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The *In Vitro* Antimalarial, Toxicological and Chemical Characteristics of Select Phytomedicines and Major Constituents

VENISHA GOVENDER (1645329)

WITS UNIVERSITY

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in the fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, South Africa

2025

Supervisor: Prof Robyn L. van Zyl
Co-supervisor: Prof Armorel D. van Eyk

Candidates' declaration

I, Venisha Govender declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

V. Govender (Signature of candidate)

31 day of October 2025 in Johannesburg

Dedication

To my family (Mom, Dad, Uncle Glen, and Kamenee). I'm not worthy of all your tireless, unending support and belief in me. Nevertheless, it humbles me. Thank you for giving me the strength to keep on going.

Presentations arising from this research project

Govender, V, van Eyk, A.D., and van Zyl R.L., The inference of OTC phytomedicines on oxidative stress, metal homeostasis, and *P. falciparum* survival. Oral presentation delivered at the University of the Witwatersrand's Research Institute for Malaria (WRIM) Research Day, April 2024.

Govender, V, van Eyk, A.D., and van Zyl R.L., The chemotherapeutic effects of OTC phytomedicines. Oral presentation delivered at the University of the Witwatersrand Division of Pharmacology Research Day, November 2023.

Govender, V, van Eyk, A.D., and van Zyl R.L., Inference on metal homeostasis in malaria by several OTC compounds. Poster presentation delivered at the 8th Southern Africa Malaria Research Conference, August 2023.

Govender, V, van Eyk, A.D., and van Zyl R.L., The inference of OTC phytomedicines on metal and oxidative stress. Poster presentation delivered at the University of the Witwatersrand School of Therapeutic Sciences Research Day, September 2023.

Abstract

Plants are an important part of medicine in treating both communicable and non-communicable diseases. As the prevalence and spread of drug-resistant malaria has increased, so too has the search for new compounds to treat this lethal disease. Phytomedicines are popular alternatives to allopathic medicine in Africa, making them promising candidates in this search.

Based on their known toxicological and chemical characteristics, there is a need to investigate the extent to which the crude extracts of the plants *Camelia sinensis*, *Viscum album*, *Siphonochilus aethiopicus*, and *Warburgia salutaris*, and their predicted constituents, inhibit chloroquine (CQ)-sensitive and -resistant strains of *P. falciparum*.

Aqueous (70 °C, MilliQ® water) and organic (dichloromethane:methanol - 1:1; acetone) crude extracts were prepared from commercially sourced *C. sinensis*, *V. album*, *S. aethiopicus*, and *W. salutaris* material. Qualitative tests were performed on each crude extract to determine the presence of alkaloids, flavonoids, anthraquinones, coumarins, phenols, saponins, and tannins, followed by quantitative HPLC fingerprinting and total flavonoid and phenolic content assays. Antiplasmodial activity of the crude extracts and major constituents were assessed against CQ-sensitive (NF54) and CQ-resistant (FCR-3) *P. falciparum* strains. Preliminary toxicology of the crude extracts and their major constituents were profiled based on their ability to induce haemolysis and cytotoxicity in K562 and HepG2 cells. Cuprous and ferrous chelation and free radical scavenging assays were undertaken to consider possible mechanisms of action of the crude extracts regarding any antiplasmodial activity. *In silico* predictions provided information on the pharmacokinetic characteristics of major constituents. Crude extract % yields ranged from the lowest yield of 3.43% (*V. album* DCM:MeOH) to 33.99% (*C. sinensis* aqueous). The crude acetone extract and the major constituent, *S. aethiopicus* acetone and ellagic acid, had the greatest antiplasmodial activity on both strains. Phenols and tannins were most abundant while anthraquinones and coumarins were absent in all extracts. The DCM:MeOH extract of *C. sinensis* appeared to have the highest amounts of secondary metabolites, while the aqueous extract of *S. aethiopicus* had the least comparatively. All extracts and major constituents induced minimal haemolysis (1-3%) and cytotoxicity (<60%), except for polygodial and *the S. aethiopicus* crude extract. None of the crude extracts had favourable cuprous or ferrous chelating properties (>60%) at 100 µg/mL, while only the *C. sinensis* DCM:MeOH extract (IC_{50} = 9.97 ± 0.17 µg/mL) had free radical scavenging properties (>95%) comparable to the standard, ascorbic acid.

S. aethiopicus organic extracts and ellagic acid have good activity antiplasmodial activity and could interact in an additive manner with standard antimalarials. Thereby further investigation into their chemical and toxicological profile, as well as the mechanism of their antiplasmodial activity is warranted.

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Table of Contents

Candidates' declaration i
Dedication ii
Presentations arising from this research project iii
Abstract iv
Acknowledgements v
List of Figures x
List of Tables xii
List of Equations xiii
Nomenclature/ List of Abbreviations, Acronyms, & Symbols xiv
CHAPTER 1 - INTRODUCTION 1
1.1 Rationale 1
1.2 Problem statement 2
1.3 Aim and Objectives 3
1.4 Outline of study 3
CHAPTER 2 – LITERATURE REVIEW 4
2.1 Malaria epidemiology 4
2.2 Malaria epidemiology in South Africa 6
2.3 *Plasmodium* parasites 6
2.3.1 *Plasmodium* Lifecycle 7
2.3.2 Clinical presentation 8
2.4 Disease management of malaria 9
2.4.1 Antimalarial drugs and resistance 13
2.4.1.1 Nucleic acid synthesis inhibitors 14
2.4.1.2 Artemisinin and its derivatives 15
2.4.1.3 Quinolines 16
2.4.1.3.1 CQ 16
2.4.1.3.2 CQ mechanism of action 16
2.4.2 Antimalarial resistance 18
2.5 Phytomedicines and malaria 19
2.5.1 Selected plants for this study 20
2.5.1.1 *Camellia sinensis* (Green Tea) 20
2.5.1.1.1 Taxonomy, distribution, & botanical description 20
2.5.1.1.2 Traditional use, phytochemistry, & biological activity 21
2.5.1.2 *Viscum album L.* (Mistletoe) 22
2.5.1.2.1 Taxonomy, distribution, & botanical description 22
2.5.1.2.2 Traditional use, phytochemistry, & biological activity 23
2.5.1.3 *Siphonochilus aethiopicus* (African Ginger) 24

2.5.1.3.1 Taxonomy, distribution, & botanical description 24
2.5.1.3.2 Traditional use, phytochemistry, & biological activity 25
2.5.1.4 *Warburgia salutaris* (Pepperbark Tree) 26
2.5.1.4.1 Taxonomy, distribution, & botanical description 26
2.5.1.4.2 Traditional use, phytochemistry, & biological activity 27
CHAPTER 3 - METHODOLOGY 29
3.1 Reagents & solvents, reference control compounds, & pure test compounds 29
3.1.1 Reagents & solvents 29
3.1.1.1 Preparation of phosphate-buffered saline used in all experiments 29
3.1.2 Reference control compounds 29
3.1.3 Pure test compounds 30
3.1.4 Crude extract preparation 31
3.1.4.1 Aqueous and organic crude extraction 31
3.2 Phytochemical analysis 32
3.2.1 Preliminary qualitative phytochemical analysis 32
3.2.1.1 Test for alkaloids 32
3.2.1.2 Test for anthraquinones 32
3.2.1.3 Test for coumarins 32
3.2.1.4 Test for flavonoids 33
3.2.1.5 Test for phenols and tannins 33
3.2.1.6 Test for saponins 33
3.2.2 Quantitative phytochemical analysis 33
3.2.2.1 Determination of total flavonoid content 33
3.2.2.2 Determination of total phenolic content 34
3.2.3 High performance liquid chromatography 34
3.2.3.1 Chromatographic conditions 35
3.2.3.2 Preliminary peak characterisation and estimation 35
3.3 Oxidative stress and metal homeostasis 35
3.3.1 2,2- Diphenyl-1-picryl-hydrazyl-hydrate (DPPH•) free radical scavenging assay 35
3.3.2 Iron chelation assay 36
3.3.3 Copper(II) chelation assay 37
3.3.4 Lipid peroxidation assay 38
3.4 *In vitro* biological activities 39
3.4.1 Antiplasmodial activity 39
3.4.1.1 *In vitro* culture maintenance of *P. falciparum* 39
3.4.1.2 Preparation of complete RPMI-1640 culture medium 40
3.4.1.3 Preparation of erythrocyte suspension 40
3.4.2 Synchronisation of *P. falciparum* culture 40
3.4.3 *Plasmodium* lactate dehydrogenase (pLDH) assay 41

3.4.3.1 Preparation of reagents 41
3.4.3.2 Preparation of experimental plates 41
3.4.3.3 Addition of pLDH reagents to experimental plates 42
3.5 Preliminary toxicity properties 42
3.5.1 Haemolysis Assay 42
3.5.2 *In vitro* cytotoxicity properties 43
3.5.2.1 Preparation of complete cell culture medium 43
3.5.2.2 *In vitro* maintenance of K562 and HepG2 cells 44
3.5.2.3 Cytotoxicity tetrazolium assay 44
3.5.2.3.1 Cell counting via the trypan blue exclusion assay 44
3.5.2.3.2 Preparation of reagents 45
3.5.2.3.3 Preparation of experimental plates 45
3.5.2.3.4 Addition of test compounds and reagents to experimental plates 46
3.5.3 *In silico* pharmacokinetic and toxicity properties 46
3.6 Data analysis and statistics 47
CHAPTER 4 - RESULTS 48
4.1 Phytochemical analysis 48
4.1.1 Qualitative phytochemical analysis 48
4.1.2 Quantitative phytochemical analysis 49
4.1.2.1 Determination of TFC & TPC 49
4.1.2.1.2 Preliminary UV-Vis HPLC chemical profiling and quantitative estimation 50
4.2 Antiplasmodial activity 52
4.3 Oxidative Stress &, metal homeostasis & lipid peroxidation inhibiting activity 56
4.3.1 DPPH• scavenging activity 56
4.3.2 Metal chelating and lipid peroxidation inhibiting activity 57
4.4 Toxicity 58
4.4.1 RBC haemolysis 58
4.4.2 MTT reducing activity 58
4.5 *In silico* studies 60
4.5.1 Drug Likeliness and ADME pharmacokinetics 60
4.5.2 Targets 62
CHAPTER 5 - DISCUSSION 65
CHAPTER 6 – CONCLUSION, RECOMMENDATIONS, & LIMITATIONS 73
References 75
Appendix A. Ethics 103
A.1: Human cell waiver 103
A.2: Ethic clearance (blood from human volunteers): approved under supervisor, Prof R. van Zyl HREC approval. 104
A.3: Biosafety clearance: approved under supervisor, Prof R. van Zyl HREC approval. 105

Appendix B. Results 106

B.1: TFC and TPC standard curves of controls 106

B.2: HPLC pure compound chromatograms 107

B.3: Antioxidant activity 109

B.4: Metal chelation 110

B.5: Lipid peroxidation 111

B.6:Haemolysis 112

Table B.6: The haemolytic activity of crude extracts and pure compounds compared to Triton™ X-100 at 100 µg/mL. 112

B.7: Cytotoxicity 113

Table B.7.1: The % MTT reduction activity of crude extracts on K562 cells compared to cisplatin, chloroquine, and quinine at 100 µg/mL. 113

Table B.7.2: The % MTT reduction activity of crude extracts on K562 cells compared to camptothecin, cisplatin, chloroquine, and quinine. 113

B.8: ADMET characteristics of standards and pure compounds 114

Table B.8.1: SwissADME and Toxtree in silico predictions of predicted major constituent drug-likeliness and toxicity. 114

Table B.8.2: SwissADME *in silico* predictions of predicted major constituent lipophilicity and pharmacokinetics 115

List of Figures

Figure 2.1: Global malaria incidence 5
Figure 2.2: Cyclical lifecycle of *Plasmodium* through mosquito vectors and human hosts 7
Figure 2.3.1. The chemical structure of current quinoline chemoprentive and chemoprophylactic antimalarials 13
Figure 2.3.2. The chemical structure of current antifolate and artemisinin chemoprentive and chemoprophylactic antimalarials 14
Figure 2.3.3. The chemical structure of current antibiotic chemoprentive and chemoprophylactic antimalarials 14
Figure 2.4: The chemical structure of CQ 16
Figure 2.5: *C. sinensis* (A) dried leaf buds (B) shrub (C) fresh leaves 21
Figure 2.6: *Viscum album* (A) Cut and dried parts of *V. album* (B) Adult plant of *V. album* (C) Fruits of *V. album* 22
Figure 2.7: *S. aethiopicus* (A) The purple flowers of *S. aethiopicus* (B) Tubers and aromatic roots of *S. aethiopicus (*C) Cone shaped rhizomes 25
Figure 2.8: *W. salutaris* (A) (B) (C) (D) (E) (Kotina *et al*., 2104) (F) (G) The fruit of *W. salutaris* ... 27
Figure 3.1: TFC assay methodology for a 96-well microtitre plate 34
Figure 3.2: Iron chelation assay methodology for a 96-well microtitre plate 37
Figure 3.3: Inhibition of lipid peroxidation assay methodology for a 96-well microtiter plate 38
Figure 3.4: *A Giemsa stained photomicrograph (1000X;* Olympus® CX41 Phase Contrast Microscope (Tokyo, Japan)*) of the NF54* P. falciparum *strain ring stage culture*. 41
Figure 3.5: Red blood cell haemolysis assay methodology for a 96-well microtitre plate..... 43
Figure 3.6: Haemocytometer quadrants, where - A1 indicates a dead cell and A2 indicates a living cell. 45
Figure 3.7: MTT assay methodology for a 96-well microtitre plate. 46
Figure 4.1: The TPC and TFC of aqueous and organic crude extracts..... 49
Figure 4.2: UV-Vis HPLC chromatograms of aqueous (A1, B1, C1, D1) , DCM:MeOH (A2, B2, C2, D2), and acetone (C1, C2, C3, C4) *C. sinensis* (A), *V. album* (B), *S. aethiopicus* (C), and *W. salutaris* (D) crude extracts. 50
Figure 4.3: HPLC-UV chromatograms showing absorbance over acquisition time of (A) S. aethiopicus acetone crude extract alone and (B) S. aethiopicus crude acetone extract spiked with 20 µg/ mL of 3,4-dihydroxybenzoic acid (1), caffeic acid (2), and naringenin (3)..... 51
Figure 4.4: The log sigmoid dose response curve of the most active crude extract compared to CQ and DHA on (A) NF54 and (B) FCR-3 P. falciparum in vitro survival (n=3). 54

Figure 4.5: The IC_{50} values of CQ, DHA, and *S. aethiopicus* acetone compared in NF54 *P. falciparum* compared to FCR-3 *P. falciparum*. (ns = P > 0.05, * = P ≤ 0.05, ** = P ≤ 0.01, **** = P ≤ 0.001, **** = P ≤ 0.0001).....56
Figure 4.6: The log sigmoid dose response curve of the % FRSA of the most and least active crude extracts compared to ascorbic acid and Trolox™.....57
Figure 4.7: The log sigmoid dose response curve of the % MTT reducing activity of the *S. aethiopicus* acetone crude extract compared to camptothecin, cisplatin, and CQ on HepG2 (A) K562 (B) cells (n=3).....59

Figure 4.8: Camptothecin, cisplatin, and *S. aethiopicus* acetone IC_{50} values on HepG2 and K562 cells. (ns = P > 0.05, * = P ≤ 0.05, ** = P ≤ 0.01, **** = P ≤ 0.001, **** = P ≤ 0.0001).....60
Figure 4.9: SWISS ADME prediction of bioavailability and distribution of select pure compounds and reference control compounds.....61
Figure 4.10: The top 15 molecular target class for *S. aethiopicus* acetone crude extracts identified constituents (A) 3,4-dihydroxybenzoic acid, (B) caffeic acid, (C) naringenin, and the standard antimalarials (E) DHA and (F) CQ. Potential targets can be employed as indicators to investigate bioactivity, possible side effects, and the possibility of repurposing therapeutically relevant compounds.....63
Figure B.1.1: The linear regression curve of rutin hydrate used to determine the TFC of the crude extracts.....106
Figure B.1.2: The linear regression curve of gallic acid used to determine the TFC of the crude extract.....16
Figure B.2.1: Chromatogram of 3,4-Dihydroxybenzoic acid.....107
Figure B.2.2: Chromatogram of caffeic acid.....107
Figure B.2.3: Chromatogram of naringenin.....108

