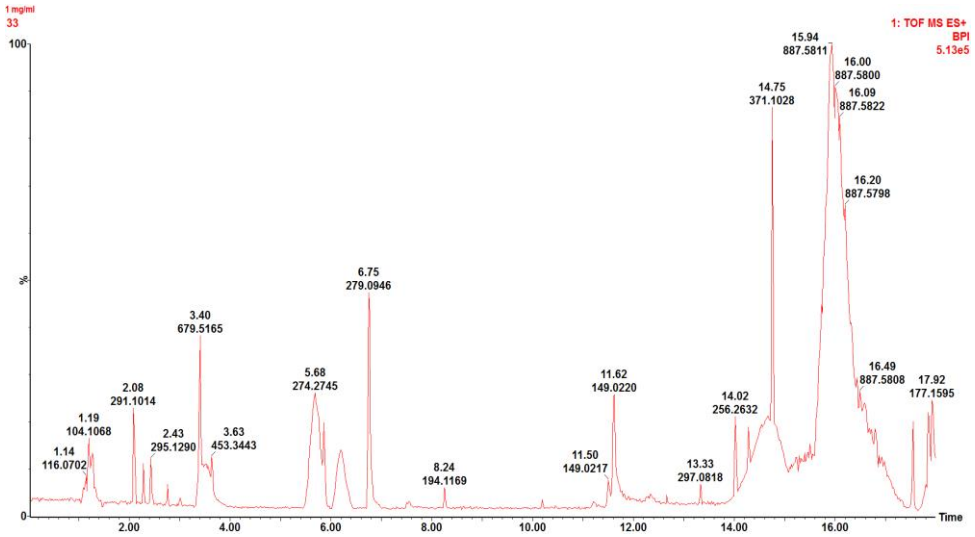


Figure 15: Total ion chromatogram of *Zingiber officinalis* oil

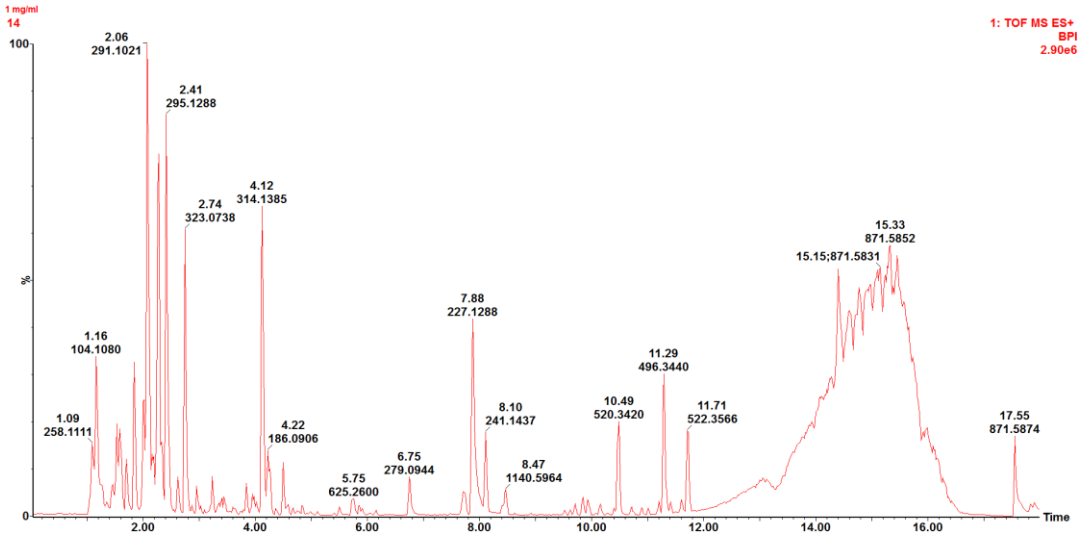
APPENDIX D

UPLC-MS Chromatograms of crude extracts of selected culinary herbs and spices

A



B



C

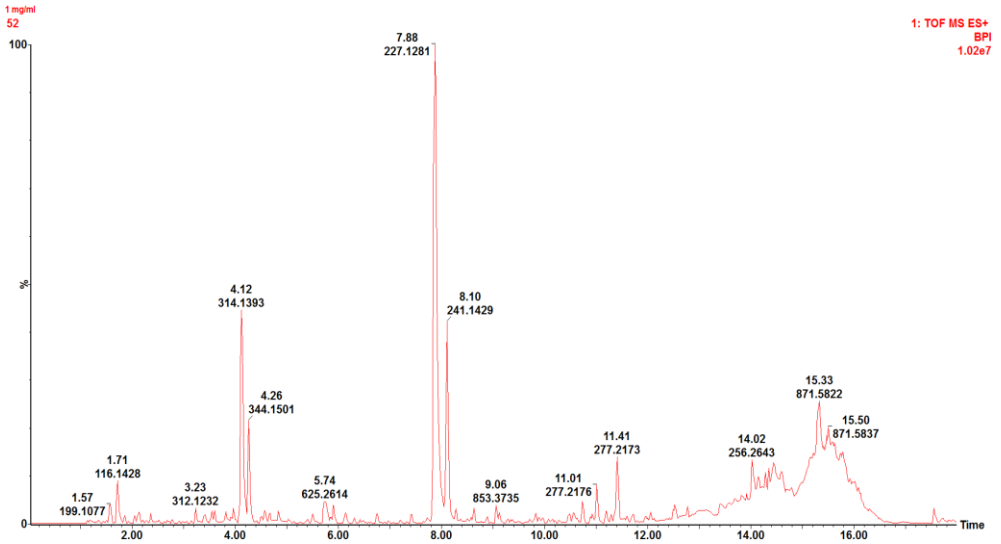
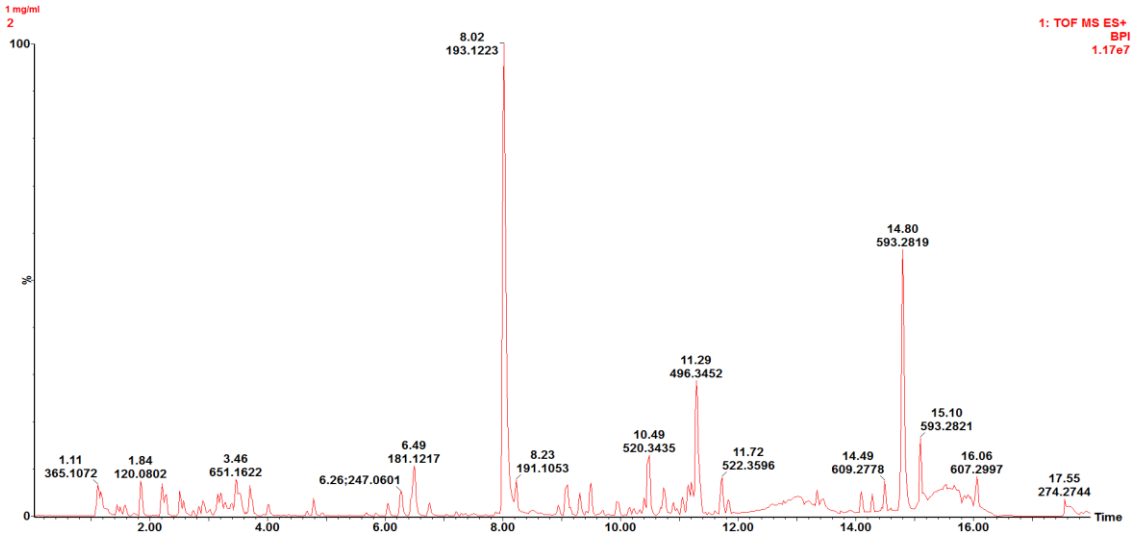


Figure 1: *Allium sativum* crude extracts, A: methanol, B: water, C: dichloromethane

A



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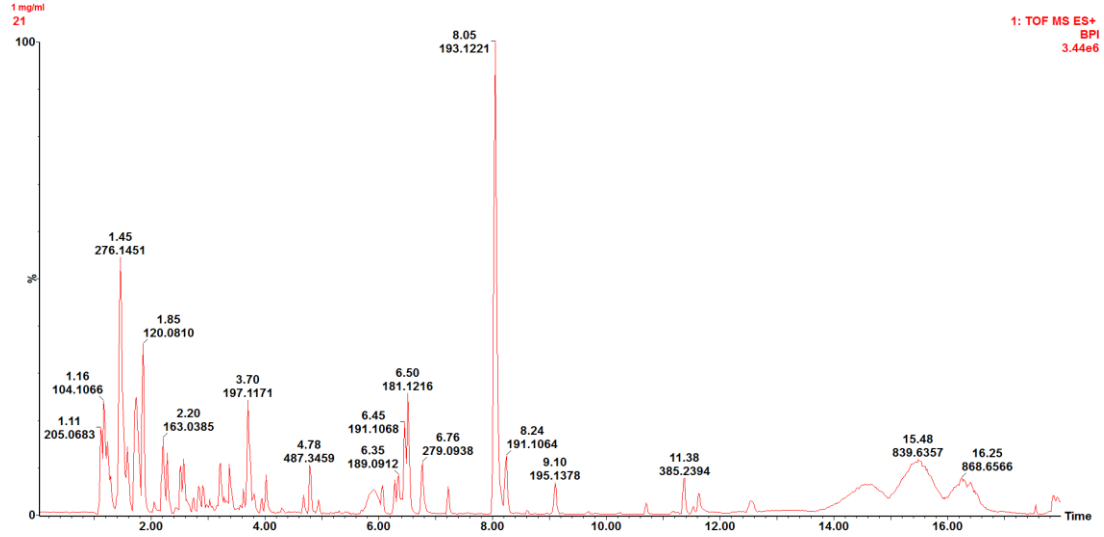