List of Tables

Table 2.1: The pharmacological and resistance characteristics of commonly used antimalarial drugs to treat *P. falciparum*. 10

Table 3.1: The molecular formulae and chemical properties of reference control compounds used in several assays throughout the study. 30

Table 3.2: The molecular formulae and chemical properties of pure compounds tested in several assays throughout the study. 31

Table 4.1: Percentage yield for each solvent used during extraction processes for *C. sinensis*, *S. aethiopicus, V. album*, and *W. salutaris*. 48

Table 4.2: Qualitative phytochemical screening of crude extracts. 49

Table 4.3: *In vitro* antiplasmodial and cytotoxic activity of selected aqueous and organic crude extracts expressed at 10 µg/ mL on both CQ-sensitive and -resistant strains and HepG2. 52

Table 4.4: *In vitro* antiplasmodial and cytotoxic (IC_{50}) activity of selected aqueous and organic crude extracts. 53

Table 4.5: IC_{50} values of the best performing crude extract and its predicted major constituents. 55

Table 4.6: The percentage DPPH free radical scavenging activity of selected crude extracts and controls. 57

Table 4.7: *In silico* predictions of predicted major constituent drug-likeliness and toxicity 60

Table 4.8: *In silico* predictions of predicted major constituent lipophilicity and pharmacokinetics. 62

Table 4.9: The top 5 predicted pure compound and standard antimalarial drug targets in *Homo sapiens* and their overlap targets in *P. falciparum*. 64

Table B.3.1: The antioxidant activity of crude extracts compared to Trolox™ and ascorbic acid at 100 µg/mL. 109

Table B.3.2: The antioxidant activity of crude extracts compared to Trolox™ and ascorbic acid. 109

Table B.4.1 The Fe (II) chelating activity of crude extracts compared to EDTA at 100 µg/mL. 110

Table B.4.2: The Cu (II) chelating activity of crude extracts compared to TMB at 100 µg/mL. 110

Table B.5: The lipid peroxidation activity of crude extracts compared to Trolox™ and ascorbic acid at 100 µg/mL. 111

Table B.6: The haemolytic activity of crude extracts and pure compounds compared to Triton™ X-100 at 100 µg/mL. 112

Table B.7.1: The %MTT reduction activity of crude extracts on K562 cells compared to cisplatin, chloroquine, and quinine at 100 µg/mL. 113

Table B.7.2: The % MTT reduction activity of crude extracts on K562 cells compared to camptothecin, cisplatin, chloroquine, and quinine. 113

Table B.8.1: SWISS ADME and Toxtree *in silico* predictions of predicted major constituent drug-likeliness and toxicity. 114

Table B.8.2: SWISS ADME *in silico* predictions of predicted major constituent lipophilicity and pharmacokinetics. 115

List of Equations

% Yield = *% Yield* = $\left(\frac{mf}{mi}\right) \times 100$ **Equation 3.1** 31

TFC = y = 0.0066x + 0.0148 **Equation 3.2** 31

TPC = y = 0.0318x + 0.0641 **Equation 3.3** 34

% Compound = $\left(\frac{\textbf{Mass of compound injected}}{\textbf{Mass of extract injected}}\right) \times \mathbf{100}$ **Equation 3.4**.....35

% Free radical scavenging activity = $\left(\frac{(\mathbf{OD_{100\%}-OD_B})-(\mathbf{OD_{TC}-OD_{CC}})}{(\mathbf{OD_{100\%}-OD_B})}\right) \times \mathbf{100}$ **Equation 3.5**.....36

% Iron chelation activity = $\left(\frac{(\mathbf{OD_{100\%}-OD_B})-(\mathbf{OD_{TC}-OD_{CC}})}{(\mathbf{OD_{100\%}-OD_B})}\right) \times \mathbf{100}$ **Equation 3.6**.....37

% Copper chelating activity = $\left(\frac{(\mathbf{OD_{100\%}-OD_B})-(\mathbf{OD_{TC}-OD_{CC}})}{(\mathbf{OD_{100\%}-OD_B})}\right) \times \mathbf{100}$ **Equation 3.7**.....37

% Inhibition of lipid peroxidation activity = $\left(\frac{\mathbf{OD_{TC}-OD_{CC}}}{(\mathbf{OD_{100\%}-OD_B})}\right) \times \mathbf{100}$ **Equation 3.8** 39

% Parasite growth inhibition = $\left(\frac{\mathbf{OD_{TC}-OD_{CC}}}{\mathbf{OD_{100\%}-OD_B}}\right) \times \mathbf{100}$ **Equation 3.9**.....42

% Haemolysis = $\left(\frac{\mathbf{OD_{TC}-OD_{CC}}}{\mathbf{OD_{100\%}-OD_B}}\right) \times \mathbf{100}$ **Equation 3.10** 43

Cell count = $\left(\frac{\boldsymbol{URQ+ULQ+LRQ+LLQ}}{\mathbf{4}}\right) \times \mathbf{2} \times \mathbf{10^4}$ **Equation 3.11**.....45

% Cell growth Inhibition = $\left(\frac{\boldsymbol{OD_{TC}-OD_{CC}}}{\boldsymbol{OD_{100\%}-OD_B}}\right) \times \mathbf{100}$ **Equation 3.12** 46

Nomenclature/ List of Abbreviations, Acronyms, & Symbols

cm^2: centimetre squared
°C: degrees Celsius
H_2O: water
g: gram
L: litre
%: percentage
µg: microgram
µL: microlitre
µm: micrometre
mg: milligram
mL: millilitre
mM: millimolar
M: molar
nm: nanometre
v/v: volume per volume
w/v: weight per volume

ACTs: artemisinin-based combination therapy
ADMET: absorption, distribution, metabolism, excretion, and toxicity
ART-R: partial artemisinin resistance
CQ: chloroquine
DCM:MeOH: dichloromethane: methanol
DNA: deoxyribonucleic acid
DHA: dihydroartemisinin
DMEM: Dulbeco's Modified Eagles Media
DMSO: dimethyl sulfoxide
DPPH: 2,2-diphenyl-1-picryl-hydrazyl
DV: digestive vacuole
EDTA: ethylenediaminetetraacetic acid
FBS: foetal bovine serum
FRSCA: free radical scavenging activity
Fe(II): ferrous iron
Fe(II)PPIX: ferrous iron protoporphyrin IX
HEPES: 4 -(2-hydroxyethyl)-1- piperazineethanesulfonic acid
HPLC: high performance liquid chromatography

IC_{50}: 50% inhibitory concentration

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

NBT: nitroblue tetrazolium

OD: optical density

pLDH: parasite lactate dehydrogenase

PES: phenazine ethosulfate

PBS: phosphate-buffered saline

PfCRT: *Plasmodium falciparu*m chloroquine resistance transporter

PfMDR1: *Plasmodium falciparum* multidrug resistance protein 1

RBCs: red blood cells

ROS: reactive oxygen species

RPMI: Roswell Park Memorial Institute

TFC: total flavonoid content

TPC: total phenolic content

TMB: ammonium tetrathiomolybdate

UV/ Vis: ultraviolet/ visible

WHO: World Health Organization

CHAPTER 1 - INTRODUCTION

1.1 Rationale

Plasmodium falciparum malaria remains a major public health, and by extension, economic burden within the African region (World Health Organization, 2024). With over 90% of global malaria case incidence and fatalities reported in Sub-Saharan Africa, the region remains the disease's epicentre (World Health Organization, 2024).

Historically, the utilisation of chemotherapy to treat and prevent malaria, led to marked decreases in malaria incidence, including elimination in several countries (World Health Organization, 2024). However, therapeutic failure associated with the emergence and spread of drug-resistant strains of *P. falciparum* has been a major contributor to its persistence in the region (Haldar *et al*., 2018; Plowe, 2022; World Health Organization, 2024). Resistance to antimalarials, most notably chloroquine (CQ) and related aminoquinolines and antifolates, has been prevalent for decades and led to their subsequent removal from clinical use (Haldar *et al*., 2018; Plowe, 2022; World Health Organization, 2023). Currently, the World Health Organization (WHO) recommends the use of artemisinin-based combination therapies (ACTs) for uncomplicated malaria caused by *P. falciparum* to provide a necessary cure and delay the onset of resistance (World Health Organization, 2023). However, over the past decade, the independent emergence of *P. falciparum* strains with markers of artemisinin partial resistance (ART-R) in Eastern Africa has been cause for concern (Bwire *et al*., 2020; Uwimana *et al*., 2020; Uwimana *et al*., 2021; Straimer *et al*., 2022). It has thus far been confirmed in four countries and suspected in four other (World Health Organization, 2024). The clinical consequences of ART-R in Africa are still unclear, but it is likely that, as in South-East Asia (Trape, 2001; Amaratunga *et al*., 2016; Mairet-Khedim *et al*., 2021; Imwong *et al*., 2017; van der Pluijm *et al*., 2019), it will challenge treatment efficacy and malaria control (Phyo *et al*., 2016; World Health Organization, 2024). Therefore, there is a critical need to explore both novel treatments and treatment combinations which evade resistance phenotypes. However, drug discovery is a time-consuming process that is primarily limited by the duration of lead optimisation and clinical trials, high costs, and strict ethical and regulatory considerations; therefore novel drugs in development are unlikely to become available within the next few years for rapid deployment in WHO regions where ART-R is prevalent or emerging (Ashley and Phyo, 2018; Tse *et al*., 2019; Hanboonkunupakarn and White, 2022; Siqueira-Neto *et al*., 2023).

Plants used in traditional medicine are of particular interest as they have been applied extensively to treating disease over time (Haruki *et al*., 2000; Chen and Hsiang, 2022; Sinha *et al*., 2023). They produce a wide range of secondary metabolites, including polyphenols, alkaloids, quinones, carotenoids, and steroids, with equally diverse mechanisms of action related to antiplasmodial

suppression or even drug resistance reversal (Haruki *et al*., 2000; Chen and Hsiang, 2022; Sinha *et al*., 2023). When combined, these mechanisms may act in a synergistic or additive manner to enhance antimalarial drug activity by increasing their efficacy in resistant parasite strains (Iwalokun, 2008; Benelli *et al*., 2017).

Limited access to and affordability of globally produced antimalarials and the growing threat of their resistance are challenges which threaten the continents' control (Penchansky and Thomas; 1981; Yenet *et al*., 2023; World Health Organization, 2024).

Therefore, in order to effectively make use of phytomedicines to develop novel antimalarial drugs and/ or drug combinations, there is a need to critically assess both their efficacy and safety as they pertain to malaria. Against this background, this study intends to contribute preliminary data on the antiplasmodial potential of aqueous and organic crude phytomedicinal extracts and their constituents. Four plant species; *Camelia sinensis*, *Siphonochilus aethiopicus*, *Viscum album*, and *Warburgia salutaris*, which have been demonstrated to have high biological activity *in vitro* against communicable diseases (Maroyi, 2013; Argüello-García, R. and Quiñonez-Bastidas, 2020; Belo *et al*., 2025; Eimam *et al*., 2025; Fouche and van Rooyan, 2025; Raj, 2025), and which are frequently used as traditional or adjunct medicines, were chosen for this purpose.

1.2 Problem statement

The emergence and spread of resistance both to previous and current effective antimalarials has threatened and continues to threaten malaria elimination progress. The emergence of ART-R in Africa indicates that the efficacy of ACTs could become compromised should resistance to partner drugs emerge. If ACTs fail in Africa, there are no antimalarials with equal efficacy to replace them and the antimalarial drugs which are in development have significant economic and temporal constraints, and are therefore unlikely to become generally available within the next few years. Phytomedicines are multifaceted with a tendency to affect biological processes, usually through several diverse mechanisms. With regards to malaria, they may compromise parasite growth, modulate host immune responses or pathogenic event progression, and even alter the susceptibility of parasite to antimalarials. Therefore, development of novel treatments and combinations of natural products and standard antimalarials which reverse and evade resistance phenotypes may be an appropriate solution. In this regard, the phytomedicines, *C. sinensis*, *Siphonochilus aethiopicus*, *Viscum album*, and *Warburgia salutaris*, which have extensive historical use in treating communicable diseases like malaria, may be reservoirs for natural products which can be aptly applied for this purpose.

1.3 Aim and Objectives

The primary aim of this study is to characterise and compare the antiplasmodial activity of 12 crudes extracts of *C. sinensis*, *S. aethiopicus*, *V. album*, and *W. salutaris* and 16 plant isolated compounds

1. Determine and compare the phytochemical composition of aqueous and organic crude extracts using various qualitative and quantitative methods.
2. Determine and compare the effect of aqueous and organic crude extracts on oxidative stress and metal homeostasis as possible mechanisms of action regarding antiplasmodial activity.
3. Determine and compare the *in vitro* antiplasmodial activity of aqueous and organic crude extracts on the CQ-sensitive (NF54) and resistant (FCR-3) *P. falciparum* strains.
4. Determine and compare the *in vitro* toxicity of aqueous and organic crude extracts on human HepG2 and K562 cells as hepatic and vascular models.

1.4 Outline of study

To achieve the aim and objectives of this study, the dissertation has been set out as follows:

- **Chapter 1** provides a brief background to the study leading into the aim of the study and the objectives used to achieve this aim.
- **Chapter 2** contextualises malaria epidemiology in Africa and South Africa. How drug resistance is challenging its eradication in these regions and how plant species might be applied to address the issues of resistance in malaria.
- **Chapter 3** describes both the qualitative and quantitative methodology used to ascertain the antiplasmodial, chemical, and toxicological profiles of crudes extracts and major constituents assessed in this study.
- **Chapter 4** outlines the results from this study. Highlighting the most important findings as they pertain to the aim and objectives. Differences in results were compared and considered significant ($P<0.05$).
- **Chapter 5** is a discussion of the interpretation of the results and their significance outside of this study.
- **Chapter 6** concludes the study indicating some limitations encountered in the study; along with recommendations arising from the study for future investigation.

CHAPTER 2 – LITERATURE REVIEW

2.1 Malaria epidemiology

Malaria is a communicable parasitic disease broadly distributed throughout tropical and sub-tropical regions of the world, where it is presently endemic in 83 countries and areas (World Health Organization, 2024). Concerted global efforts have largely been successful in the quest for elimination, where over the last two decades, an estimated 2.1 billion malaria cases and 11.7 million malaria deaths were averted globally (World Health Organization, 2024). Since 2000, 15 countries (Morocco (2010), Turkmenistan (2010), Armenia (2011), Maldives (2015), Kyrgyzstan (2016), Sri Lanka (2016), Paraguay (2018), Uzbekistan (2018), Algeria (2019), Argentina (2019), China (2021), El Salvador (2021), Azerbaijan (2023), Belize (2023), Tajikistan (2023)) have been certified malaria free by the WHO and a further 9 (Egypt (2000), Kazakhstan (2004), Syrian Arab Republic (2007), Iraq (2011), Georgia (2012), Türkiye (2012), Oman (2013), Malaysia (2020), Cabo Verde (2021)) have attained 3 consecutive years of zero indigenous cases but have yet to be certified (World Health Organization, 2024).