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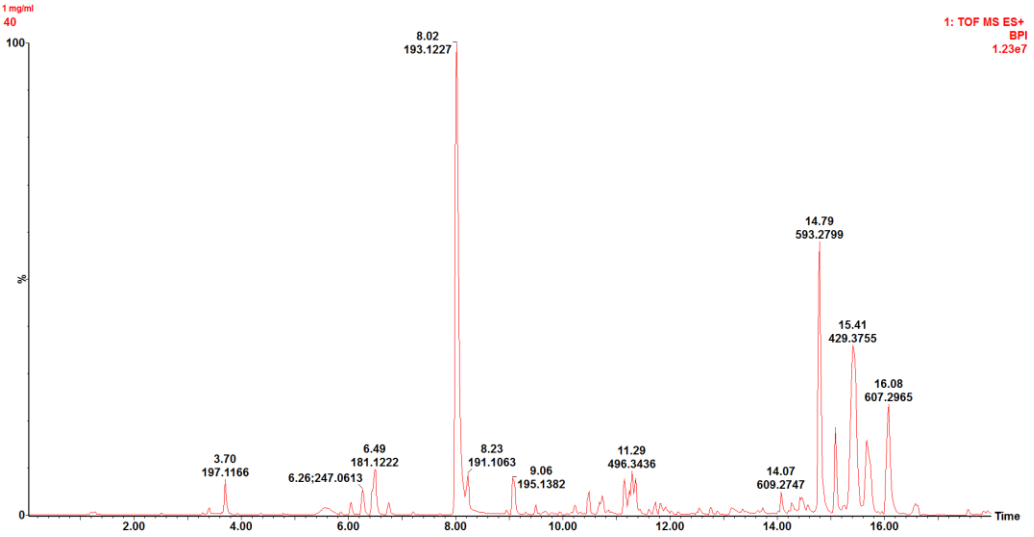
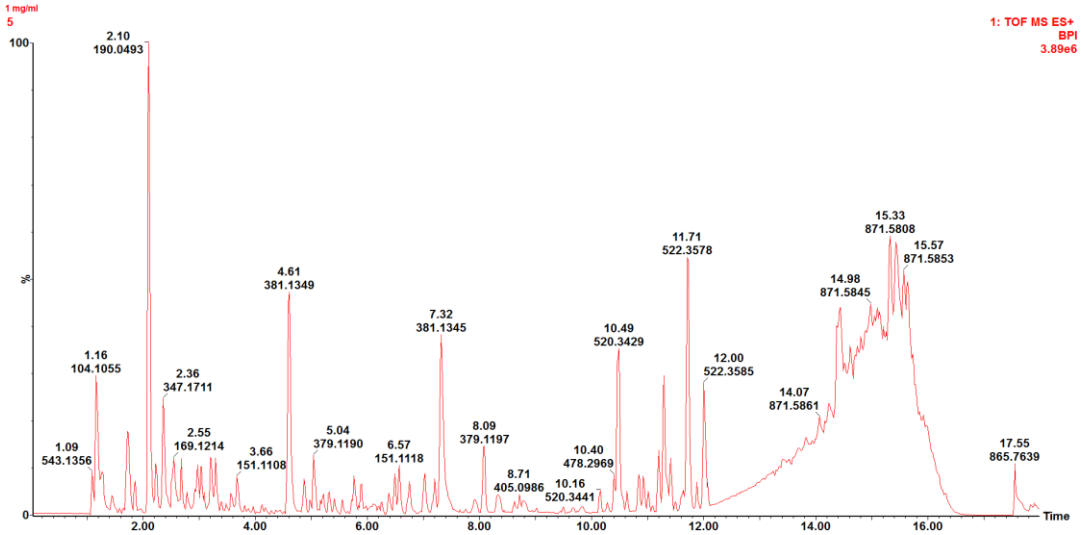
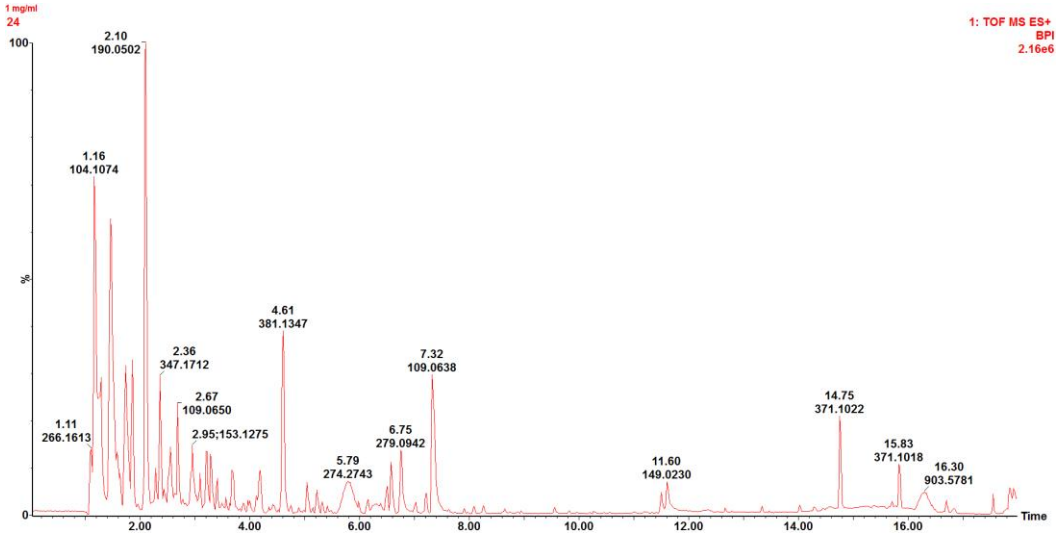


Figure 2: *Apium graveolens* extracts, A: methanol, B: water, C: dichloromethane

A



B



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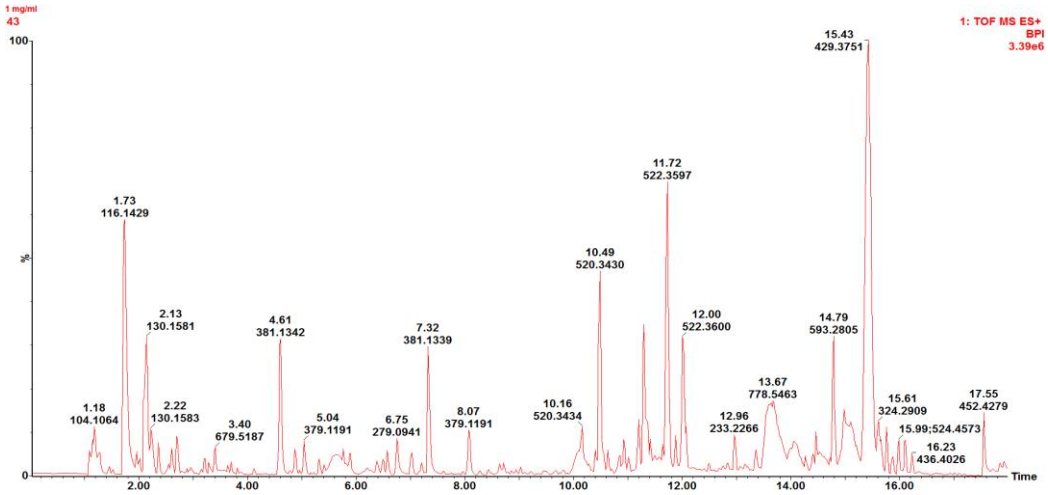


Figure 3: *Anethum graveolens* extracts, A: methanol, B: water, C: dichloromethane

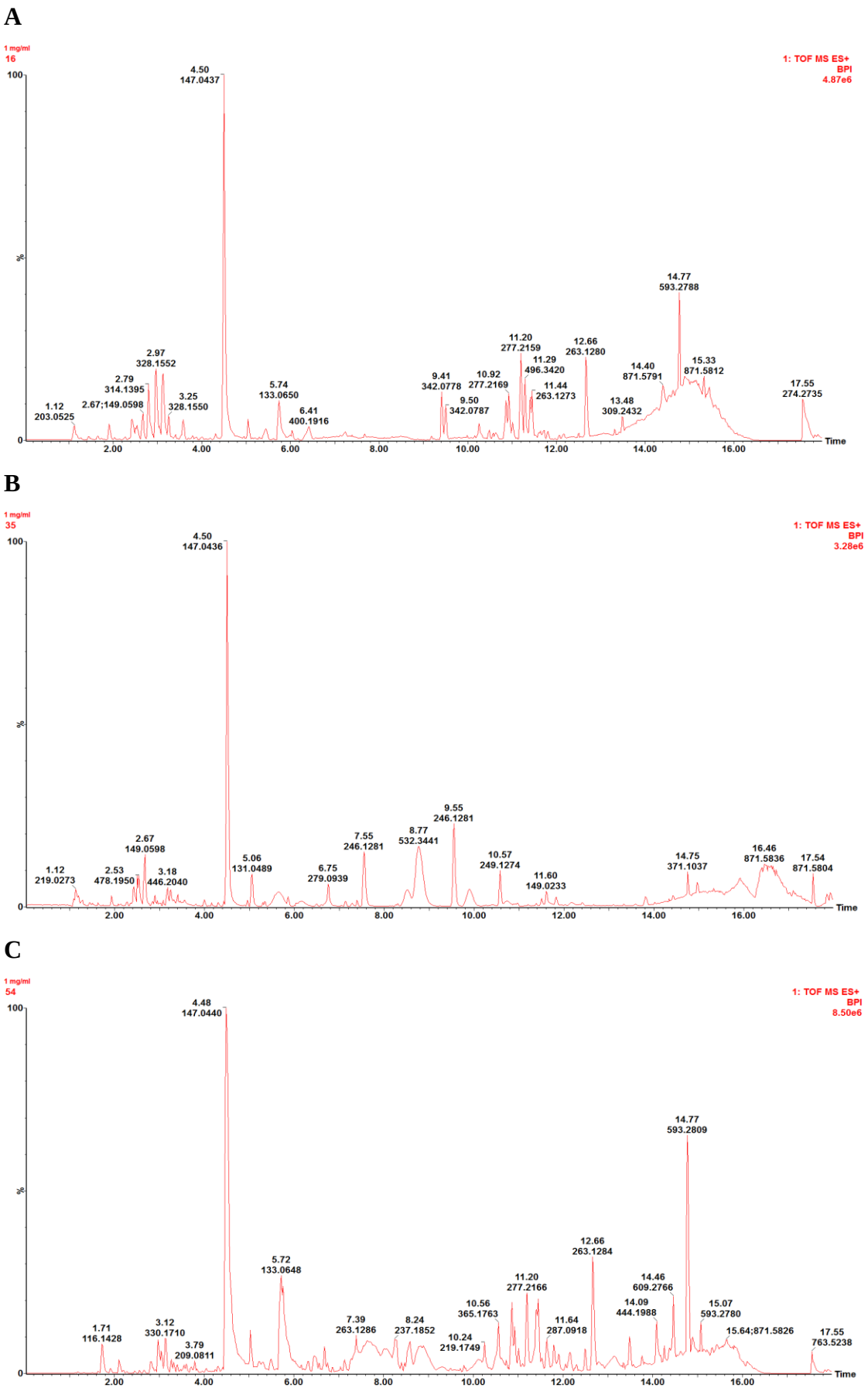
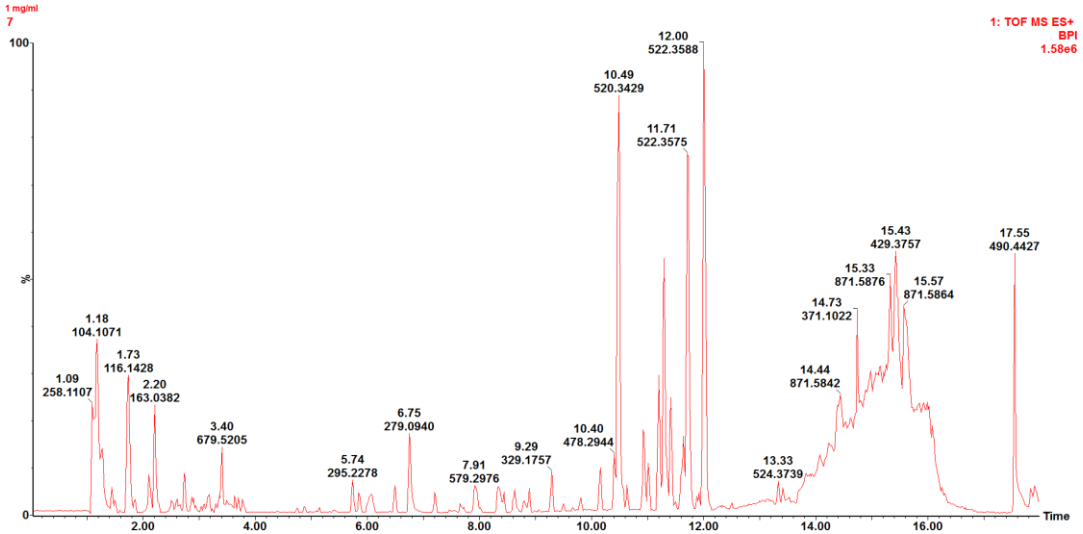


Figure 4: *Cinnamomum zeylanicum* extracts, A: methanol, B: water, C: dichloromethane

A



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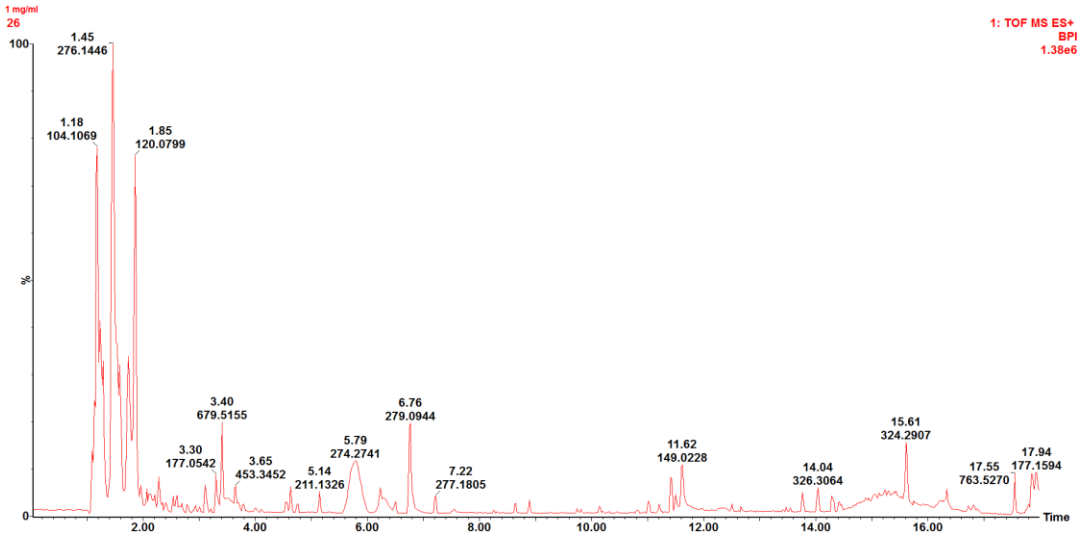

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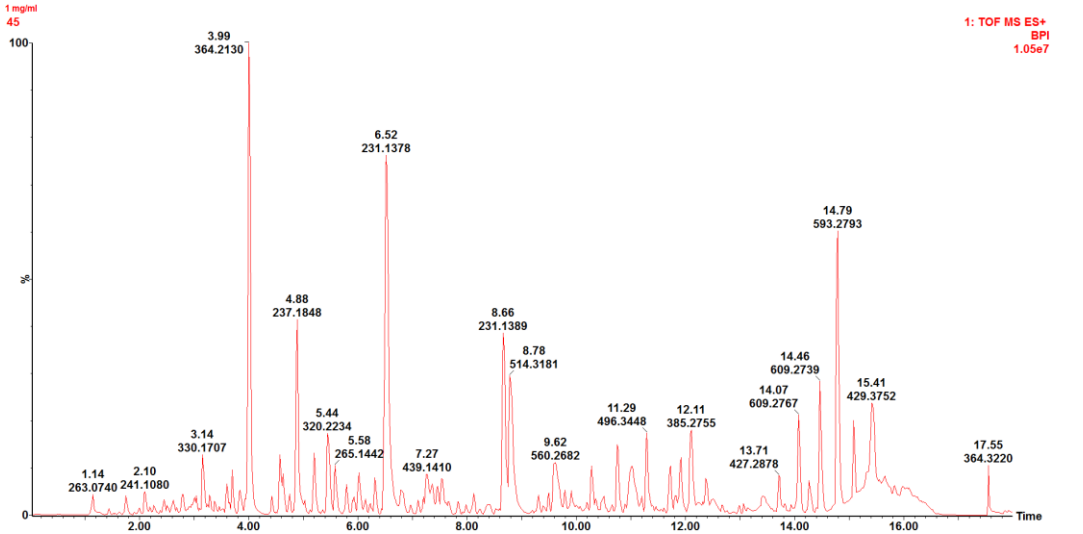
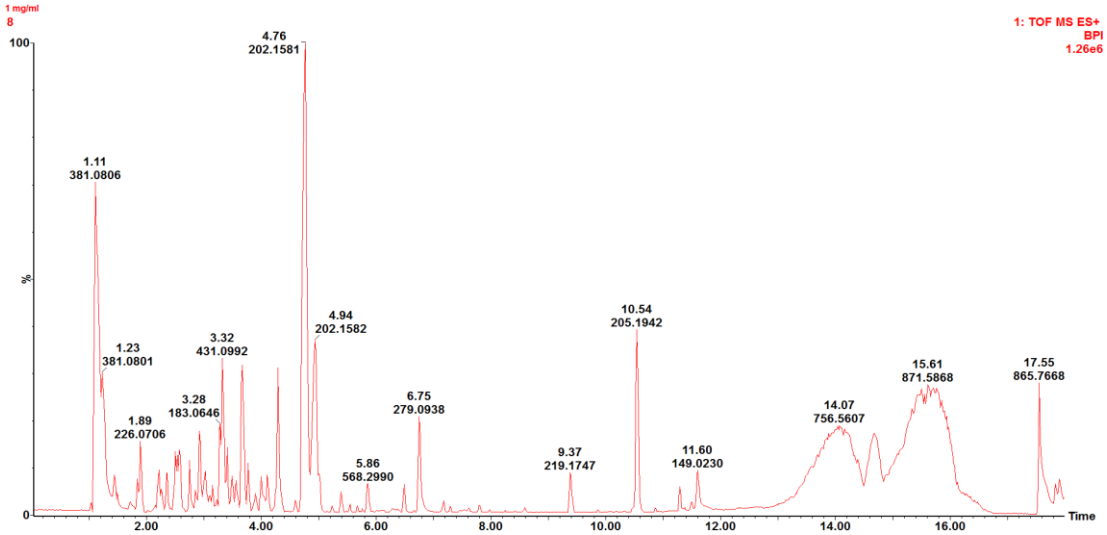


Figure 5: *Coriandrum sativum* extracts, A: methanol, B: water, C: dichloromethane

A



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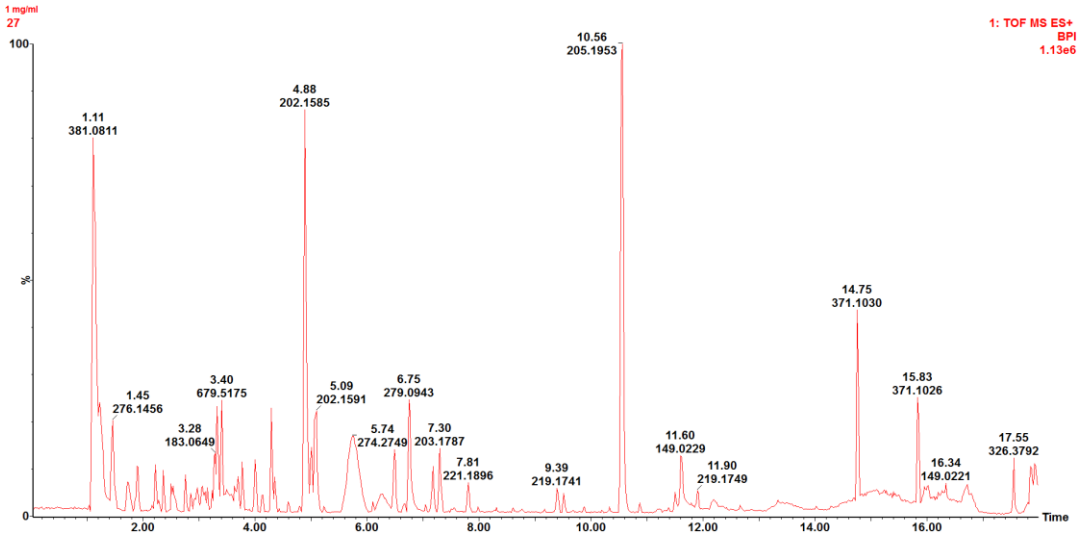

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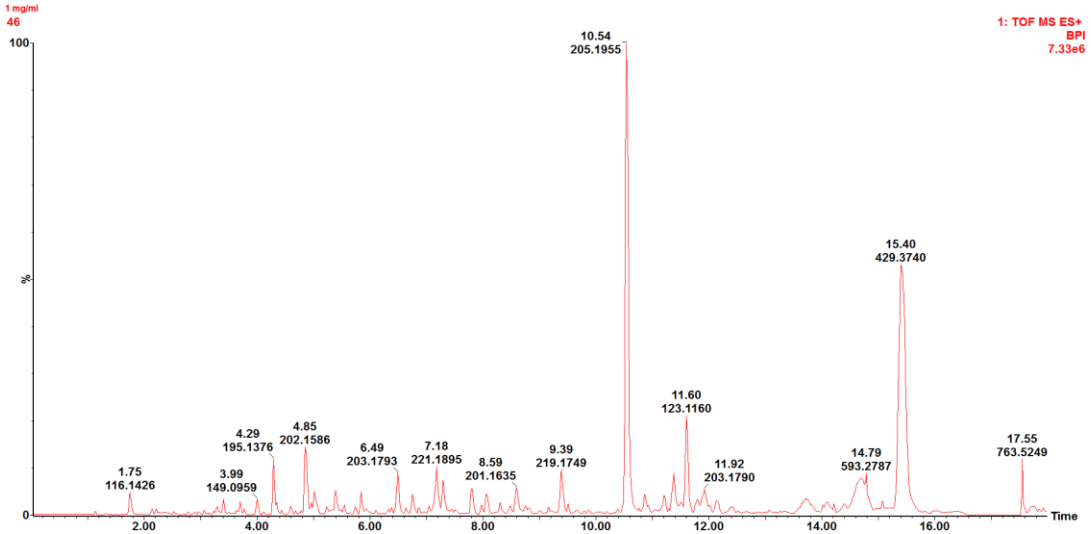
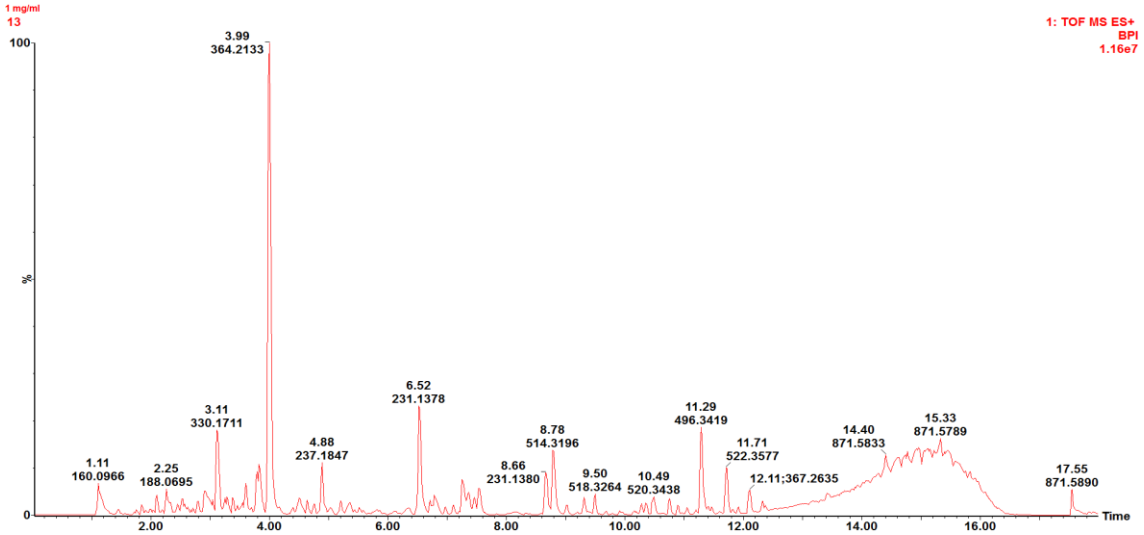