Elimination efforts have disproportionately restricted the distribution of malaria to the WHO African region, where it remains a serious global public health burden (World Health Organization, 2024). This in part has been due to the vectorial capacity of the three anopheline species endemic to the region (*Anopheles gambiae* sensu stricto, *An. arabiensis*, and *An. funestus*). Emerging vector species such as *An. stephensi* further complicate malaria control on the continent (World Health Organisation, 2024). In 2023, 263 million clinical cases resulting in 597,000 deaths were estimated, where the WHO African region accounted for 94% of cases and 95% of deaths globally (World Health Organization, 2024). According to the 2024 WHO World Malaria Report, of the current 85 malaria endemic countries, just 29 accounted for 93.6% and 95.4% of malaria cases and deaths globally. Four of which, Nigeria (30.9%), the Democratic Republic of Congo (11.3%), Niger (5.9%), and the United Republic of Tanzania (4.3%), accounted for almost half of deaths globally (World Health Organization, 2024).

Socioeconomic status also has a profound impact on malaria mortality and morbidity, where low socioeconomic status limits access to diagnostic services and healthcare (World Health Organization, 2024). Such delays in diagnosis and treatment often leads to progression of severe infection and further transmission amongst low socioeconomic status populations (World Health Organization, 2024). Socioeconomic factors also majorly influence at-risk populations, such as infants, children under 5 years of age, pregnant women, and immunocompromised patients (Sarfo *et al*., 2023; World Health Organization, 2024). Children under the age of 5 years in sub-Saharan Africa constitute two-thirds of malaria mortality (Sarfo *et al*., 2023; World Health Organization, 2024).

Currently, 3.2 billion people, approximately 40% of the world's population, live in malaria affected areas and are still at risk of contracting the disease (World Health Organization, 2024) (Figure 2.1).

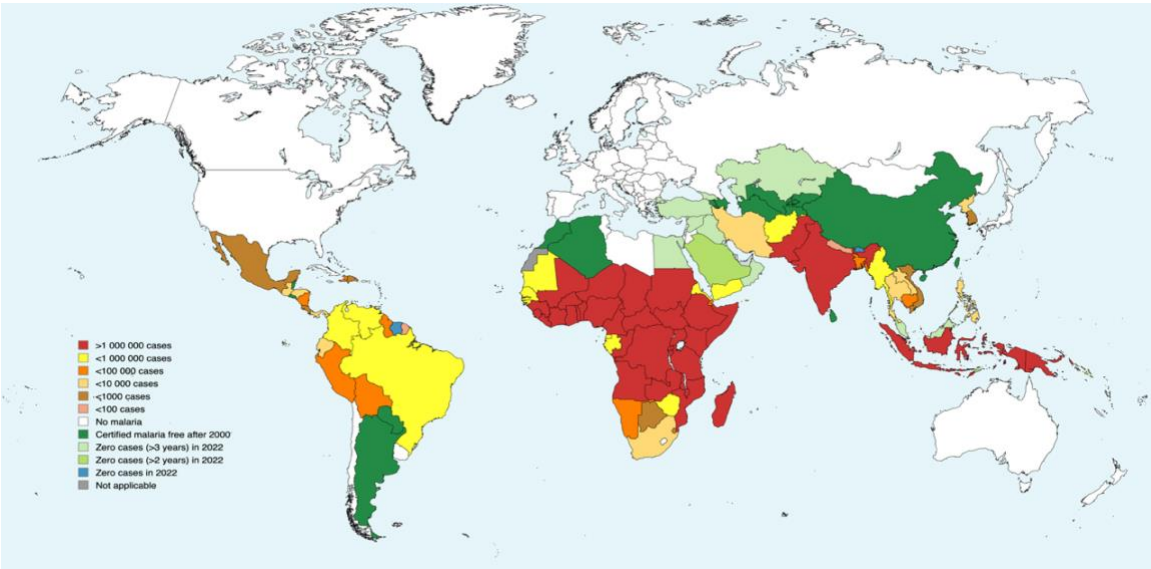


Figure 2.1: Global malaria incidence (Adapted from the WHO World Malaria Report 2024).

Other affected regions include Southeast Asia, the Eastern Mediterranean region, Western Pacific, and the Americas, which constitute the remaining global malaria cases (World Health Organization, 2024).

The negative impact of malaria extends further to the economy. There is concurrent poverty and economic burden on both individuals and the healthcare systems in malaria-endemic countries (Andrade *et al*., 2022). For example, it has been reported that Africa loses close to $12 billion of its gross domestic product annually, while also causing stagnation in economic growth by as much as 1.3% (Andrade *et al*., 2022). Since 2001, countries in sub-Saharan Africa have spent on average $300 on malaria case management, an amount that is unacceptable in low-income countries whose income resources are already overburdened and stretched by concurrent social and political issues (Andrade *et al*., 2022).

Although, progress towards elimination is ongoing, whereby 2 billion cases and 11.7 million deaths resulting from malaria have been prevented since 2000 (World Health Organization, 2024). Current WHO estimates of key indicators of global malaria burden have not improved since 2015 and are reversing in some countries (World Health Organization, 2024). Several challenges regarding malaria control and elimination remain, including population growth – it has been postulated that by 2030, 30% more people in malaria endemic African countries will require protection and treatment for malaria (World Health Organization, 2024). Increasing commodity costs present another challenge, due to widespread prevalence of malaria vector species resistance to pyrethroids

(Ranson *et al*., 2011; Trape *et al*., 2011; World Health Organization, 2024), and an increased demand for bed nets co-treated with the synergist piperonylbutoxide, which are 29% more costly than conventional pyrethroid treated bed nets (World Health Organization, 20240. *P. falciparum* acquired histidine-rich protein 2 mutations which allow them to evade detection by the most common and cheapest rapid diagnostic tests also pose an issue as new rapid diagnostic tests will most likely be introduced at higher costs which may limit their use in populations most likely to require them (World Health Organization, 2024).

2.2 Malaria epidemiology in South Africa

South Africa is located on the southernmost edge of malaria distribution in Africa, where transmission is restricted to low-altitude border regions of the Limpopo, Mpumalanga, and KwaZulu- Natal provinces (Mabona *et al*., 2024). Malaria in South Africa follows a seasonal trend with high transmission during the rainy season from September to May and peak post December and April holiday seasons (Mabona *et al*., 2024). Infections are almost exclusively caused by *P. falciparum* and are transmitted by *An. funestus* and *An. arabiensis* (Mabona *et al*., 2024). Over the past two decades, South Africa has shown consistent improvement in reducing both morbidity and mortality rates associated with malaria (Mabona *et al*., 2024; World Health Organization, 2024). To date, South Africa has achieved an 87% reduction in cases, declining from 64 622 in 2000 to 8126 in 2020, and a 91% reduction in mortality, which dropped from 459 deaths in 2000 to 58 deaths in 2020 (National Department of Health, 2019; World Health Organization, 2024). In the past year, South Africa's morbidity and mortality stood at an estimated 4500 and 38, respectively. South Africa is currently one of 25 countries identified by the WHO with the potential to eliminate malaria by 2025 (Mabona *et al*., 2024; World Health Organization, 2024). However, the issue of drug resistance and as well as malaria importation from neighbouring countries still poses a threat (Mabona *et al*., 2024). As such, measures to eliminate *P. falciparum*, the predominant and deadliest species found in South Africa, as well as *An. funestis* and *An. arabiensis* need to be investigated (Mabona *et al*., 2024).

2.3 *Plasmodium* parasites

P. falciparum is not only responsible is for the vast majority of malaria-associated deaths in South Africa, it is the predominant species across the African region (Sato, 2021; World Health Organization, 2024). *P. vivax*, while less lethal than *P. falciparum*, is one of only two species (along with the rarer *P. ovale*) which are known to cause relapsing infections after the parasite has been cleared, and whose treatment options are limited (Sato, 2021). In South Africa, *P. vivax* incidence occur at 0.1 per 1000 people per year while *P. ovale* is not known to occur (World Health Organization, 2024). The other human-infecting species include *P. malariae* and most recently the zoonotic *P. knowlesi* (Sato, 2021; World Health Organization, 2024). *P. vivax*, *P. ovale*, and *P.*

malariae, typically result in milder infections, while *P. knowlesi* has been known to cause severe malaria in South East Asia (World Health Organization, 2024).

2.3.1 *Plasmodium* Lifecycle

The *Plasmodium spps*. display complex life cycles whereby they undergo several morphological stages involving cyclical infections through female *Anopheles* mosquito vectors (sporogonic cycle) and vertebrate hosts (exoerythrocytic and intraerythrocytic cycles) (Figure 2.2) (Sato, 2021; CDC, 2024). Infection occurs when during a blood meal, *Plasmodium* infected female *Anopheles* spp. bite human hosts, transmitting motile sporozoites into their bloodstream (Sato, 2021; CDC, 2024). The sporozoites circulate briefly before going on to infect host hepatocytes, where the asexual lifecycle and proliferation (schizogony) ensues over the next 7-10 days (Sato, 2021; CDC, 2024). After schizogony occurs, infected hepatocytes rupture releasing thousands of now mature daughter merozoites back into circulation where they begin to infect erythrocytes (Sato, 2021; CDC, 2024). In *P. vivax*, this stage may remain dormant for years before merozoites are released (Sato, 2021; CDC, 2024). Once infected, the intraerythrocytic cycle begins; merozoites replicate asexually into ring stage trophozoites and mature schizonts, alternately rupturing and reinfecting erythrocytes (Sato, 2021; CDC, 2024). A single intraerythrocytic cycle is repeated every 48 hours in *P. falciparum* (Sato, 2021; CDC, 2024).

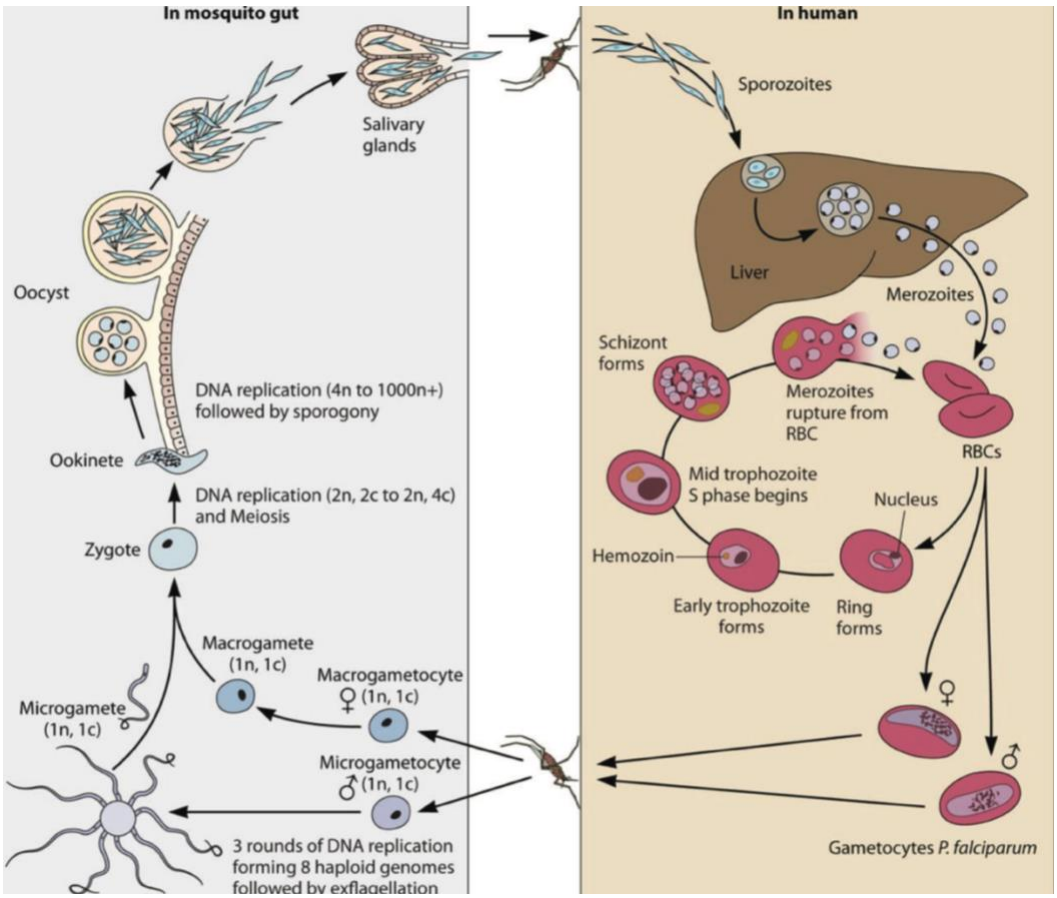


Figure 2.2: Cyclical lifecycle of *P. falciparum* through mosquito vectors and human hosts (Adapted from Lee *et al*., 2014).

After several asexual cycles some of the merozoites released when an erythrocyte ruptures, go on to differentiate (in response to stress or other cues) into male and female gametocytes within the bone marrow or spleen by female *Anopheles* spp. during a blood meal, they undergo zygote formation within the vector gut (Sato, 2021; CDC, 2024). The mature gametocytes subsequently return to circulation upon maturity where, following their ingestion by female *Anopheles* spp. during a blood meal, they undergo zygote formation within the vector gut (Sato, 2021; CDC, 2024). Morphological changes elongate the cell and convert it into the motile ookinete stage, which invade the vector gut wall and develop into oocytes (Sato, 2021; CDC, 2024). When the oocytes rupture, sporozoites are released and infect the vector salivary glands from which they are able to infect a new human host upon the vectors next blood meal, beginning the cycle anew (Sato, 2021; CDC, 2024).

2.3.2 Clinical presentation

Clinical manifestations of malaria typically appear 7-10 days post-infection, upon the parasite's completion of their exo-erythrocytic stage and migration into systemic circulation (Piccaluga and Ignatius, 2023). WHO treatment guidelines vary depending on the classification of malaria as either asymptomatic, uncomplicated, or severe (World Health Organization, 2023). Disease severity varies by *Plasmodium* species and host variables, such as immune response and related exposure to *Plasmodium* spp. (Wassmer *et al*., 2015). In hyperendemic regions many adults and children who have previously survived infections acquire partial resistance, which upon reinfection leads to milder symptoms (World Health Organization, 2024). Subsequently, these individuals may be asymptomatic carriers of malaria who drive morbidity by creating a reservoir for infection (World Health Organization, 2024). However, this partial resistance disappears over months to years without constant exposure, which explains why children and older populations frequently develop severe malaria (World Health Organization, 2024).

Malaria in non-immune individuals generally produces fever (Piccaluga and Ignatius, 2023). In its classical definition, uncomplicated malaria results in periodic febrile episodes, defined by the intraerythrocytic cycle of *Plasmodium spp*. (Piccaluga and Ignatius, 2023). However, fevers can be irregular and accompanied by other symptoms (Piccaluga and Ignatius, 2023). *P. falciparum* malaria generally presents as a cluster of non-specific symptoms (including fever, shivering, headache, diarrhoea, nausea, vomiting, joint and muscle pain, and tachycardia and tachypnoea), and may mimic other disease and *vice versa*, including but not limited to, influenza, viral hepatitis, meningitis, encephalitis, septicaemia, typhoid or tick bite fever, and gastroenteritis (National Department of Health, 2019; World Health Organization, 2023). For this reason, the WHO recommends the use of either light microscopic examination of a thin blood smear or a rapid diagnostic test to confirm a malaria diagnosis (World Health Organization, 2023).

Although acute and generally treatable, non-immune patients with uncomplicated malaria are at significant risk of disease progression to severe malaria, as life-threatening complications can develop rapidly (National Department of Health, 2019). These complications occur almost invariably because of delayed diagnosis or treatment, underdosing with effective drugs, or ineffective therapy due to resistance (Piccaluga and Ignatius, 2023). Clinical symptoms of severe malaria are noticeably more life-threatening where multi-organ involvement is frequent, and if left untreated, is often fatal (Piccaluga and Ignatius, 2023). Furthermore, although acute symptoms of severe malaria contribute to most of the disease burden, severe malaria disease symptoms can often lead to long term sequalae (National Department of Health, 2019). An example of this includes the neurological sequalae that can result from cerebral malaria, such as the presence of epileptic seizures in children, months, or even years, following an episode of cerebral malaria (National Department of Health, 2019). Additionally, cognitive impartment is common in cerebral malaria survivors, affecting their cognitive abilities such as motor, sensory, memory, and language skills (National Department of Health, 2019). Another important long-term effect of severe malaria is chronic anaemia caused by repeated malaria infections in children living in areas of high transmission intensity (National Department of Health, 2019, World Health Organization, 2023). In the case of pregnancy, placental parasite accumulation can lead to maternal anaemia; complications may result in both maternal and foetal death (World Health Organization, 2024). Placental infection can also lead to poor foetal growth and low birthweight, which in turn lead to retardation of child growth and poor cognitive outcomes (World Health Organization, 2024).

2.4 Disease management of malaria

Along with vector control and the RTS, S/(AS01) (Mosquirix™) and R21/ Matrix-M vaccines, current malaria elimination strategies rely on chemotherapy, for the rapid, complete clearance of malaria parasites to prevent uncomplicated cases from progressing to severe disease or death (World Health Organization, 2023). Chemotherapy is employed either as a chemopreventive or chemoprophylactic strategy. Chemoprophylaxis entails the administration of sub-therapeutic doses of antimalarials to prevent new infections in non-immune travellers to malaria endemic regions. While chemoprevention involves the administration of the full treatment course of antimalarial drugs according to a predetermined schedule, regardless of whether an individual has been infected with *a Plasmodium* spp. or not. There are several methods of chemoprevention currently recommended by the South African National Department of Health (2019). Ultimately, management of malaria with chemotherapy should be complemented by robust case management, which is dependent on several factors including epidemiology, parasite biology, and clinical presentation of the disease (World Health Organization, 2023).

Table 2.1. The pharmacological and resistance characteristics of commonly used antimalarial drugs to treat *P. falciparum* (Fry and Pudney, 1992; O'Neil, 2010; Combrinck et al., 2013; Wang *et al.*, 2015a; Tilley *et al*., 2016; Tibon *et al*., 2020; Sato, 2021; World Health Organization, 2024).

Antimalarial class	Drug	Use & targets	Mechanism of action	Resistance polymorphisms
Quinolines	**CQ**	Non-*P. falciparum* malaria Erythrocytic stage (trophozoites & schizonts)	Inhibition of haem detoxification	pfcrt (K76T, C72S, A220S, N326D, I356L) pfmdr (in combination with Pfcrt mutation only) (N86Y, Y184F, D1246Y)
	Quinine	Severe malaria Erythrocytic stage (trophozoites & schizonts)	Inhibition of haem detoxification	pfcrt (K76T)
	Mefloquine	Treatment or prophylaxis as monotherapy or in combination with artesunate Erythrocytic stage (trophozoites & schizonts)	Inhibition of haem detoxification	pfcrt76Thr primary mediator; pfmdr1 86Tyr and 1246Tyr; other SNPs in these two genes contribute, mainly outside Africa

				pfmdr (N86, Increased gene copy number)
	Lumefantrine	Treatment in combination with artemether Erythrocytic stage (trophozoites & schizonts)	Not precisely known Inhibition of haem detoxification	Currently unvalidated Lumefantrine select for pfmdr1 mutation (N86)
	Primaquine	Erythrocytic stage (trophozoites & schizonts)	Generation of reactive oxygen species or by electron transport chain inhibition	*Pv*mdr
	Atovaquone	Treatment or prophylaxis in combination with proguanil All *Plasmodium* species Erythrocytic schizonticide	Inhibition of parasite respiratory reaction via the cytochrome bc1 complex	*pf*.cytochrome b (Y268N/S/C)
Antifolates	**Proguanil**	Treatment or prophylaxis in combination with atovaquone Erythrocytic stage (schizonts)	Class II antifolate Inhibition of dihydrofolate reductase via cycloguanil which disrupts deoxythymidylate synthesis	Acquisition of *pf*dhfr mutations (108Asn, 51Ile, 59Arg, and 164Leu)
Artemisinins	**Artesunate**	Erythrocytic stage Treatment in combination with amodiaquine, mefloquine, or pyronaridine; severe malaria (intravenous)	Suggested to produce free radicals in the presence of haem, which cause damage to parasite proteins and other macromolecular structures	High failure rates in parts of South East Asia and North East India (K13PD mutations)

	Artemether	Erythrocytic stage Treatment in combination with lumefantrine		
Antibiotics	**Doxycycline**	Erythrocytic stage	Inhibition of aminoacyl-tRNA binding to MRA ribosomal complexes	SNPs in *pf*mdt and *pf*etQ
	Clindamycin	Erythrocytic stage	Inhibition of protein synthesis pathways	Resistance not documented

2.4.1 Antimalarial drugs and resistance

Over the past century several drug classes have been approved, distributed, and used to treat and prevent malaria (World Health Organization, 2024). Current antimalarial drugs can be broadly classified into three major classes according to their mechanisms of action, and include compounds which affect deoxyribonucleic acid production pathways, create oxidative stress, and which alter different aspects of haemoglobin digestion (World Health Organization, 2023) (Figure 2.3.1, Figure 2.3.2, and Figure 2.3.3).

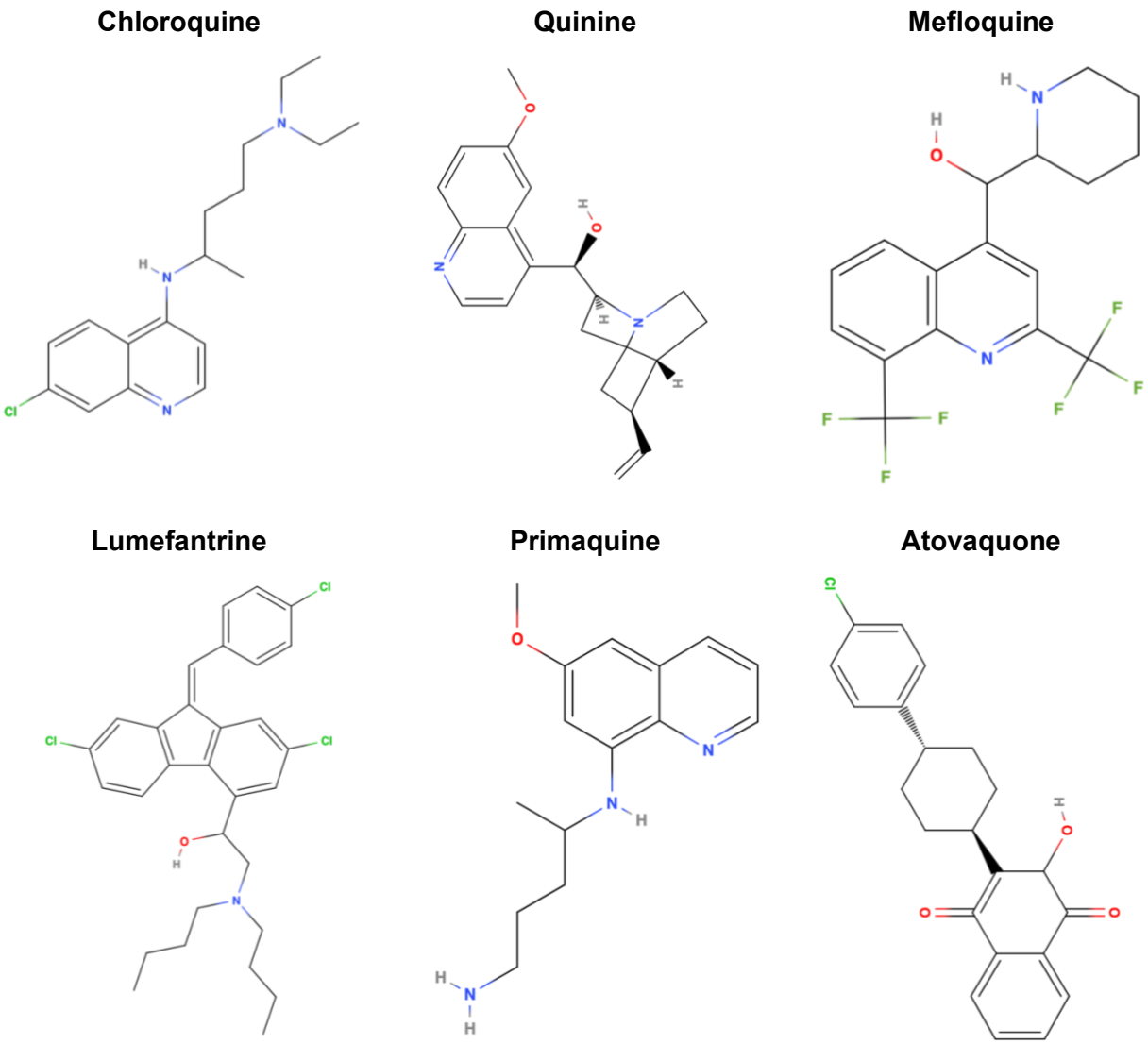


Figure 2.3.1: The chemical structure of current quinoline chemopreventive and chemoprophylactic antimalarials.

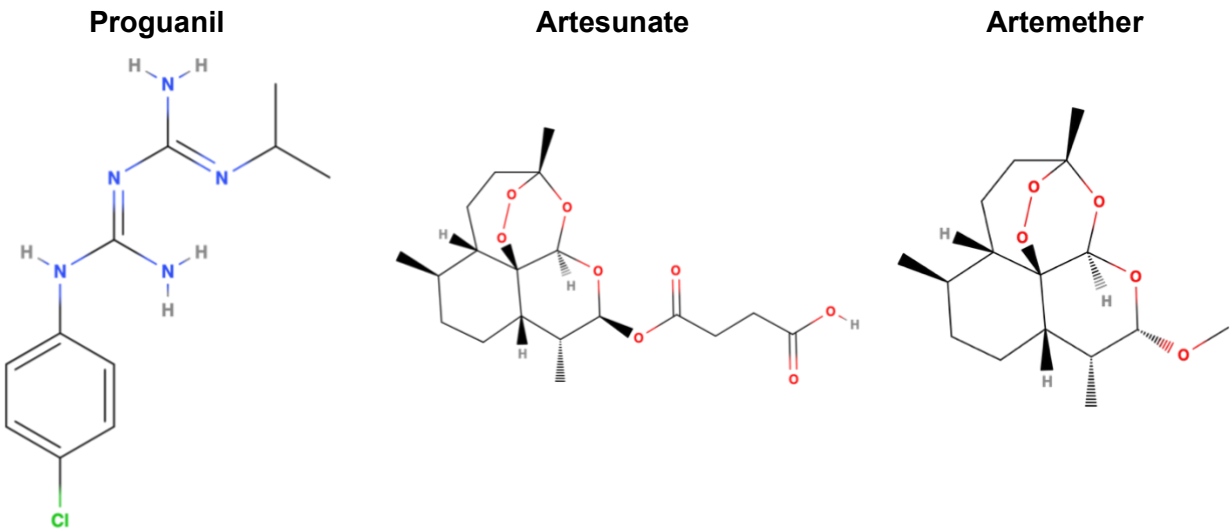


Figure 2.3.2: The chemical structure of current antifolate and artemisinin chemopreventive and chemoprophylactic antimalarials.

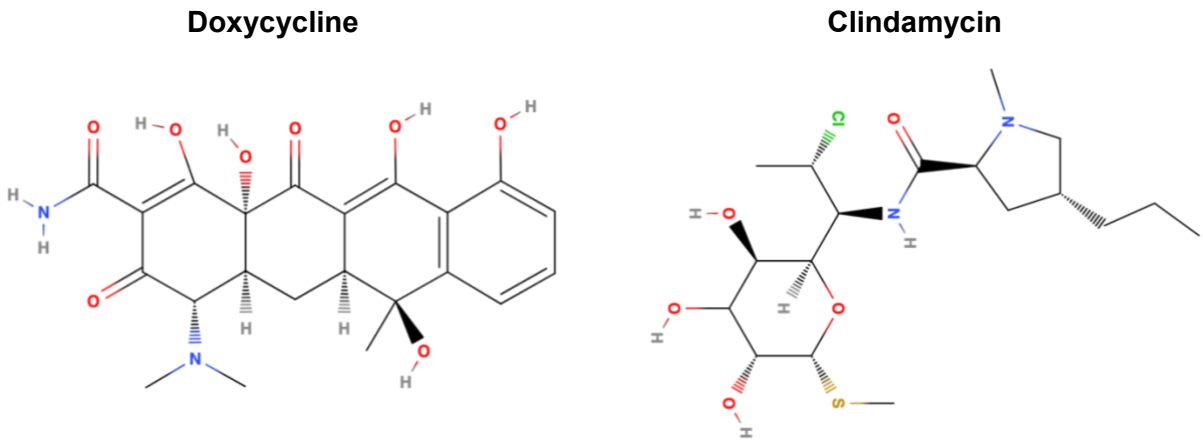


Figure 2.3.3: The chemical structure of current antibiotic chemopreventive and chemoprophylactic antimalarials.

Some of these drugs are used solely for the treatment of the infection, while others are more commonly prescribed for prophylaxis; and, several are utilised for both (World Health Organization, 2023). The South African Department of Health recommends the prophylactic drugs, doxycycline, mefloquine, atovaquone-proguanil, and to treat active infections, artemether-lumefantrine, quinine-clindamycin or artesunate (National Department of Health, 2019).

2.4.1.1 Nucleic acid synthesis inhibitors

Drugs from this class of antimalarials either target enzymes involved in the folate production pathway of the parasite (folate antagonists) or the enzymes of the mitochondrial electron-transport chain which are integral to parasite purine and pyrimidine synthesis (naphthoquinones) (World Health

Organization, 2023). Consequently, disruptions of these pathways interfere with DNA production and parasite replication (World Health Organization, 2023).

The most widely used antifolate preparation is a combination of two separate drugs – pyrimethamine, and the sulfonamide compound sulfadoxine. Pyrimethamine targets the enzyme dihydrofolate reductase (as does the antimalarial drug, cycloproguanil), while sulfadoxine disrupts the function of dihydropteroate synthase, which when combined inhibits parasite DNA production at two crucial stages (World Health Organization, 2023; Masserey *et al*., 2025). Sulfadoxine-pyrimethamine is effective against schizonts in both the exoerythrocytic and intraerythrocytic stages of the parasite life cycle (World Health Organization, 2023; Masserey *et al*., 2025). However, resistance to sulfadoxine-pyrimethamine is widespread as a result of mutations in both target enzymes which has limited it effectiveness in South Africa, Southeast Asia, Southern China, and the Amazon Basin; resistance in malaria endemic regions of Africa vary (World Health Organization, 2024; Masserey *et al*., 2025).

Proguanil is another antifolate which remains useful and is usually co-administered with atovaquone (World Health Organization, 2023). Atovaquone, a naphthoquinone, decouples electron transport in the mitochondrion and collapses the membrane potential of the organelle, which prevents regeneration of ubiquinone. A dihydroorotate dehydrogenase coenzyme, essential to the parasite pyrimidine pathway (Olanlokun *et al*., 2021; World Health Organization, 2023).

2.4.1.2 Artemisinin and its derivatives

ART and its derivatives, which are fast acting and highly potent, can reduce *P. falciparum* by up to 100 000-fold every intraerythrocytic cycle (World Health Organization, 2023; Zheng *et al*., 2024). However, ART and its derivatives have a short plasma half-life (<1-2 hours) associated with high levels of parasite recrudescence, which has prompted their use in combination with slower-acting, longer-lasting partner drugs which utilise a different mechanism of action, known as ACTs (World Health Organization, 2023; Zheng *et al*., 2024). ACTs are administered as a three-day treatment course, to both cover two intraerythrocytic cycles and minimise the potential of resistance

In Africa, where *P. falciparum* is the predominant species, antimalarial chemotherapy emphasises ACTs for the treatment of uncomplicated *P. falciparum* (World Health Organization, 2023; Zheng *et al*., 2024). The WHO recommends 6 ACTs as first-line treatment in child and adults with uncomplicated *P. falciparum* malaria. Treatment of severe malaria requires intravenous infusions, of which the drug of choice is artesunate for at least 24 hours, after which a 3-day ACT oral should be provided (Dondorp *et al*., 2005; Dondorp *et al*., 2010; World Health Organization, 2023; Zheng *et al*., 2024).