Figure 6: *Cymbopogon citratus* extracts, A: methanol, B: water, C: dichloromethane

A



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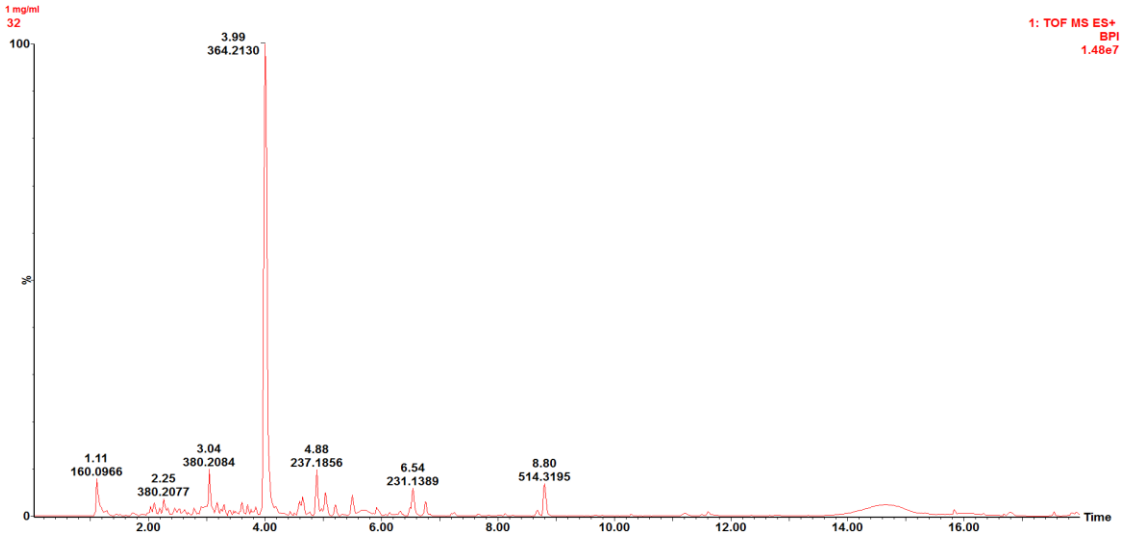


C

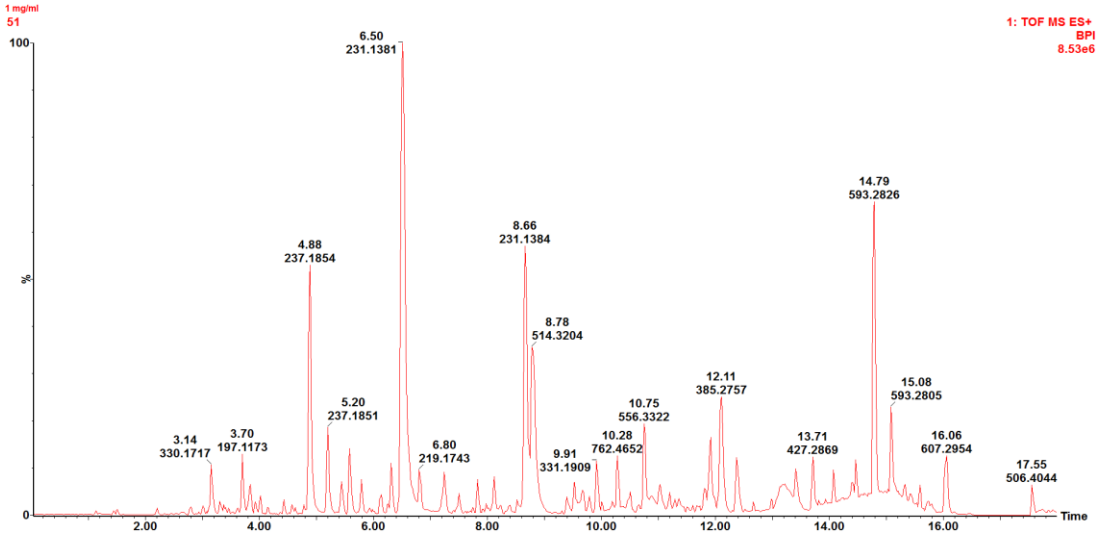


Figure 7: *Laurus nobilis* extracts, A: methanol, B: water, C: dichloromethane

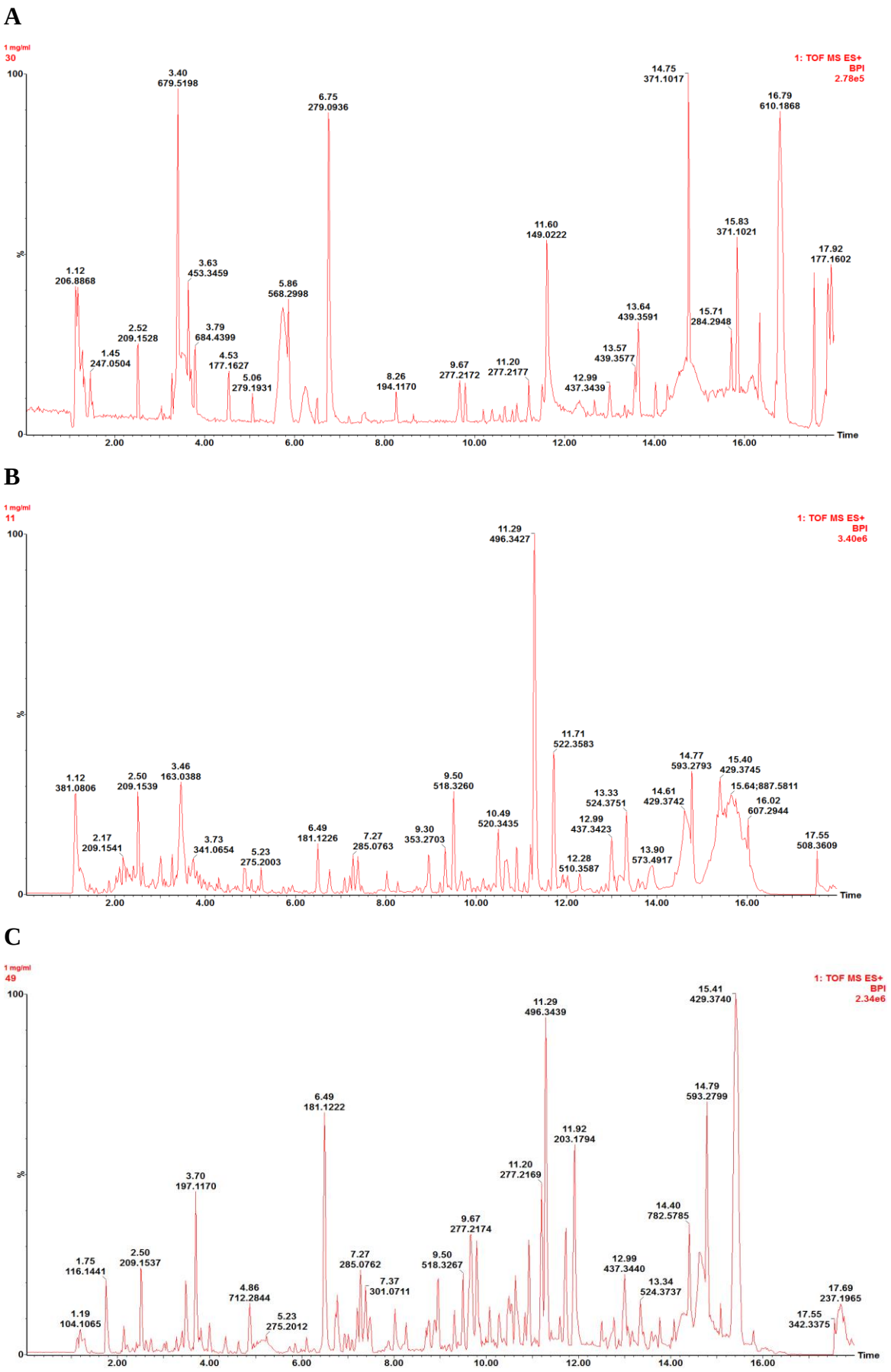
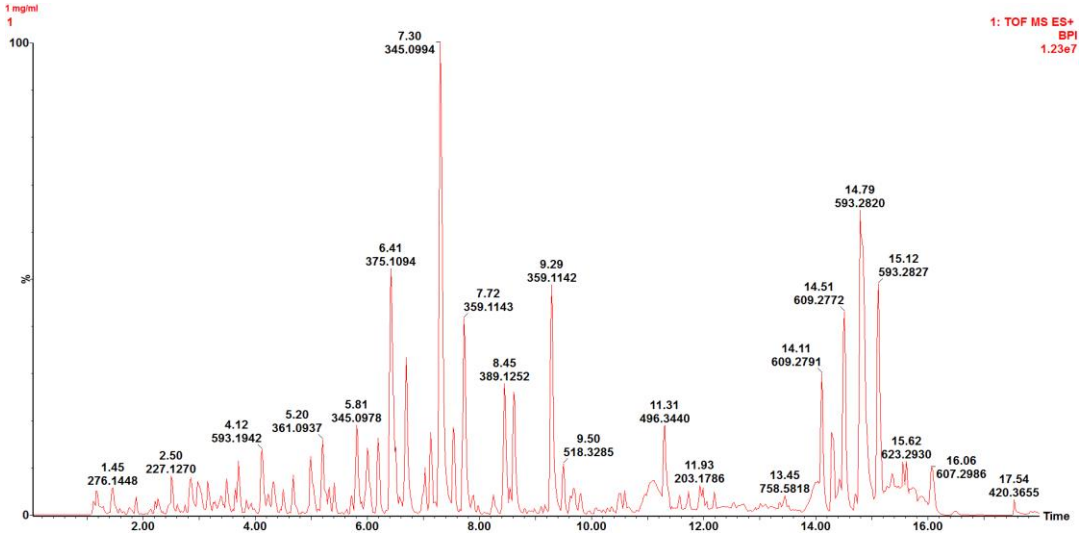
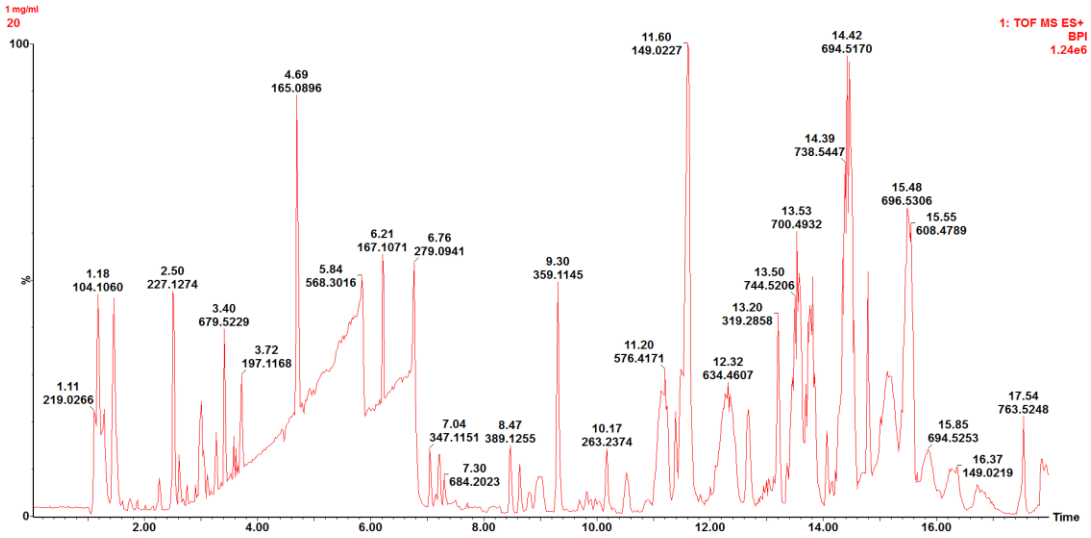