2.4.1.3 Quinolines

Members of the quinoline antimalarial class can be categorised as either 4-aminoquinolines, 8-aminoquinolines, or aryl-amino alcohols. 4-Aminoquinolines compounds included CQ, amodiaquine, piperaquine, and pyronaridine.

2.4.1.3.1 CQ

CQ is a synthetic analogue of quinine which became first-line therapy for malaria in the 1940s (Loeb *et al*., 1946). Structurally, CQ is divided into 3 distinct parts: an aromatic ring with a chlorine atom at position 7, a quinine ring with an amino side chain at position 4, and a lateral flexible side chain (Figure 2.4) .

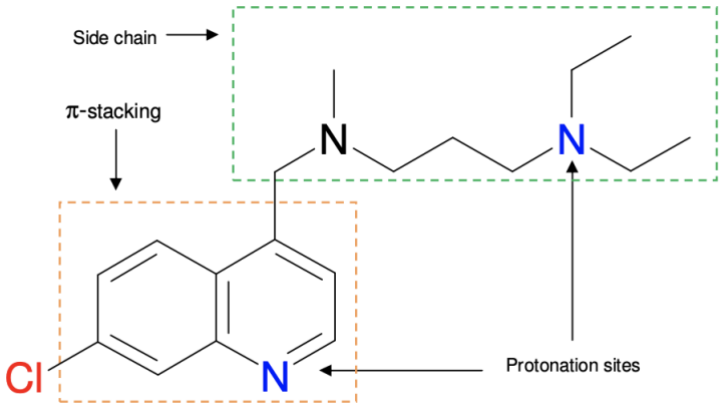


Figure 2.4: The chemical structure of CQ (Mushtaque, 2015).

Since its inception, CQ has been the most used single drug to treat malaria and remained popular until the onset of resistance due to its rapid action, simplified dosing, affordability, and decreased toxicity compared to quinine (Trape *et al*., 1987). Today, while most strains of *P. vivax*, *P. malariae*, and *P. ovale* remain sensitive to CQ, more than 80% of *P. falciparum* field isolates are reported to be resistant to CQ (Ginsburg *et al*., 2005; World Health Organization, 2020; World Health Organization, 2023). Consequently, CQ-resistance is associated with cross resistance to other quinoline drugs, such as quinine and amodaiquine (Tinto *et al*., 2006; World Health Organization, 2020).

2.4.1.3.2 CQ mechanism of action

During the asexual intraerythrocytic stage of the *Plasmodium* lifecycle, haemoglobin is catabolised within the parasite's acidic digestive vacuole (DV) as a necessary nutrient supply for growth and development, releasing free haem (Fe(II) protoporphyrin IX [Fe(II)PPIX]) as a by-product. As Fe(II)PPIX has the tendency to partition into cell membranes and catalyse the formation of reactive oxygen species (ROS) (Chou and Fitch, 1981; Vincent, 1989), *Plasmodium* spp. have evolved a complex mechanism of Fe(II)PPIX detoxification, which involves the immediate oxidation of free

Fe(II)PPIX into inert haemozoin crystals (Pagola *et al*., 2000; Egen *et al*. 1994; Egan 2002; Oliveira *et al*., 2002).

It is generally accepted that CQ acts by interfering with the Fe(III)PPIX detoxification process in parasites as they grow within host erythrocytes (Francis, 1997; Bray *et al*., 2005). CQ, a weakly basic drug, is neutral in the cytoplasm of the parasite, allowing it to readily diffuse across parasite membranes. When CQ molecules enter the parasite DV, where the pH is 5.2, they become protonated, trapping them within the compartment where they accumulate (approximately 1000-fold relative to its concentration in the parasite cytoplasm) (Homewood *et al*., 1972; Yayon *et al*., 1985). Within the DV, CQ inhibits *Plasmodium* spp. growth by pi-pi stacking its 4-aminoquinoline core into the Fe(II)PPIX ring structure or by clamping into the grooves of haemozoin crystals, hindering the deposition of oncoming free Fe(II)PPIX molecules (Shelnut, 1983; Egen *et al*., 1994). It has also been speculated that CQ complexes with free Fe(II)PPIX, which then accumulate at the DV membrane, possibly perforating the membrane and leading to the spillage of the complex into the parasite cytoplasm. Inhibition of Fe(II)PPIX detoxification is therefore expected to lead to severe oxidative damage within the parasite, ultimately resulting death.

The 8-aminoquinoline compounds, primaquine and tafenoquine, are primarily used to treat relapsing malaria. The WHO recommends a standard 14-day course of primaquine to prevent the relapse of *P. vivax* or *P. ovale* malaria (excluding glucose-6-phosphate dehydrogenase-deficient patients, pregnant women, and infants <6 months-old). A short-course, standard dose primaquine treatment is also recommended as it may lead to better adherence. Additionally, primaquine is also a gametocytocidal agent, which in low-transmission areas, is recommended by the WHO as a single dose with an ACT to treat *P. falciparum* patients to reduce the transmissibility of treated *P. falciparum* infections, while CQ in combination with either primaquine or tafenoquine is the treatment option for radical cure of *P. vivax* (World Health Organization, 2023). Once the infected individual is able to tolerate oral therapy, artemether-lumefantrine or quinine plus either doxycycline or clindamycin, should be administered (World Health Organization, 2023). Mixed infections of *P. falciparum* with either *P. vivax* or *P. ovale* should be treated with the standard therapy for uncomplicated severe *P. falciparum* infections, along with a follow-up course of primaquine.

The WHO recommends intermittent preventive therapy, with monthly sulfadoxine-pyrimethamine and amodiaquine-sulfadoxine-pyrimethamine to prevent malaria in high-risk groups and seasonal malaria (World Health Organization, 2023). Additionally, malaria chemoprophylaxis is a routine practice for travellers to Africa from regions not endemic for malaria, most commonly with atovaquone-proguanil, mefloquine, or doxycycline, a tetracycline antibiotic (World Health Organization, 2023).

2.4.2 Antimalarial resistance

Antimalarial drug resistance, as defined by the WHO, is the ability of a parasite strain to survive and/or multiply despite the administration and absorption of medicine given in doses equal to or higher than those usually recommended but within the tolerance of the subject, provided drug exposure at the site of action is adequate (World Health Organization, 2023).

Since the wide-scale deployment of antimalarials throughout the 20th century, *P. falciparum* parasites have undergone tremendous selection pressure to evolve mechanisms of resistance (Trape *et al*., 1998; Butler *et al*., 2010; Duffey *et al*., 2021). This phenomenon has rendered antimalarials such as CQ, less effective, significantly curtailing its role in the control of malaria (Peters, 1982; Payne, 1987; Trape *et al*., 1998). Resistance has since continued to evolve, risking the clinical efficacy of current first-line antimalarials (Leroy *et al*., 2019; van der Pluijm *et al*., 2019).

Artemisinin derivatives were used as monotherapies in South East Asia for decades until their use was discouraged in 2006 but is likely to have continued in the years following (Yeung *et al*., 2008; World Health Organization, 2023). Use of artemisinin monotherapies, and of their associated partner drugs at subtherapeutic doses and/or in substandard formulations has likely contributed to the emergence of ART-R in South East Asia, which was first described in Cambodia in 2008 (Noedl *et al*., 2008; Dondorp *et al*., 2009; Rogers *et al*., 2009). ART-R is characterised by delayed clearance of parasites after treatment (defined as a parasite clearance half-life of >5.5 h or persistent parasitaemia 3 days after the onset of therapy) and enhanced *in vitro* survival of parasites after artemisinin exposure (Noedl *et al*., 2008; Dondorp et al., 2009). In the Greater Mekong Subregion, concurrent ACT partner drug resistance has led to high failure rates following ACT treatment (Huang *et al*., 2012; Phyo *et al*., 2012; Kyaw *et al*., 2013; van der Pluijm *et al*., 2019). Dihydroartemisinin-piperaquine failure rates reached high levels in Cambodia and Southern Vietnam, while artesunate mefloquine failed at the Myanmar-Thai border region (Miotto *et al*., 2015; Phyo et al., 2016; van der Pluijm *et al*., 2019; World Health Organization, 2024). Within the Greater Mekong Subregion, all currently available ACTs have shown efficacy below 90% at some point within the past two decades (Miotto *et al*., 2015; Phyo et al., 2016; van der Pluijm *et al*., 2019; World Health Organization, 2024).

Until quite recently, several studies from across Africa, showed a low prevalence of pfkelch13 propeller domain mutations and a very low prevalence of mutations associated with ART-R resistance in South East Asia (Conrad *et al*., 2014; Cooper *et al*., 2015; Kamau *et al*., 2015; Taylor *et al*., 2015; Ménard *et al*., 2016). This has since changed since the emergence and expansion of a validated ART-R marker (pfkelch13 R561H) in Rwanda (Uwimana *et al*., 2020). Equally worryingly, analysis of microsatellites in the flanking regions of pfkelch13 gene showed a common origin for this parasite population, different from the R561H haplotype identified in the Greater Mekong Subregion (Uwimana *et al*., 2020). While this Rwandan ART-R parasite lineage has the phenotypic hallmarks

of ART-R, it has not yet affected the clinical efficacy of the current first-line ACT in Rwanda, artemether-lumefantrine (Uwimana *et al*., 2020). The *de novo* emergence ART-R associated pfkelch13 mutations have since been observed multiple times both clinically and *in vitro* in Eastern Africa (Ariey *et al*., 2014; Lubell *et al*., 2014; Lu *et al*., 2017; Uwimana *et al*., 2020; Balikagala *et al*., 2021; Ndwiga *et al*., 2021; Conrad *et al*., 2023; Fola *et al*., 2023; Mihreteab *et al*., 2023; Moser *et al*., 2021; Uwimana *et al*., 2020). Including in Guyana (Mathieu *et al*., 2020), New Guinea (Miotto *et al*., 2020), and Uganda (Ikeda *et al*., 2018; Asua *et al*., 2021). In addition, there have been recent reports of decreasing artemether-lumefantrine efficacy detected in Angola and Burkina Faso that appear unrelated to ART-R (Dimbu *et al*. 2021; Gansané *et a*l., 2021).

It is important to note that ART-R exerts clinical consequences primarily when resistance to partner drugs is also seen (Slater *et al*., 2016). This is particularly problematic as resistance to the majority of ACT partner drugs has been reported in East Africa, which highlights the need to develop novel drug combinations which evade or reverse resistance (Slater *et al*., 2016; Martin *et al*., 2018; Leroy *et al*., 2019). In South Africa, the combination of atovaquone and proguanil is still highly effective with no reported cases of resistance to the either drug (NICD, 2022). Similarly for the other prophylactic and treatment drugs used in South Africa, no resistance has been reported by the NICD surveillance team. With ACTs safe to be administered as a three-day treatment course, to both cover two intra-erythrocytic cycles and minimise the potential of resistance (National Department, 2019).

However, resistance, both to artemisinin's' and their partner drugs, is likely to emerge and spread in new regions were ACTs have previously been effective (Rosenthal *et al*., 2019). Therefore, investigating novel source of antimalarials is a priority. Phytomedicines, which have been useful in treating disease in humans for centuries, remains at the forefront of this search.

2.5 Phytomedicines and malaria

Phytomedicines refers to an umbrella term for plant derived materials or preparations with therapeutic benefits which contain raw or unprocessed ingredients from one or more plants. Prior to the advent of chemotherapeutic drugs, plants formed the basis of various traditional and folk medicine, which constituted the dominant means of preventing and treating malaria for centuries (Sinha *et al*., 2023). Scientific inquiry into such phytomedicines demonstrated that their medicinal value lay in the secondary metabolites which they possessed, and which had definite physiological actions on the human body (Sinha *et al*., 2023). Innovations in analytical techniques during the last part of the 19th and early 20th centuries allowed for the identification and isolation of such compounds, which were developed into the first antimalarial drugs (Yetein *et al*., 2013; Memvanga *et al*., 2015; Sinha *et al*., 2023). Since then, natural products from plant sources have served as an important reservoir for the discovery of new drugs (Sinha *et al*., 2023).

The discovery of antimalarial drugs from plant sources began almost 200 years ago, when the antimalarial drug quinine, a cinchona alkaloid, was first isolated from *Cinchona* bark, and remained the only known effective antimalarial drug until the 1930s. The structure of quinine eventually served as a template for the development of several synthetic congeners as potent antimalarial agents including CQ, primaquine, and mefloquine. The current first-line treatment for malaria, artemisinin, was isolated from the tree *Artemisia annua* (Collaboration Research Group for Qinghaosu, 1977; World Health Organization, 2024). Subsequently, as chemotherapeutic drugs established itself within mainstream medicine, the practice of treating disease with phytomedicines, which could not compete with the dramatic effects achieved from novel chemotherapeutic drugs, went into decline (Sinha *et al*., 2023). Amongst the general public however, culturally salient, lower cost phytomedicines which were more broadly accessible, remained in common use (Ocloo *et al*., 2014). Today, although widely used amongst high income populations as an adjunct to contemporary drugs for various health related benefits, phytomedicines are still considered primary healthcare amongst low-income populations on account of availability and accessibility challenges of current health systems (World Health Organization, 2013; Al-Harrasi *et al*., 2022). Consequently, the tendency to self-diagnose malaria and subsequently self-medicate, has remained a systemic issue in malaria endemic regions (World Health Organization, 2023; Enioutina *et al*., 2017; Seile *et al*., 2022). Currently, much of the sub-Saharan population, relies on phytomedicines as remedy against fever and other acute symptoms of malaria, while some populations use phytomedicines in association with antimalarials such as CQ to enhance its activity (Rasoanaivo *et al*., 1998; Waako *et al*., 2005; Ijarotimi *et al*., 2010). However, the therapeutic implications of such practices are uncertain as, pharmacokinetic and pharmacodynamic interactions, are generally unknown (Lin, 2014). Thus far, the potency of some of these phytomedicines against *P. falciparum* malaria has been demonstrated both *in vitro* and *in vivo* (Gessler *et al*. 1994; Nandakumar et al., 2006; Gathirwa *et al*. 2008; Somsak *et al*., 2018; Afieroho *et al*., 2019; Oluba *et al*., 2020).

2.5.1 Selected plants for this study

2.5.1.1 *Camellia sinensis* (Green Tea)

2.5.1.1.1 Taxonomy, distribution, & botanical description

Camelia sinensis, is an evergreen shrub within the family *Theaceae*, used to brew tea, an aromatic beverage prepared by steeping its dried leaves in boiling water (Figure 2.5; Namita *et al*., 2012).

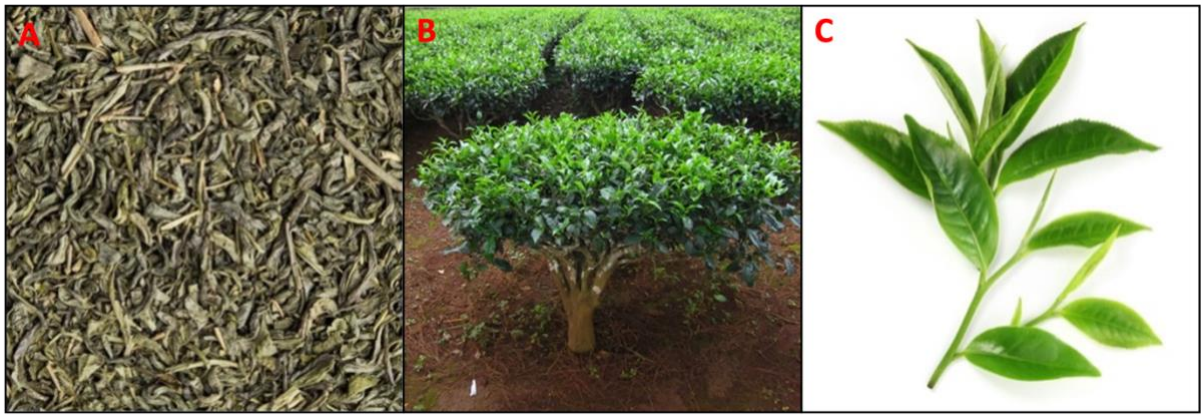


Figure 2.5: *C. sinensis* (A) dried leaf buds (B) shrub (C) fresh leaves (Namita *et al*., 2012).

C. sinensis is further classified into two main varieties: *C. assamica*, which is a larger plant with larger leaves, and *C. sinensis*, which has a smaller leaf size. For centuries, tea has been widely consumed throughout East Asia as a popular non-alcoholic beverage and traditional medicine (Namita *et al*., 2012). Nowadays, tea in the form of commercial products is consumed by nearly 3 billion people worldwide (Xie *et al*., 2021)

Tea types are most commonly categorised as either green, white, oolong, black, and pu-erh teas. The first four tea types differ in degree of enzymatic oxidation related to their processing; green tea is nonfermented, white tea is slightly fermented, oolong tea is semifermented, and black tea is fully fermented (Xie *et al*., 2021). Pu-er tea processing involves additional microbial fermentation. Green tea is processed by heating fresh leaves, which inactivates their oxidative enzymes; polyphenol oxidase and peroxidase which are ubiquitous in the leaves (Mahmood *et al*., 2010). The leaves are then rolled or shaped and then dried to reduce moisture content to create the final dried tea product (Mahmood *et al*., 2010; Namita *et al*., 2012).

2.5.1.1.2 Traditional use, phytochemistry, & biological activity

The non-fermented nature of green tea gives rise to chemical profiles primarily based on polyphenols (catechins), alkaloids, amino acids, carbohydrates, lipids, pigments, minerals, and vitamins (Mahmood *et al*., 2010; Namita *et al*., 2012; Xie *et al*., 2021). In addition to post-harvesting processing, plant variety, growth conditions, the age of the plant, climatic conditions, and season among others, contribute to differences in individual constituents present in any given batch of tea (Mahmood *et al*., 2010; Namita *et al*., 2012; Xie *et al*., 2021).

Polyphenols, generally comprise approximately 30-40% of the extractable solids of dried green tea leaves and include flavonoids, phenolic acids, alkaloids, and catechins and their derivatives, (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epigallocatechin-3-gallate (EGCG), which are the most abundant (Zhao *et al*., 2022). Other active

constituents include caffeine, polysaccharides, volatile oils, lipids, amino acids, minerals, and other trace elements, such as fluoride and aluminium, and other uncharacterised compounds (Mahmood *et al*., 2010; Zhao *et al*., 2022).

Although black tea is the most consumed product worldwide, green tea has risen in popularity in recent decades. This is primarily due to both scientific and public opinion that its consumption prevents oxidative stress related chronic disease such as cardiovascular diseases, Alzheimer's, diabetes, and certain cancers (Khan *et al*., 2009; Namita *et al*., 2012; Xing *et al*., 2019). Studies have suggested that the activity of green tea polyphenols are mostly due to their powerful antioxidant activities which are reported to attenuate the disease process of cancer as well as pathogenic organisms (Oderinde *et al*., 2019; Akinwunmi *et al*., 2021). The antimalarial activity of *C. sinensis* extracts has studied to a lesser degree and offers an incomplete picture with conflicting results as to their antimalarial activity (Oderinde *et al*., 2019; Akinwunmi *et al*., 2021).

2.5.1.2 *Viscum album L.* (Mistletoe)

2.5.1.2.1 Taxonomy, distribution, & botanical description

V. album L. (Santalaceae, formally Viscaceae or Loranthaceae), the most common species of its genus, is mainly present throughout Northern and Central Europe, Northwest Africa, and Southwest and Central Asia (Nazaruk and Orlikowski, 2016; Christenhusz *et al*., 2017; Valle *et al*., 2021). Commonly known as European mistletoe or simply mistletoe, *V. album* grows as an evergreen woody shrub, which persists for several seasons, on different host trees (Christenhusz *et al*., 2017) (Figure 2.6).



Figure 2.6: *V. album* (A) Cut and dried parts of *V. album* (B) Adult plant of *V. album* (C) Fruits of *V. album* (Christenhusz *et al.,* 2017).

It produces yellowish-green flowers and fruits which appear as translucent whitish berries, in late autumn and early winter (Christenhusz *et al*., 2017). The shrubs attach to the branches and stems

of host trees by a swelling called a haustorium, through which they are supplied water, minerals, and organic compounds from their host (Christenhusz *et al*., 2017). Additionally, in common with all mistletoes, green leaves and stems, enable *V. album* to concomitantly photosynthesise (Christenhusz *et al*., 2017). They frequently colonise apple (*Malus mali)*, poplar (*Populus*), and oak (*Quercus*) trees but are able to colonise several others (Valle *et al*., 2021). Depending on the host tree, individual subspecies may be differentiated, e.g. *Viscum album* L. subsp. *album* L. is seen on deciduous trees, *Viscum album* subsp. *abietis* (Wiesb.) Abrom. on fir, and *Viscum album* subsp. *austriacum* (Wiesb.) Volim mainly on pine (Nazaruk and Orlikowski, 2016). Usually visible from November to March, *V. album* does not lose their leaves through European winters and in fact remain photosynthetically active at temperatures below freezing (Christenhusz *et al*., 2017). Their flowers and characteristic white berries, which contain a single green seed lacking a seed coat but surrounded by a sticky pulp, ripen in winter and are a nutritional source for birds and insects (Christenhusz *et al*., 2017).

V. album has a long history of use in folk medicine where it has been used to treat anxiety, insomnia, epilepsy, internal bleeding, several cardiovascular pathologies, ulcers, rheumatism, and certain types of cancer (Christenhusz *et al*., 2017; Valle *et al*., 2021). In fact, *V. album* extracts have been widely used in cancer therapy in and around central Europe for over 100 years. *In vitro* studies and clinical trials using *V. album* extracts have since definitively demonstrated anticancer, immunomodulatory, cytotoxic, and apoptotic activities (Tröger *et al*., 2013; Loef and Walach, 2020).

2.5.1.2.2 Traditional use, phytochemistry, & biological activity

Several chemical and pharmacological studies have identified various major compounds in *V. album*, such as viscotoxins, lectins, flavonoids, biogenic amines, lignans, and mucilages (Peñaloza *et al*., 2020; Melo *et al*., 2023). The content of which depends mainly on host tree metabolism as well as the time of harvesting (Nazaruk and Orlikowski, 2016; Valle *et al*., 2021; Melo *et al*., 2023).

Lectins and viscotoxins have been recognised for their direct ability to induce cytotoxic effects in tumour cells (Tepe *et al*., 2021; Valle *et al*., 2021; Wider *et al*., 2022), while triterpenes enhance the activity of natural killer cells via antitumour and apoptotic properties (Seifert *et al*., 2008; Sevastre *et al*., 2010; Sevastre *et al*., 2012; Sárpataki *et al*., 2015; de Oliveira Melo *et al*., 2018; Valle *et al*., 2021). The overall therapeutic effects of *V. album* are generally more apparent in whole extracts as compared to purified lectins and viscotoxins alone (Valle *et al*., 2021). Such preparations are used in addition to conventional treatment to reduce side effects of chemotherapy or to support the convalescence of patients, particularly in Germany (Christenhusz *et al*., 2017). It has been found to be a safe treatment with mild to moderate adverse drug reactions in some cancer patients (Steel *et al.,* 2014). Various *V. album* preparations may be obtained as injectable prescriptions, e.g.,

abnobaviscum®, Cefalektin®, Eurixor®, Helixor®, Iscador®, Iscucin®, Isorel®, and Lektinol® (Büssing *et al*., 2000; Christenhusz *et al*., 2017). Combination extracts which possess water insoluble constituents have also demonstrated comparable inhibition of cell proliferation, cytotoxicity, and apoptosis induction (Christenhusz *et al*., 2017).

Previous studies have focused on testing *V. album* extracts for potential anticancer, antidiabetic, anti-inflammatory, immunomodulatory, antimicrobial, and cardiac activity (Steele *et al*., 1991; Gardin *et al*., 2009); Steele *et al*., 2014; Kleszken, 2022). However, to date, no studies regarding specific antimalarial activity of *V. album L.* have been conducted. Antimalarial activity has however been demonstrated in other species of mistletoe such as African mistletoes (*Agelanthus dodoneifolius, Globimetula braunii*) (Builders *et al*., 2012; Olanlokun *et al*., 2021).

2.5.1.3 *Siphonochilus aethiopicus* (African Ginger)

2.5.1.3.1 Taxonomy, distribution, & botanical description

Siphonochilus aethiopicus (Schweinf.) B. L. Burtt. is a rhizomatous herb, belonging to the family Zingiberaceae, of which 53 genera, and more than 1200 species including ginger (*Zingiber officinale* Roscoe) and turmeric (*Curcuma longa* L.) belong to (Crouch *et al*., 2000). The species has a tropical Eastern and Southern distribution (Benin, Ethiopia, Eswatini, Malawi, Mozambique, Niger, Nigeria, Northern Ghana, Senegal, South Africa, Tanzania, Zimbabwe) where populations naturally occur in dams, shaded lowland areas, or on hill slopes (Gordon-Gray *et al*., 1989; Makhuvha *et al*., 1997). In South Africa, it is commonly referred to as “African or Natal ginger” in English, “wildegemmer” in Afrikaans, and “Isiphephetho or Indungulo” in Zulu (Crouch *et al*., 2000). It can only be found in Mpumalanga and Limpopo, as unsustainable harvesting practices and overexploitation have led to local extinctions, most notably in KwaZulu-Natal (Gordon-Gray *et al*., 1989; Makhuvha *et al*., 1997). Although, *S. aethiopicus* has yet to be evaluated for the International Union for Conservation of Nature’s (IUCN) Red List (Hilton-Taylor *et al*., 1998), South Africa currently has it listed as critically endangered according to its own national Red List (Lötter *et al*., 2006).

*S. aethiopicu*s is a bisexual forest floor plant with spindle-shaped roots and large deciduous leaves which develop from a small, cone-shaped rhizome (Figure 2.7).



Figure 2.7: *S. aethiopicus* (A) The purple flowers of *S. aethiopicus*, (B) tuber and aromatic roots of *S. aethiopicus* (C) Cone shaped rhizomes (Adebayo *et al*., 2021; Viljoen *et al*., 2023).

The leaves and rhizomes are somewhat aromatic, with a smell similar to *Z. officinale* Roscoe. Short-lived, white to bright pink or purple flowers borne at ground level are proceeded by berry-like fruits which, similarly, are borne either above or below ground (Kiew, 1980; Pooley, 1998). Like most members of the Zingiberacea family, *S. aethiopicus* has a dormant phase (June to November), during which the plants are leafless, with no roots present on their rhizomes (Kiew, 1980; Pooley, 1998).

2.5.1.3.2 Traditional use, phytochemistry, & biological activity

The roots, tubers, and rhizomes of *S. aethiopicus* are used extensively across Africa as a spice in cooking and to treat physical and mental health (Makgopa and Koma, 2009; Sodi *et al*., 2011; Rasethe *et al*., 2022). Chewing or brewing fresh rhizomes as either hot or cold infusions are popular method of treating chronic and infectious respiratory, febrile, and nervous conditions (van Wyk, 2008; Seile *et al*., 2022). These include asthma, influenza, coughs and sore throats, influenza, colds, fever, hysteria, mental disturbance, and nightmares (van Wyk, 2008; Seile *et al*., 2022). Other documented traditional uses include the treatment of general pain, toothache, rheumatism, neuralgia; dysmenorrhoea, and as an infertility and vaginal thrush cure amongst women (Watt and Breyer-Brandwijk, 1962; van Wyk and Gericke, 2000; Makgopa and Koma, 2009; Sodi *et al*., 2011; Adebayo *et al*., 2021; Rasethe *et al*., 2022).

Monoterpenes, sesquiterpenoids of the furanoid type, and diarylheptanoids are abundant in the roots and rhizomes of *S. aethiopicus* (Igoli *et al*., 2012; Igoli *et al*., 2021). Siphoonochilone is the major compound and constitutes 20% of the essential oil composition in addition to the compounds 1,8-cineole, α-terpineol, δ-3-carene, camphene, myrcene, β-elemene, γ-elemene, sibnene, β-pinene, phellandrene, (*E*)- β-ocimene, *cis*-allocimene, and *trans*-allocimene (Holzapfel *et al*., 2002; Viljoen *et al*., 2002). The compounds epicurzerenone, furanodienone, 8(17),12*E*-labdadiene-15,16-dial, 15-hydroxy-8(17),12*E*-labdadiene-16-al, 16-oxo-8(17), 12*E*-labdadiene-15-oic acid. Igoli *et al*. (2021)

have also recently reported on the isolation of the first diarylheptanoid, 2,3-diacetyl-7-(3",4"-dihydroxy-5"-methoxyphenyl)-1-(4'-hydroxy-3'-methoxyphenyl)-5-heptene, along with the flavonoids 3,4',7-trimethyl kaempferol and 3,7-dimethoxyquercetin, from *S. aethiopicus* extracts (Igoli *et al*., 2012; Igoli *et al*., 2021). The furanoterpernoids have been demonstrated to exert antiasmathic and antiallergic activity, while 8(17), 12*E*-labdadiene-15,16-dial and furanodienone exert antitrypanosomal activity (Holzapfel *et al*., 2002; Viljoen *et al*., 2002).

Many of the traditional uses of *S. aethiopicus* as an antiseptic, analgesic, anti-inflammatory, antifungal, antimicrobial, and antimalarial has been supported *in vitro* (Viljoen *et al*., 2000; Zschocke and van Staden, 2002; Light *et al*., 2002; Motsei *et al*., 2003; Stafford *et al*., 2005; Lategan *et al*., 2009; Coopoosamy *et al*., 2010; Fouche *et al*., 2013).

2.5.1.4 *Warburgia salutaris* (Pepperbark Tree)

2.5.1.4.1 Taxonomy, distribution, & botanical description

Warburgia salutaris (Bertol f.) Chiov. is one of four species in its genus in the family Canellaceae, a small family of evergreen, aromatic trees. Canellaceae species are predominantly distributed across the Americas and Asia; *Warburgia* is its only genus which occurs within Africa (Leonard and Viljoen, 2015). Three species, *W. elongate* Verdc., *W. ugandensis* Sprague (with two subspecies, *W. ugandensis* and *W. longifolia* Verdc.), and *W. stuhlmannii* Engl., are found throughout East Africa (Democratic Republic of Congo (DRC), Ethiopia, Kenya, Malawi, Tanzania, Uganda) (Leonard and Viljoen, 2015). *W. salutaris* has a more South East distribution; including Lesotho, eSwatini, Malawi, Mozambique, Zimbabwe, and South Africa (Leonard and Viljoen, 2015). *W. salutaris* has been known to occur in bushveld, woodland, and coastal forests (Mander *et al*., 1995; Botha *et al*., 2004; Leonard and Viljoen, 2015; Nkqenkqa and Mundembe, 2023).

In Southern African, where the bark is highly valued, unsustainable harvesting practices and overexploitation, as well as habitat degradation and loss due to urban expansion and agricultural development, have contributed to population fragmentation and about a 50% decline in wild populations (Cunningham, 1993; Botha *et al*., 2004; Maroyi, 2008; Senkoro *et al*., 2020). Several localised extinctions have occurred outside of protected areas and as a result *W. salutaris* is listed as endangered, facing a high risk of global extinction in the wild, according to the current International Union for Conservation of Nature (IUCN) Red List (under criteria A2acd), (Hilton-Taylor *et al*., 1998; Maroyi, 2013). South Africa, which also has *W. salutaris* listed as endangered according to its own national Red List, is reported to have the largest natural population of *W. salutari*s in Southern Africa (Scheepers *et al*., 2011; Dludlu *et al*., 2017).