Figure 8: *Melissa officinalis* extracts, A: methanol, B: water, C: dichloromethane

A



B



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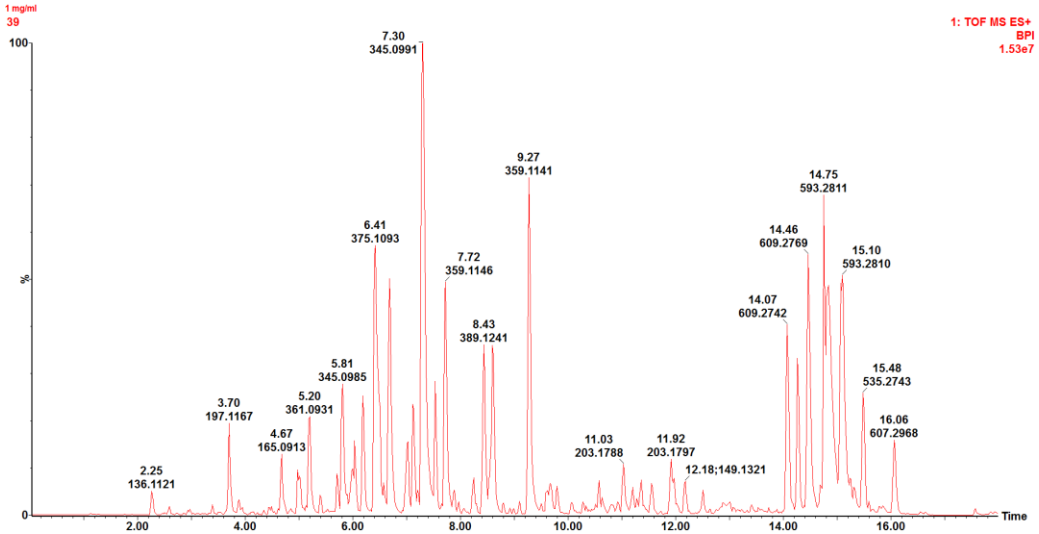
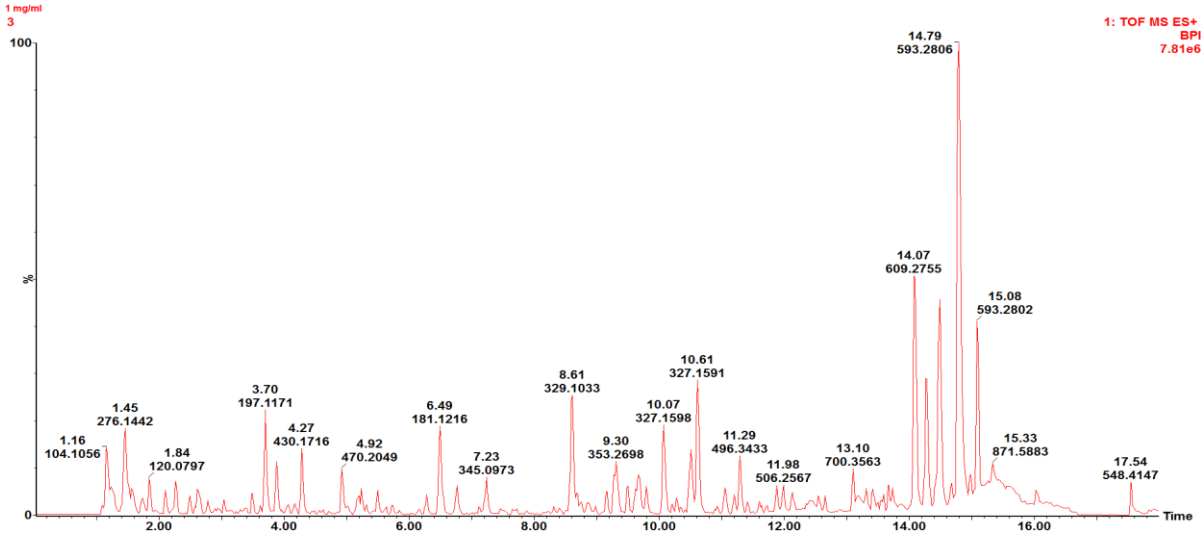
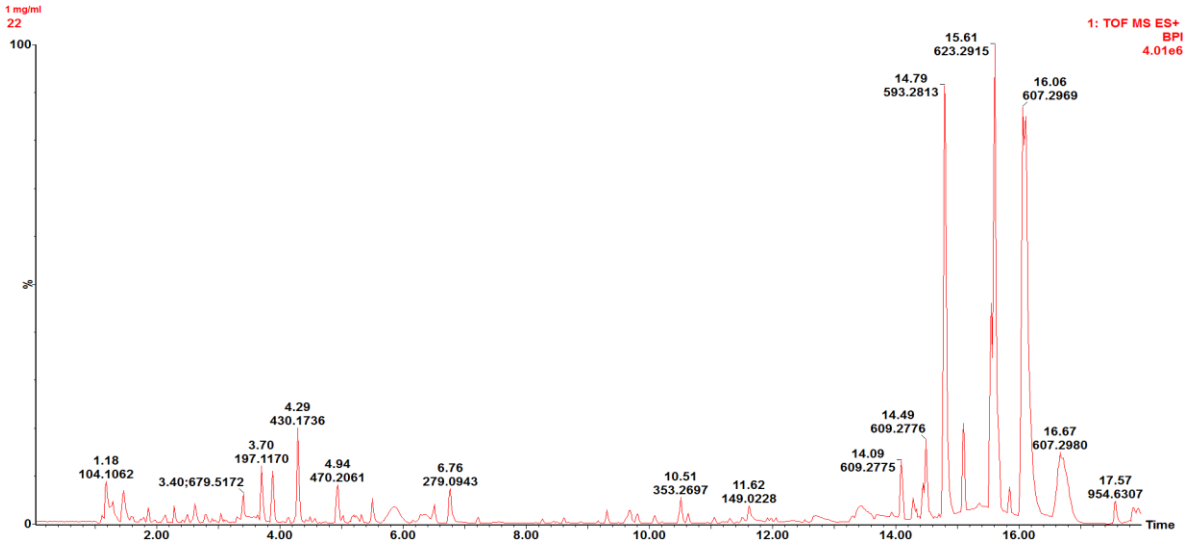


Figure 9: *Mentha piperita* extracts, A: methanol, B: water, C: dichloromethane

A



B



C

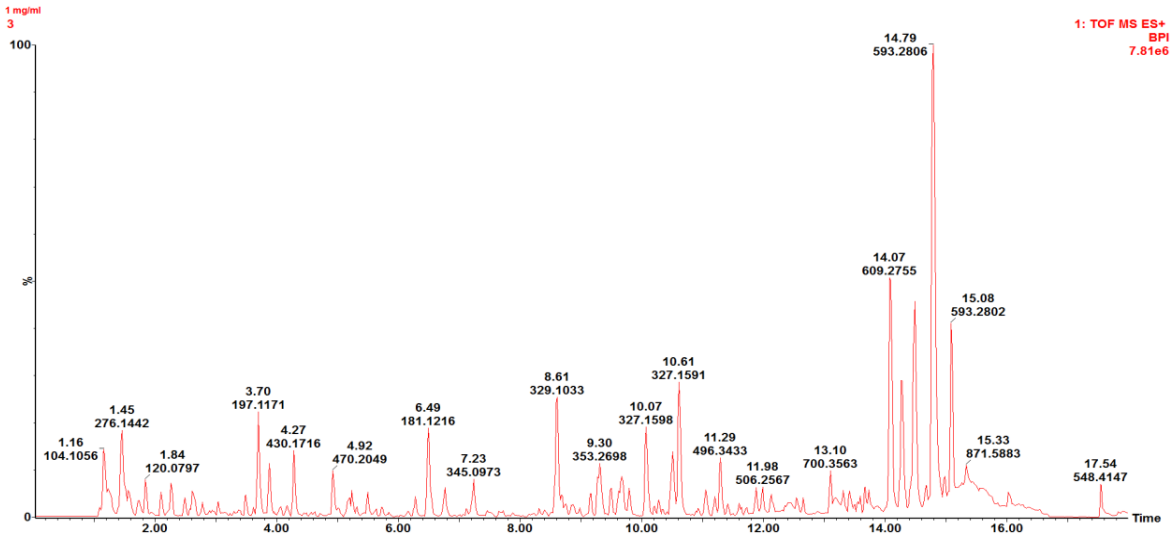


Figure 10: *Ocimum basilicum* extracts, A: methanol, B: water, C: dichloromethane