All parts of *W. salutaris*, but especially its bark, have a peppery smell and taste, hence its common English name “pepperbark tree” (Mander *et al*., 1995, 2013). It has a short main stem and shiny brown bark with rough longitudinal marks, which become cracked and darker brown with age (Mander *et al*., 1995) (Figure 2.8).



Figure 2.8: *W. salutaris* (A) (B) (C) Dried stem bark of *W. salutaris* (D) (E) Dried leaves of *W. salutaris* (Kotina *et al*., 2014) (F) (G) The fruit of *W. salutaris* (Leonard and Viljoen, 2015).

The leaves are alternate, long, and narrow; greener on top and paler below, with small white to greenish flowers which grow during April and May (Mander *et al*., 1995; Kotina *et al*., 2014). Waxy green berry fruits, which turn purple upon ripening, are produced between October and December (Figure 2.8) (Mander *et al*., 1995).

Specific pharmacological properties of the species have been described literature which validate its used as in traditional medicine (Maroyi *et al*., 2013; Leonard and Viljoen, 2015).

2.5.1.4.2 Traditional use, phytochemistry, & biological activity

Historically, *W. salutaris* has been hugely influential within a medicinal and spiritual capacity as a general health tonic and in in ritual functions to cleanse evil spirits and bring good luck (Mukamuri and Kozanayi, 1999; Semenya and Maroyi, 2018). Infusions or decoctions of its bark have been used for a variety of physical indications as a topical and systemic agent to treat abdominal and back pain, coughs, colds, headaches, bladder infections, tooth aches, sexually transmitted infections, and fever, and malaria (van Wyk *et al*., 2009; Maroyi *et al*., 2013; Leonard and Viljoen, 2015). The leaves, roots, and stalks also have a history of use as a traditional medicine (Leonard and Viljoen, 2015).

The chemical profile of *W. salutaris* is characterised by phenols, drimane and colorotane sesquiterpenoids (α-hydroxycinnamosmolide, isopolygodial, mukaadial, muzigadial, polygodial, salutarisolide, ugandensidial, and warburganal), and furans and furanones; of which, ugandensidial, is reported to be the most abundant compound in *W. salutaris* bark (Mashimbye *et al*., 1999; Rabe

and van Staaden, 2000; Drewes *et al*., 2001; Madikane *et al*., 2007; Maroyi, 2013; Nyaba *et al*., 2018; Khumalo *et al*., 2019). Alkaloids, flavonoids, saponins, steroids, tannins, and essential oil constituents have also previously been extracted from the bark and leaves of *W. salutaris* (Mohanlall and Odhav, 2009; Khumalo *et al*., 2019).

Studies of *W. salutaris* extracts and individual constituents have demonstrated antibacterial, antimycobacterial, antifungal, antifeedant, anti-inflammatory; cytotoxic, phytotoxic, pisicidal, molluscicidal, and antiplasmodial activities (Madikane *et al*., 2007; Maroyi, 2013; Leonard and Viljoen, 2015; Nyaba *et al*., 2018). Warburganal, polygodial, and muzigadial, the most studied *W. salutaris* constituents, have demonstrated antibacterial, antifungal, insect antifeedant, antiulcer, molluscicidal, and anticancer activity (Kubo *et al*., 1976; Kubo *et al*., 1983; Fukuyama *et al*., 1980; Kubo and Taniguchi, 1988; Appleton *et al*., 1992; Rabe and van Staaden, 2000; Montenegro *et al*., 2014; Leonard and Viljoen, 2015). *W. salutaris* leaf extracts, which possess the similar warburganal and polygodial ratios as its bark extracts, have demonstrated anti-inflammatory activity against IL-1b in RAW264.7 cells (Drewes *et al*., 2001; Khumalo *et al*., 2024). Aqueous and ethanol leaf extracts tested exerted immunomodulatory activity, where the ethanol extracts inhibited cytokine activity in LPS-induced RAW264.7 cells.

CHAPTER 3 - METHODOLOGY

3.1 Reagents & solvents, reference control compounds, & pure test compounds

3.1.1 Reagents & solvents

The highest quality of chemicals were purchased from commercial companies such as Sigma-Aldrich®, Glentham Life Sciences, Fluka, ACE Chemical, and Riedel-de-Haën. Water used in the study was obtained from a Millipore™ water (H_2O) purification system (Elix® Essential 3, 5, 10, 15 (UV)/RiOs™ Essential 5, 8, 16, 24). All methanol, dichloromethane, and dimethyl sulfoxide (DMSO) used throughout the study were of HPLC grade quality.

3.1.1.1 Preparation of phosphate-buffered saline used in all experiments

Phosphate-buffered saline (PBS) was prepared using reagents purchased from Sigma-Alrich®, with potassium chloride (4 mM), 2 mM potassium phosphate monobasic, 4 mM sodium chloride, 4 mM sodium phosphate dibasic dehydrate were dissolved in autoclaved MilliQ® water and the pH adjusted to 7.4 with 10 mM sodium hydroxide. The PBS solution was autoclaved and stored at 4 °C until required for use in haemolysis (section 3.5.1) and pLDH (section 3.4.3), cytotoxicity (section 3.5.2.3) assays.

3.1.2 Reference control compounds

The thirteen compounds which used as reference controls in various assays, were of the highest quality and were purchased from Riedel-de Haën, Sigma-Aldrich, and Rochelle Chemicals (Table 3.1). Stock solutions (10 mM) were prepared in 100 µL aliquots and stored at -70 ºC until required. Stock solutions (10 mM) were diluted in 100% DMSO unless otherwise indicated. The final working solution contained 1% (v/v) or lower of DMSO to ensure limited cell/parasite death in either Roswell Park Memorial Institute (RPMI)-1640 media for haemolysis and pLDH assays, or complete Dulbecco's Modified Eagles Medium (DMEM) cell culture media for cytotoxicity assays

Table 3.1. The molecular formulae and chemical properties of reference control compounds used in several assays throughout the study.

Compound	Formula	MW (g/mol)	Solvent
L(+)-Ascorbic acid	$C_6H_8O_6$	176.12	MilliQ® H_2O
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™)	$C_{14}H_{18}O_4$	250.29	DMSO
Ethylenediaminetetraacetic acid disodium salt (EDTA)	$C_{10}H_{14}N_2Na_2.2H_2O$	372.24	MilliQ® H_2O
Ammonium tetrathiomolybdate	$H_8N_2MoS_4$	260.28	DMSO
Quinine hydrochloride dihydrate	$C_{20}H_{24}N_2O_2.HCl.2H_2O$	396.91	DMSO
CQ diphosphate salt	$C_{18}H_{26}ClN_3.2H_3PO_4$	515.86	MilliQ® H_2O (10%), DMSO (90%)
Dihydroartemisinin	$C_{15}H_{24}O_5$	284.35	DMSO
3,4,5-Trihydroxybenzoic acid (Gallic acid)	$C_7H_6O_5$	170.12	DMSO
Rutin hydrate	$C_{27}H_{30}O_{16}.xH_2O$	610.52	DMSO
Triton™ X-100	*t*-Oct-C_6H_4-$(OCH_2CH_2)_xOH$, x= 9-10	647.00	MilliQ® H_2O
(S)-(+)-Camptothecin	$C_{20}H_{16}N_2O_4$	348.35	DSMO
Cis-Diamineplatinum (II) dichloride	$Pt(NH_3)_2Cl_2$	300.05	DMSO
Verapamil hydrochloride	$C_{27}H_{38}N_2O_4.HCl$	491.07	DMSO

3.1.3 Pure test compounds

Sixteen pure compounds of the highest quality were purchased from Glentham Life Sciences and Sigma Aldrich (South Africa) for testing in various assays (Table 2.2). Stock solutions (10 mg/mL) were prepared in 100 µL aliquots and stored at -20 ºC until required. Stock solutions were diluted in RPMI-1640 media for haemolysis and pLDH assays, and complete DMEM cell culture media for cytotoxicity assays for screening and IC_{50} value determination. DMSO concentrations were <1% of the final in-reaction concentration to prevent interference in parasite and cell viability. Dilutions were freshly prepared prior to the beginning of each assay and for all repeats.

Table 1.2 The molecular formulae and chemical properties of pure compounds tested in several assays throughout the study.

Compound	Formula	MW (g/mol)	Solvent
Syringic acid	$C_9H_{10}O_5$	198.17	DMSO
(+)-Catechin hydrate	$C_{15}H_{16}O_7$	308.28	DMSO
Hesperetin	$C_{16}H_{14}O_6$	302.28	DMSO
Ferulic acid	$C_{10}H_{10}O_4$	194.18	DMSO
Kaempferol	$C_{15}H_{10}O_6$	286.24	DMSO
Polygodial	$C_{15}H_{22}O_2$	234.33	DMSO
Oleic acid	$C_{18}H_{34}O_2$	282.46	DMSO
Naringenin	$C_{15}H_{12}O_5$	272.25	DMSO
Caffeic acid	$C_9H_8O_4$	180.16	DMSO
Sinapic acid	$C_{11}H_{12}O_5$	224.21	DMSO
Ellagic acid	$C_{14}H_6O_8$	302.19	DSMO
(-)-Epicatechin	$C_{15}H_{14}O_6$	290.27	DMSO
4-Hydroxybenzoic acid	$C_7H_6O_3$	138.12	DMSO
(-)-Epigallocatechin	$C_{15}H_{14}O_7$	306.27	DMSO
(-)-Epicatechin gallate	$C_{22}H_{18}O_{10}$	442.37	DMSO
3,4-Dihydroxybenzoic acid	$C_7H_6O_4$	154.12	DMSO

3.1.4 Crude extract preparation

Aqueous and organic extracts were each prepared from commercial products containing the plants *Camelia sinensis, Siphonochilus aethiopicus, Viscum album*, and *Warburgia salutaris*, which were acquired commercially.

3.1.4.1 Aqueous and organic crude extraction

Briefly, dried and ground samples (1:10 (w/v)) were weighed and extracted in MilliQ® H_2O at 40 °C, stirring continuously for 3 hours. The same process was repeated when preparing organic extracts in acetone and dichloromethane methanol added in a 1:1 (v/v) ratio. After 3 hours all extracts were filtered with filter paper and left uncovered overnight in a cool dark fume hood to minimise decay and evaporate to dryness. To further minimise extract decay, extracts were solubilised as soon as dried. Dried extracts were weighed, their percentage yields calculated using Equation 1, and solubilised in DMSO (≥99.0%; Sigma Aldrich) to stock solutions of 100 mg/mL.

$$\% \text{ Yield } = \left(\frac{mf}{mi}\right) \times 100 \qquad \textbf{Equation 3.1}$$

Where m_f is the final mass of the extract following evaporation and m_i is the initial mass of the dried plant materials (Nciki, 2016).

The crude extract stocks were then centrifuged at 252 x g in a Thermo Scientific Heraeus™ Megafuge™ 40 Centrifuge, filtered through 0.22 µm nylon filters, and stored in 100 µL aliquots microcentrifuge tubes at -20 °C until required for further analysis. Depending on the assay, the crude extract stocks were further diluted in either DMSO, complete RPMI-1640 media, or complete cell culture media for screening and subsequent IC_{50} value determination, if warranted. Dilutions were freshly prepared prior to the beginning of each assay and for all repeats.

3.2 Phytochemical analysis

3.2.1 Preliminary qualitative phytochemical analysis

Qualitative phytochemical screening of crude extracts (5 mg/mL) was investigated using colorimetric methods at room temperature (RT °C) unless otherwise stated in accordance with modified methods described by Bibi *et al*. (2010), Ganesan *et al*. (2023), Ramaan *et al*. (2006), Sofowora (1982), Evans (2009), and Solihah *et al*. (2012). Extracts were screened for the presence of alkaloids, anthraquinones, coumarins, flavonoids, saponins, and tannins. The degree of presence of secondary metabolites was reported as: + Mildly present; ++ Moderately present; +++ Highly present; or – Absent.

3.2.1.1 Test for alkaloids

Dragendorff's reagent, potassium iodide and bismuth subnitrate (ACE Chemicals) in 1% hydrochloric acid, was prepared for the detection of alkaloids (Bibi *et al*., 2010). Crude extracts (1 mL) were mixed with 1 mL 1% hydrochloric acid before gently being heated in a steam bath (60 ºC; 15 minutes) and then filtered through a 0.2 µm filter (GVS™) (Solihah *et al*., 2012). Dragendorff's reagent (3 drops) was added to the filtrate. Heavy metal ions present in the reagent bonds with nitrogen present in alkaloids to form ion pairs which produce an insoluble orange-red precipitate indicating the presence of alkaloids.

3.2.1.2 Test for anthraquinones

To detect the presence of anthraquinones the method described by Sofowora (1982) was used. Briefly, 3 mL of crude extracts was shaken with 3 mL benzene, filtered through a 0.22 µm filter and the filtrate shaken with 5 mL ammonia (10%). The formation of a rose pink or red colour in the ammonia phase (lower layer) indicated the presence of anthraquinone glycosides.

3.2.1.3 Test for coumarins

As described by Ganesan *et al*. (2023), 2 mL of crude extracts and 2 mL sodium hydroxide (10%) were mixed. The formation of a yellow colour indicated the presence of coumarins.

3.2.1.4 Test for flavonoids

The presence of flavonoids was detected following the method described by Evans (2009). Extracts were mixed with 1 mL sodium hydroxide (2%) solution. Formation of an intense yellow colour which reverted to colourless upon the addition of a few drops of concentrated HCl indicates the presence of flavonoids.

3.2.1.5 Test for phenols and tannins

As described by Raaman (2006), 500 µL of crude extracts were added to 1 mL MilliQ® H_2O and 2 drops of 2% (w/v) $FeCl_3$ solution. The formation of a blue-green to black colour indicated the presence of phenols and tannins.

3.2.1.6 Test for saponins

The presence of saponins was detected using the methods by Solihah *et al*. (2012). The crude extracts (200 µL) were vigorously shaken with 5 mL MilliQ® H_2O for 5 minutes. The formation of a stable foam which persisted for at least 15 minutes or the formation of an emulsion when olive oil (3 drops/ tube) was added indicated the presence of saponins.

3.2.2 Quantitative phytochemical analysis

3.2.2.1 Determination of total flavonoid content

The total flavonoid content (TFC) was estimated using the aluminium chloride colourimetric method, where $AlCl_3$ forms acid stable complexes with specific flavonoid structures which can then be quantified spectrophotometrically (Figure 3.1; Christ and Müller, 1960; Pękal and Pyrzynska, 2014). The absorbance was measured at 510 nm on an ultraviolet-visible (UV-Vis) plate reader (Thermo Scientific™ MultiSkan™ Go). The calibration curve used for quantification was constructed by performing a 1:2 serial dilution of rutin hydrate in MilliQ® water (3.125 – 200 µg/mL). TFC in the crude extracts was expressed as µg of rutin hydrate equivalents (RE) per g of dried plant material and calculated based on the standard curve derived by linear regression in Equation 3.2.

$$TFC = y = 0.0066x + 0.0148 \quad (R^2 = 0.9952) \qquad \textbf{Equation 3.2}$$

Where y is the total flavonoid concentration within the crude extract measure at 510 nm measured as rutin hydrate equivalents, 0.0066 is the slope of the rutin hydrate standard curve, x is the absorbance of crude extracts at 510 nm, and 0.0148 is the y-intercept of the rutin hydrate standard curve.

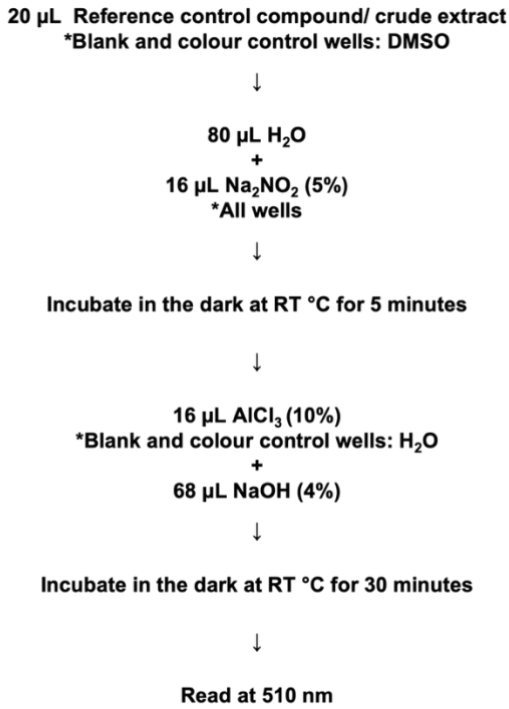


Figure 3.1. TFC assay methodology for a 96-well microtitre plate (Pękal and Pyrzynska, 2014).