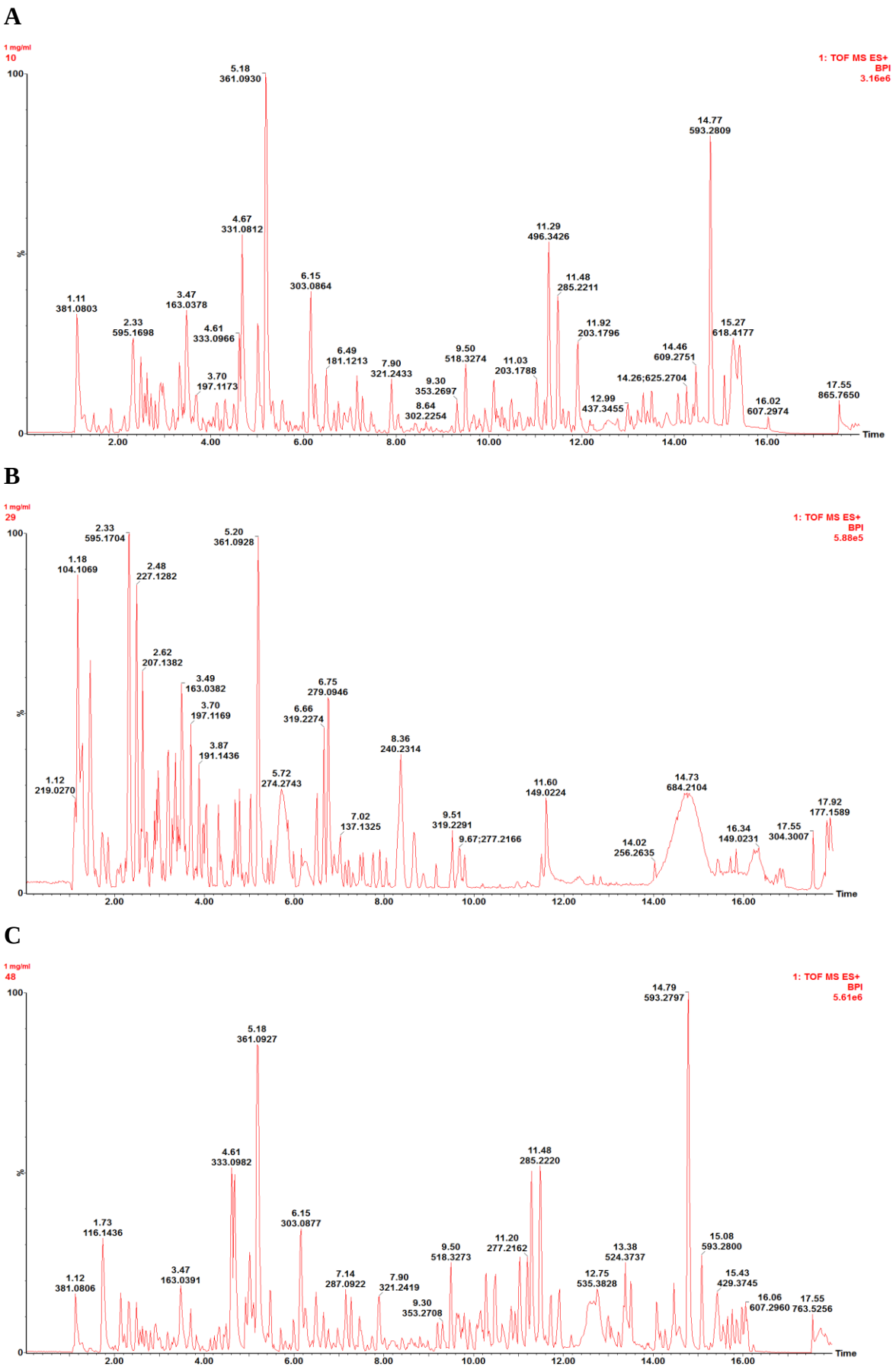


Figure 11: *Origanum marjorana* extracts, A: methanol, B: water, C: dichloromethane

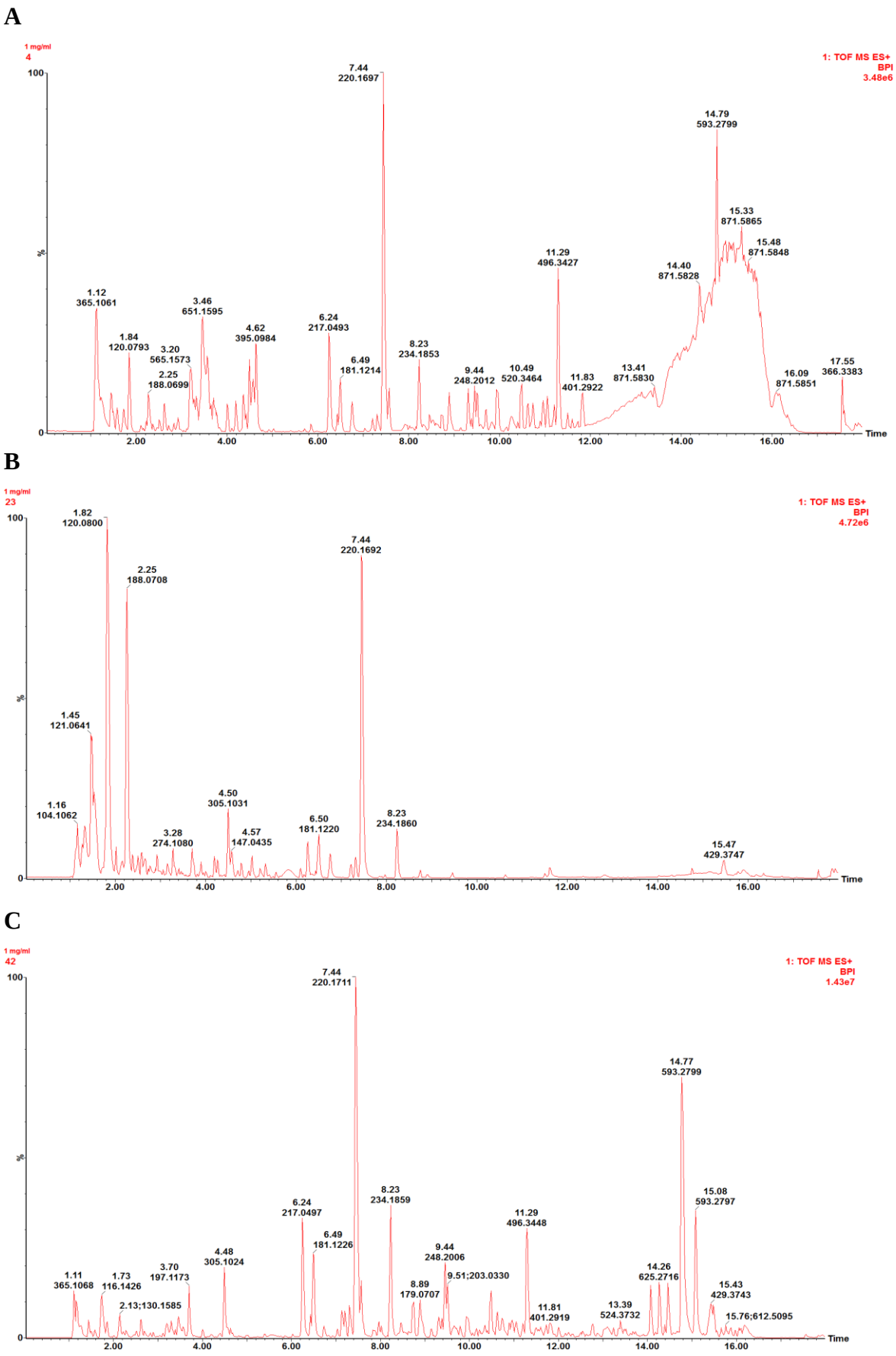


Figure 12: *Petroselinum crispum* extracts, A: methanol, B: water, C: dichloromethane

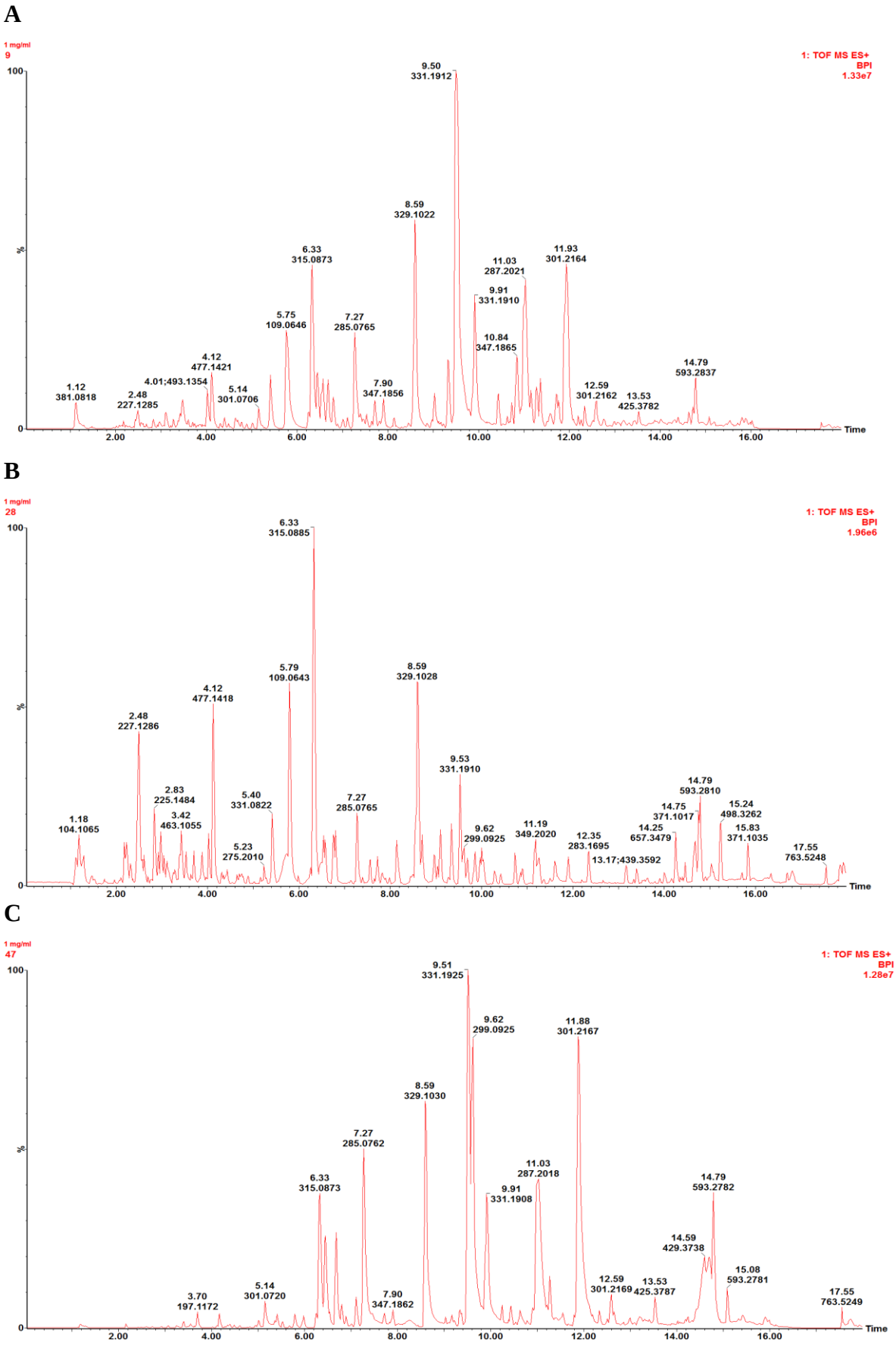


Figure 13: *Rosmarinus officinalis* extracts, A: methanol, B: water, C: dichloromethane

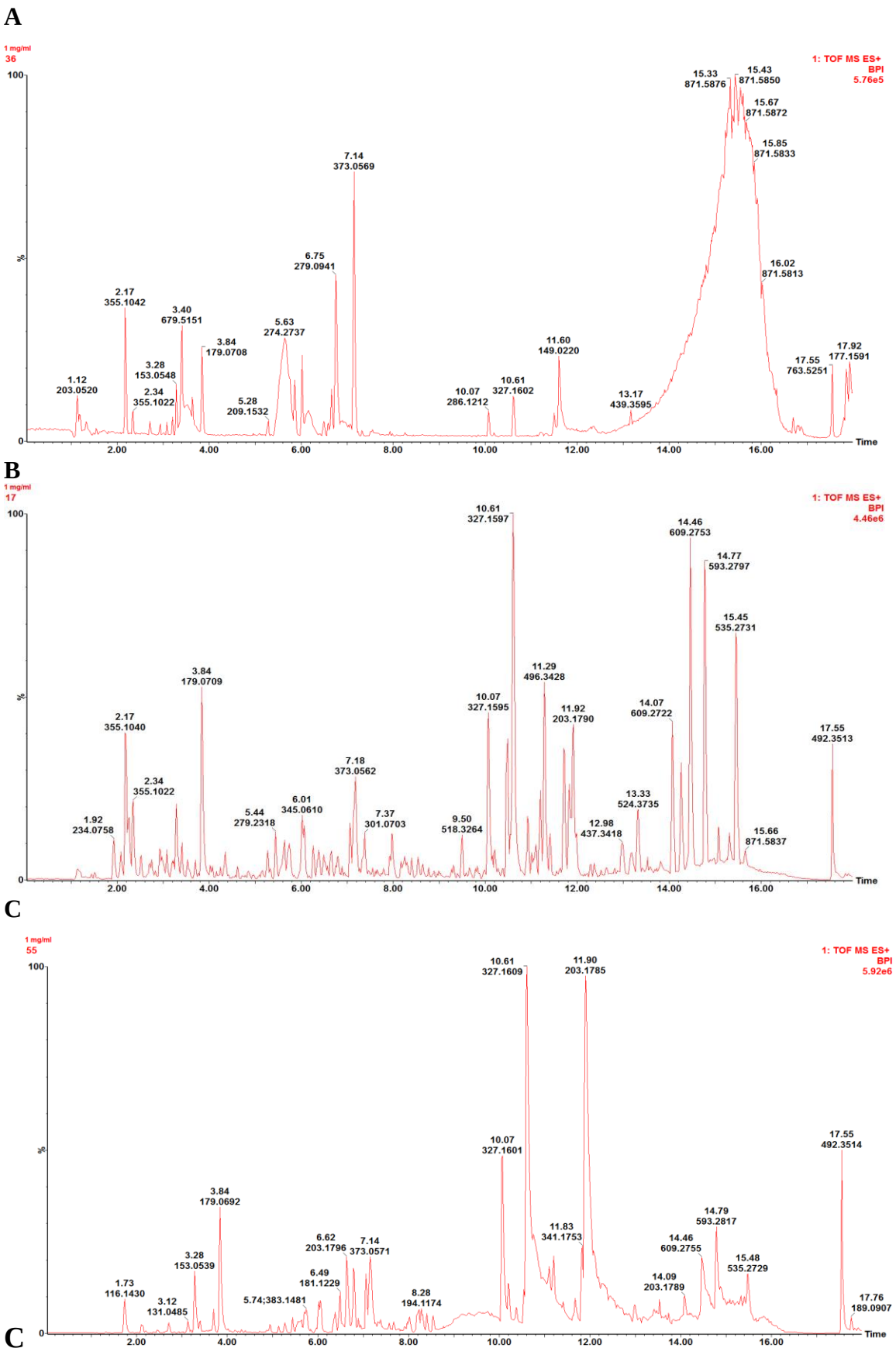
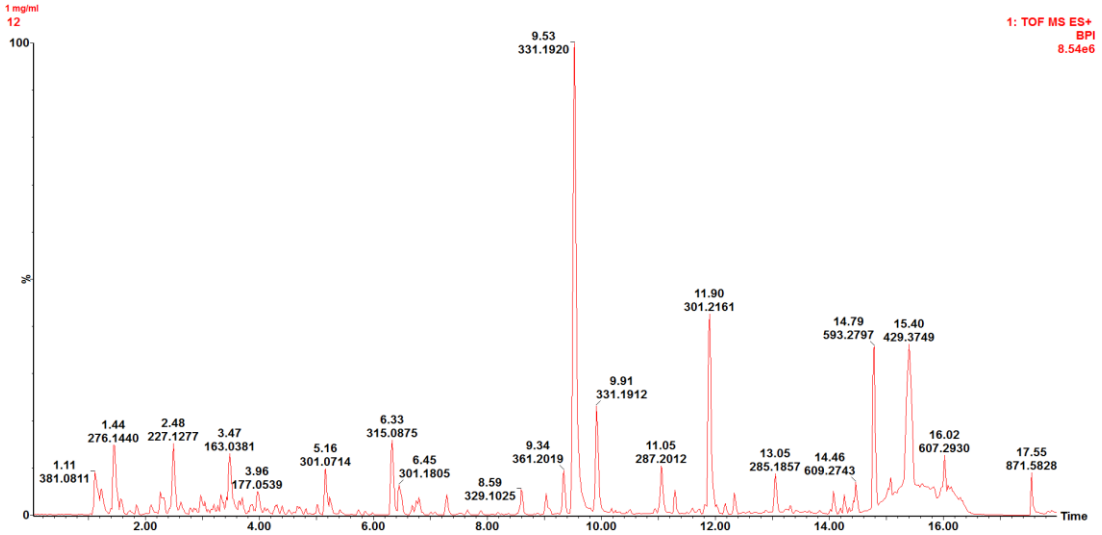
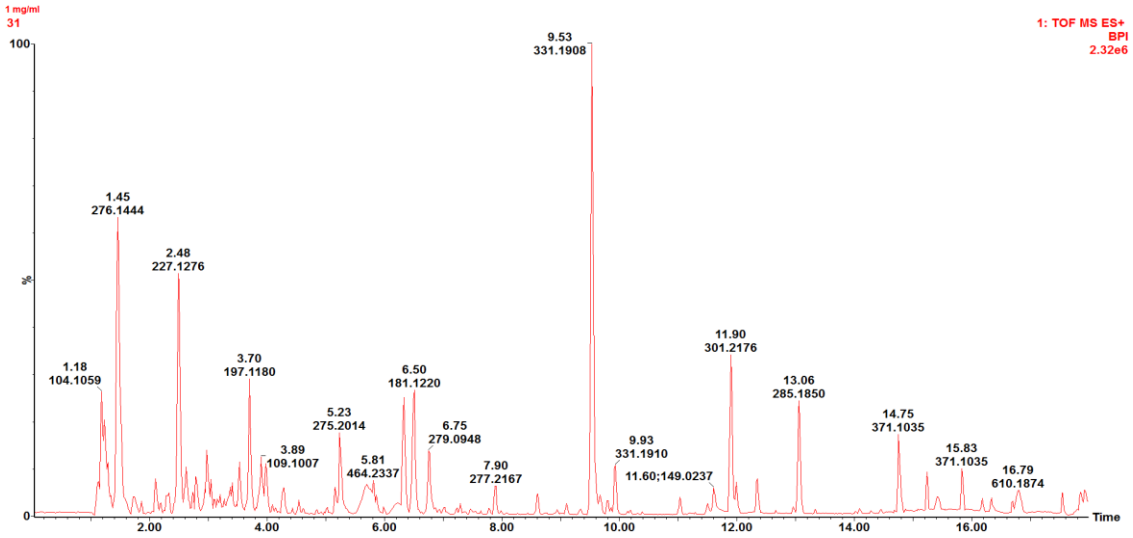


Figure 14: *Syzygium aromaticum* extracts, A: methanol, B: water, C: dichloromethane

A



B



C

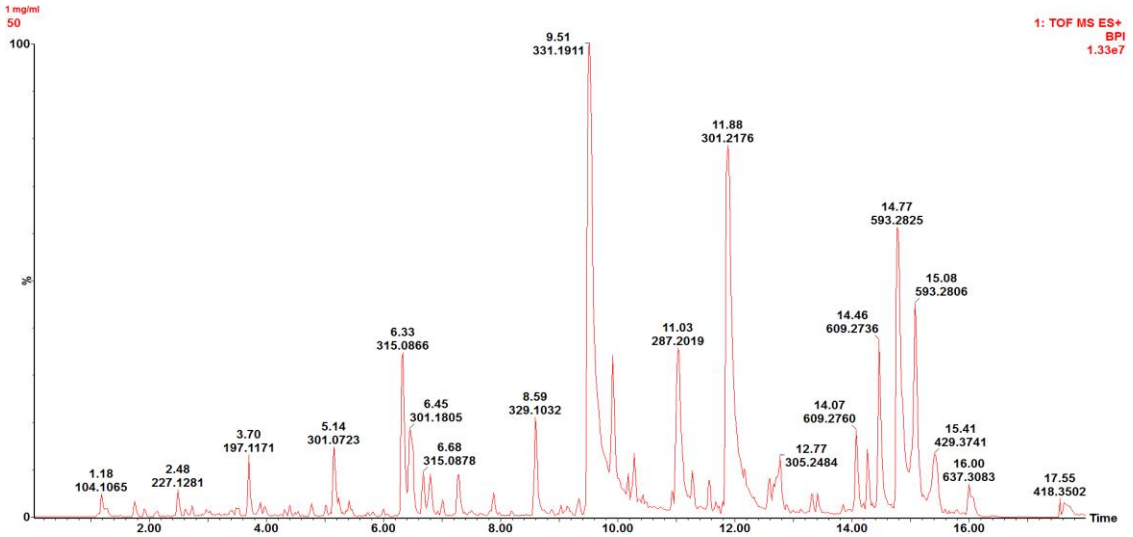


Figure 15: *Salvia officinalis* extracts, A: methanol, B: water, C: dichloromethane

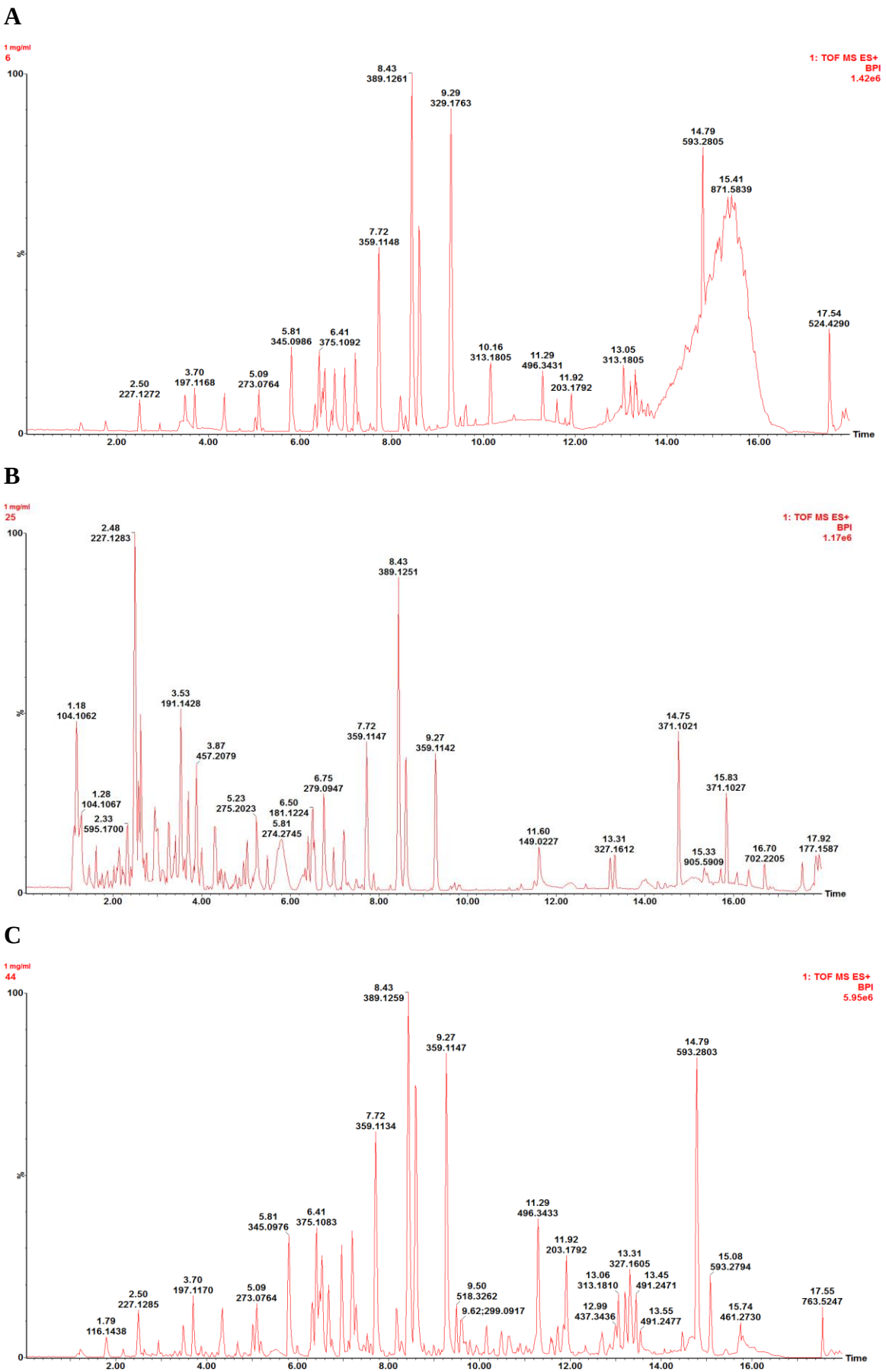


Figure 16: *Thymus vulgaris* extracts, A: methanol, B: water, C: dichloromethane

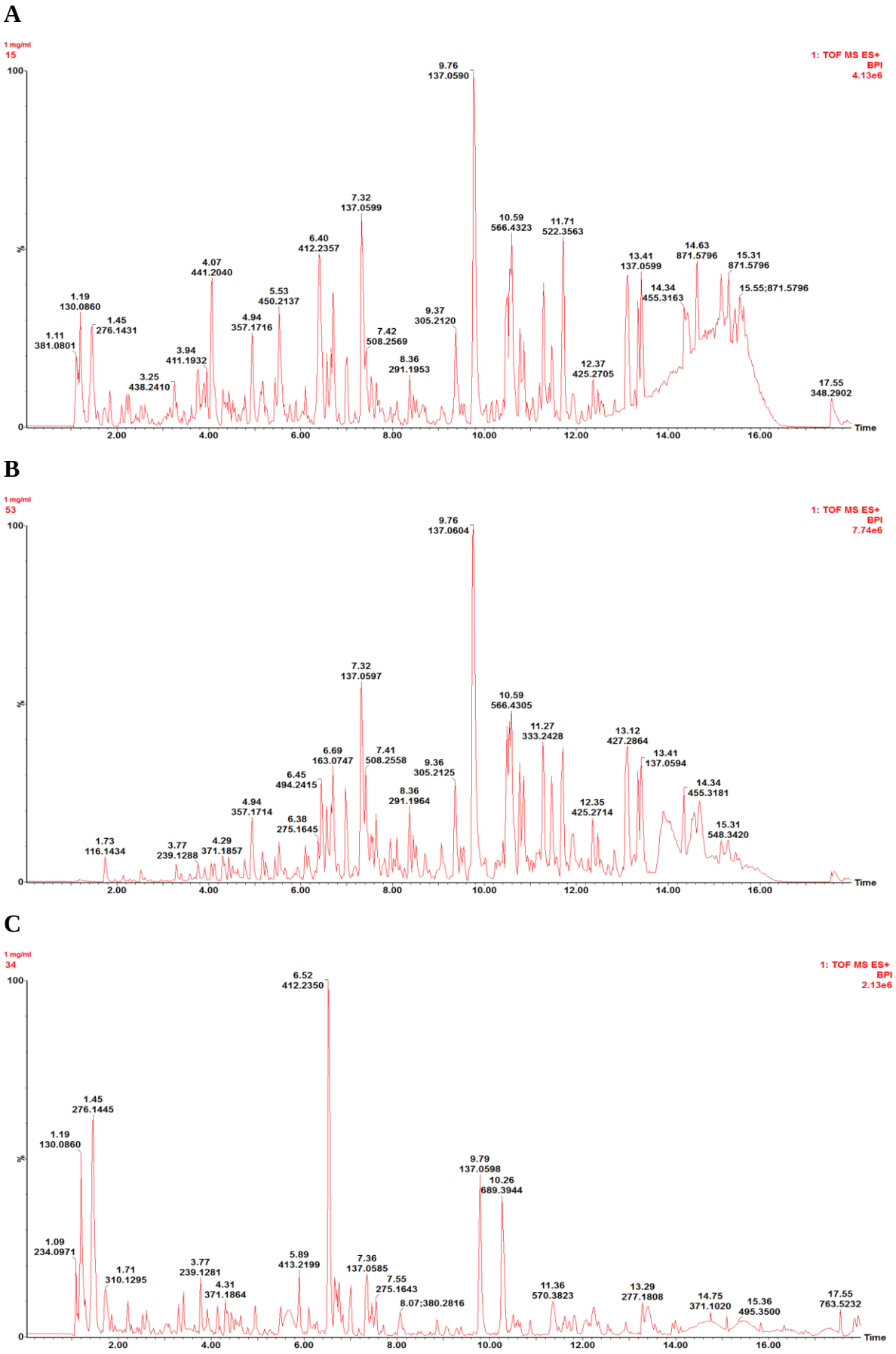


Figure 17: *Zingiber officinalis* extracts, A: methanol, B: water, C: dichloromethane

APPENDIX E

LIST OF SPICE/ HERB MATERIALS

Name and description of plant material	Supplier	Batch number
Allium sativum (garlic) powder	WARREN CHEM specialities	SHI0189/2714
Anethum graveolens (dill)	Bridget Kitley's nursery	N/A
Apium graveolens (celery)	Bridget Kitley's nursery	N/A
Cinnamomum zeylanicum (cinnamon) powder	BRENNTAG	02/18
Coriandrum sativum (coriander)	Bridget Kitley's nursery	N/A
Cymbopogon citratus (lemon grass)	Bridget Kitley's nursery	N/A
Laurus nobilis (bay laurel)	Bridget Kitley's nursery	N/A
Melissa officinalis (lemon balm)	Bridget Kitley's nursery	N/A
Mentha piperita (peppermint) leaves powder	WARREN CHEM specialities	P211743
Ocimum basilicum (basil)	Bridget Kitley's nursery	N/A
Origanum marjorana (marjoram)	Bridget Kitley's nursery	N/A
Petroselinum crispum (parsley)	Bridget Kitley's nursery	N/A
Rosmarinus officinalis (rosemary) fine powder	WARREN CHEM specialities	20175
Salvia officinalis (sage)	PHARMA GERMANIA	1882
Syzygium aromaticum (clove) powder	WARREN CHEM specialities	12109
Thymus vulgaris (thyme) powder	WARREN CHEM specialities	325 MILL
Zingiber officinalis (ginger) root peeled powder	WARREN CHEM specialities	3638 MILL

APPENDIX F

LIST OF ESSENTIAL OILS

Name of essential oil	Supplier	Lot number
Apium graveolens (celery) oil	PRANA MONDE CC	10289
Cinnamomum zeylanicum (cinnamon) oil	PRANA MONDE CC	26839
Coriandrum sativum (coriander) oil	PRANA MONDE CC	12501
Cymbopogon citratus (lemon grass) oil	PRANA MONDE CC	00217
Laurus nobilis (bay laurel) oil	PRANA MONDE CC	26062
Melissa officinalis (lemon balm) oil	PRANA MONDE CC	N/A
Mentha piperita (peppermint) oil	PRANA MONDE CC	10867
Ocimum basilicum (basil) oil	PRANA MONDE CC	22928
Origanum marjorana (marjoram) oil	PRANA MONDE CC	27093
Petroselinum crispum (parsley) oil	PRANA MONDE CC	15044
Rosmarinus officinalis (rosemary) oil	PRANA MONDE CC	24887
Salvia officinalis (sage) oil	PRANA MONDE CC	25035
Syzygium aromaticum (clove) oil	PRANA MONDE CC	24563
Thymus vulgaris (thyme) oil	PRANA MONDE CC	11398
Zingiber officinalis (ginger) oil	PRANA MONDE CC	26093

APPENDIX G

ETHICS WAIVER

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Tobias Health Sciences Building, 2nd floor Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-160129-1 29/01/2016

***TO WHOM IT MAY CONCERN*:**

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Prof S van Vuuren, T M Mapeka (Student no 1247148).**

Project title: An optimal poly-herbal formulation with antimicrobial and anti-oxidant properties.

Reason: This is a laboratory study using bacterial cultures. The bacterial strains include *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Enterobacter faecalis* (ATCC 29212), *Shigella sonnei* (ATCC 99290), *Escherichia coli* (ATCC 25922), and *Salmonella typhimurium* (ATCC 1403) There are no human participants.

UNIVERSITY OF THE WITWATERSRAND
OFFICE OF THE DEPUTY VICE CHANCELLOR RESEARCH
29 JAN 2016
RESEARCH OFFICE HREC (Medical)
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Rhulani Mkansi.