3.2.2.2 Determination of total phenolic content

The total phenolic content (TPC) was estimated using the Folin-Ciocalteu colourimetric method (Singleton and Rossi, 1965). Briefly, 100 µL Folin-Ciocalteu reagent (0.2 N) was added to 20 µL crude extracts (0.1, 1, 10, 100 µg/mL) or standard gallic acid solutions, thoroughly mixed, and allowed to stand at RT °C for 5 minutes. Thereafter, a saturated sodium carbonate (7.5% w/v) solution was added, mixed in thoroughly, and allowed to stand in the dark at RT for 2 hours. Absorbance was measured at 750 nm using a UV-Vis plate reader (Thermo Scientific™ MultiSkan™ Go). The calibration curve used for quantification was constructed using different concentrations of gallic acid standard solutions (3.125 – 200 µg/mL, taking the 1:2 gallic acid : MilliQ® water dilution into account). TPC was expressed as a µg of gallic acid equivalents (GAE) per µL of each crude extract and calculated based according to a standard curve derived using Equation 3.3.

TPC = y = 0.0318x + 0.0641 (R^2 = 0.9993) **Equation 3.3**

Where *y* is the total phenolic concentration within the crude extract measure at 750 nm measured as gallic acid equivalents, 0.0318 is the slope of the gallic standard curve, *x* is the absorbance of crude extracts at 750 nm, and 0.0641 is the y-intercept of the gallic acid standard curve.

3.2.3 High performance liquid chromatography

Chromatographic analyses of crude extracts were performed on a PerkinElmer Flexar™ high performance liquid chromatography (HPLC) system equipped with a binary pump, autosampler,

column oven, and a UV-Vis detector. Separation was carried out using a Kinetex® RP-C18 (150 mm x 4.6 mm; 5 µm particle size) column from Phenomenex (California, USA) at a constant temperature of 25 °C, and a wavelength of 270 nm to detect compounds present in the extracts. Instrument controls and data acquisition and processing were carried out on Chromera® software (Version 4.2.0.6415).

3.2.3.1 Chromatographic conditions

Composition of crude extracts was determined as previously described methods by Nciki (2016). Filtered crude extracts (2.5 mg/mL) (0.45 µm) were injected at a volume of 10 µL, at a constant flow rate of 1 mL/minute for 45 minutes using a gradient mobile phase consisting of HPLC-grade MeOH and a 0.1% (v/v) formic acid (FA) aqueous solution (Nicki, 2016). The gradient elution was set to: 0.5 – 10 minutes, 1% MeOH:99% FA; 10 – 15 minutes, 5% MeOH:95% FA; 15 – 20 minutes, 15% MeOH: 75% FA; 20 – 40 minutes, 20% MeOH: 80% FA; 40 – 45 minutes, 60% MeOH:40% FA; 45 – 55 minutes, 1% MeOH:99% FA.

3.2.3.2 Preliminary peak characterisation and estimation

Preliminary characterisation and estimation of selected pure compounds was done on the crude extract which had the best antiplasmodial activity to provide a tentative indication of compound amounts. Pure compounds were selected based on their likelihood to be present in a given extract as observed in literature. Selected pure compounds (20 µg/mL) were characterised by first gauging their retention times (Section 3.2.3.1). The standard addition procedure was utilised to estimate the quantity of the compound in the extract (Al-Obaidi *et al*., 2019). The active extracts were then injected into the HPLC system unspiked and spiked (where final concentration of pure compounds were 20 µg/mL) with compounds individually or all together (if retention time were found to be sufficiently different) (Section 3.2.3.1) into the selected crude extract (5 mg/mL). The estimated amount of pure compound in the extract was calculated from the peak areas using Equation 3.4.

$$\% \text{ Compound } = \left(\frac{Mass\ of\ compound\ injected}{Mass\ of\ extract\ injected}\right) \times 100 \qquad \textbf{Equation 3.4}$$

Where % compound is the estimated concentration of pure compound in the crude extract (Nciki, 2016).

3.3 Oxidative stress and metal homeostasis

3.3.1 2,2- Diphenyl-1-picryl-hydrazyl-hydrate (DPPH•) free radical scavenging assay

The ability of compounds to bind free radicals was assessed using the colourimetric method proposed by Blois (1958). The assay is based on the transfer of electrons to DPPH•, a stable free radical which appears violet in solution (Blois, 1985). A fresh DPPH• stock solution (100 µM) was

prepared in HPLC grade methanol 12 hours prior to all experiments and stored at 4 °C for a maximum of seven days. DPPH• was incubated with test compounds for 30 minutes at RT °C in the dark (Gülçin *et al*., 2005). The plate was read using an UV-Vis plate reader (Thermo Scientific™ MultiSkan™ Go) at an absorbance of 562 nm. Test compounds were initially screened at 100 µg/mL and if shown to possess >60% activity were assessed to determine their IC_{50} values. The percentage free radical scavenging activity (FRSCA) was calculated using Equation 3.5. DPPH• FRSCA of the test compounds were compared against the positive control, ascorbic acid. Negative (test compound solvent in MeOH) and colour control controls (crude extracts in MeOH) were included.

$$\% \text{ Free radical scavenging activity } = \left(\frac{(OD_{100\%} - OD_B) - (OD_{TC} - OD_{CC})}{(OD_{100\%} - OD_B)}\right) \times 100 \qquad \textbf{Equation 3.5}$$

Where $OD_{100\%}$ is the optical density of the 100% FRSA control, OD_B is the optical density of the blank, OD_{TC} is the optical density of the test compound, and OD_{cc} is the optical density of the colour control.

3.3.2 Iron chelation assay

The ability of compounds to chelate Fe^{2+} was assessed using the colorimetric method proposed by Carter (1971), with modifications. The assay is based on the ability of Fe^{2+} to react with ferrozine which forms a purple-coloured complex and can be monitored spectrophotometrically. However, compounds which can chelate Fe^{2+} disrupt the formation of the Fe^{2+}-ferrozine complex which is measured as a decrease in absorbance at 562 nm. Test compounds were initially screened at 100 µg/mL and if shown to possess >60% activity were assessed to determine their IC_{50} value. Ammonium acetate, ferrozine and $FeCl_2.4H_2O$ were prepared fresh at the onset of each experiment. Following incubation, the plate was read using a UV-Vis spectrophotometer (Thermo Scientific™ MultiSkan™ Go) at an absorbance of 562 nm before the percentage iron chelating activity was calculated using Equation 3.6 (Figure 3.2). Percentage iron chelation of the test compounds was compared against the positive control, ethylenediamine-tetra-acetic acid (EDTA). Negative (test compound solvent + $FeCl_2$ + ferrozine solvents) and colour control controls (crude extracts with no ferrozine) were included.

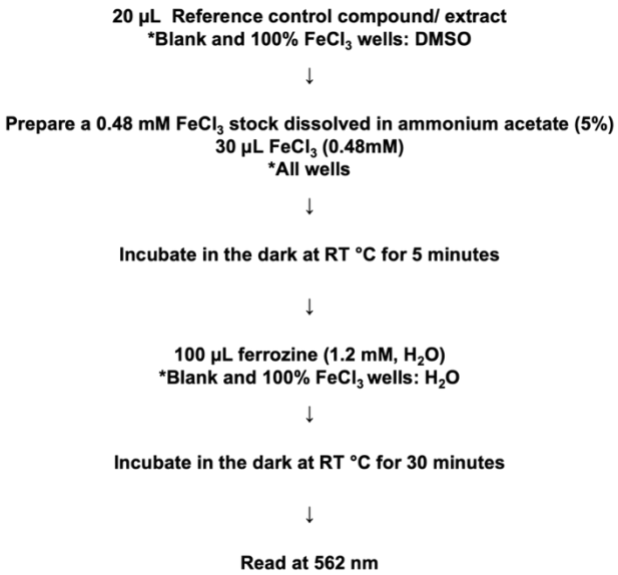


Figure 3.2. Iron chelation assay methodology for a 96-well microtitre plate (Carter, 1971).

$$\% \text{ Iron chelating activity } = \left(\frac{(OD_{100\%} - OD_B) - (OD_{TC} - OD_{CC})}{(OD_{100\%} - OD_B)}\right) \times 100 \qquad \textbf{Equation 3.6}$$

Where $OD_{100\%}$ is the optical density of the 100% iron chelating control, OD_B is the optical density of the blank, OD_{TC} is the optical density of the test compound, and OD_{cc} is the optical density of the colour control.

3.3.3 Copper(II) chelation assay

A copper(II) chelation assay was used to determine the copper chelating properties of the test compounds, using the adjusted protocol described by Říha *et al.* (2013). Copper(II) sulfate ($CuO_4S.5H_2O$; 5 mM) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH = 7.4; 15 mM) were prepared in MilliQ® H_2O. The working 1 mM copper solution (50 µL) and 100 µL HEPES were incubated in the dark for 15 minutes with 50 µL test or reference compounds, 50 µL of 5 mM hydroxylamine to oxidise remaining Cu^{2+}, and subsequently 50 µL of 5 mM neocuproine as a colourimetric detector of the remaining Cu(I) ions (Syed and Syeda, 2007; Říha *et al.*, 2013). The plate was read using a UV-Vis spectrophotometer (Thermo Scientific™ MultiSkan™ Go) at an absorbance of 450 nm. The percentage copper chelation was calculated using Equation 3.7. Percentage $CuO_4S.5H_2O$ of test compounds were compared against the positive control, TMB. Negative (test compound solvent + $CuO_4S.5H_2O$ + HEPES + hydroxylamine + neocuproine solvent) and colour control controls (crude extracts + $CuO_4S.5H_2O$ + HEPES + hydroxylamine + neocuproine solvent) were included.

$$\% \text{ Copper chelating activity } = \left(\frac{(OD_{100\%} - OD_B) - (OD_{TC} - OD_{CC})}{(OD_{100\%} - OD_B)}\right) \times 100 \qquad \textbf{Equation 3.7}$$

Where $OD_{100\%}$ is the optical density of the 100% copper chelating control, OD_B is the optical density of the blank, OD_{TC} is the optical density of the test compound, and OD_{cc} is the optical density of the colour control.

3.3.4 Lipid peroxidation assay

Reactive oxygen species have been implicated in the pathogenesis and progression of malaria as a disease (Vasquez, *et al*., 2021). The lipid peroxidation assay as outlined by Gülçin *et al*. (2004) was used to assess if the crude extracts have any activity on such a pathway as a potential mechanism of action. Inhibition of lipid peroxidation by the crude extracts was measured as described by Gülçin *et al*. (2004) (Figure 3.3).

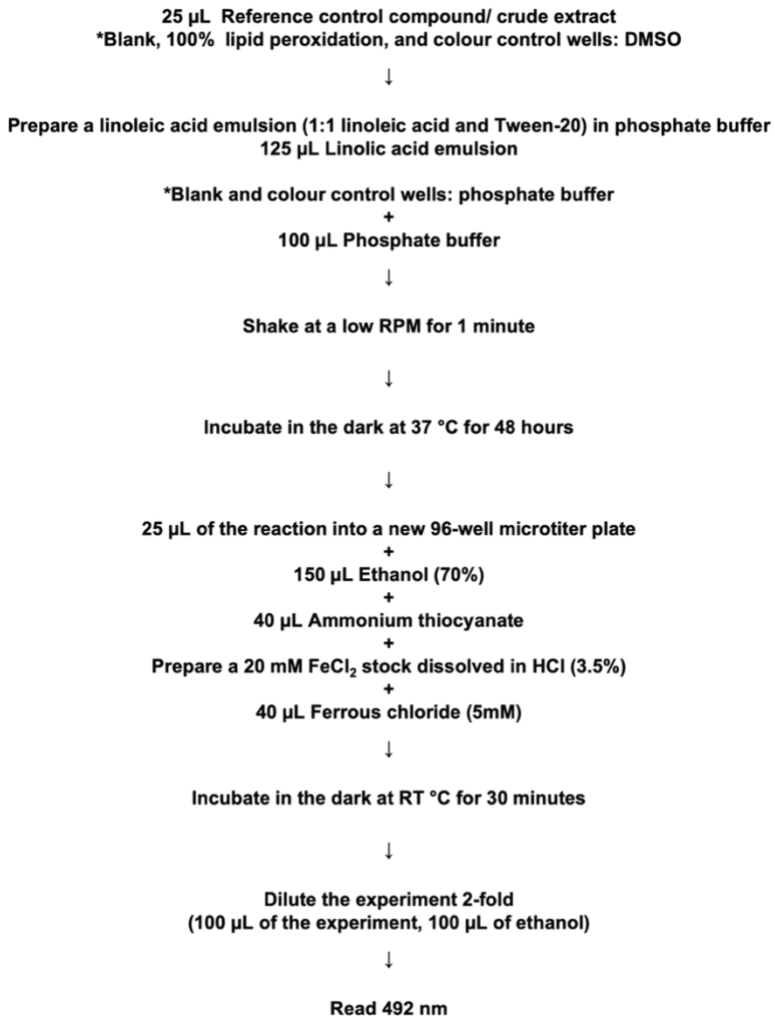


Figure 3.3. Inhibition of lipid peroxidation assay methodology for a 96-well microtitre plate (Gülcin *et al*., 2004).

Crude extracts were initially screened at 100 µg/mL and if shown to possess >60% activity were assessed to determine their IC_{50} values (0.001, 0.1, 10, 25, 50, 100, 200 µg/mL). Crude extracts were reacted with a linoleic acid emulsion and sodium phosphate buffer (pH 7.0) before ammonium

thiocyanate and ferrous chloride were added to elicit a colour change that could be measured at 500 nm using a UV/ Vis spectrophotometer (Thermo Scientific™ MultiSkan™ Go). The percentage lipid peroxidation inhibition of crude extracts were compared against Trolox™ (0.1 – 100 µg/mL), the positive control; with appropriate controls included: negative (test compound solvent + linoleic emulsion + phosphate buffer + hydroxylamine + neocuproine solvent) and colour control controls (crude extracts + $CuO_4S.5H_2O$ + HEPES + hydroxylamine + neocuproine solvent) were included. The percentage lipid peroxidation inhibition was calculated using Equation 3.8.

$$\% \text{ Inhibition of lipid peroxidation activity } = \left(\frac{OD_{TC} - OD_{CC}}{(OD_{100\%} - OD_{B})}\right) \times 100 \qquad \textbf{Equation 3.8}$$

Where $OD_{100\%}$ is the optical density of the 100% inhibition of lipid peroxidation control, OD_B is the optical density of the blank, OD_{TC} is the optical density of the test compound, and OD_{cc} is the optical density of the colour control.

3.4 *In vitro* biological activities

HepG2 cells, a human liver cell line originally derived from a human hepatocellular carcinoma, K562 cells, a human lymphoblast cell line originally derived from a myeloid leukaemia, and the *P. falciparum* chloroquine-sensitive (NF54) and chloroquine-resistant (FCR-3) strains, were obtained within the Division of Pharmacology, at the University of the Witwatersrand.

3.4.1 Antiplasmodial activity

The *P. falciparum* chloroquine-sensitive (NF54) and chloroquine-resistant (FCR-3) strains were originally obtained from the Wellcome Trust and stored within the Division of Pharmacology, at the University of the Witwatersrand. Appropriate human ethics and biosafety clearances were obtained to conduct this research (Appendices A.1, A.2, and A.3).

3.4.1.1 *In vitro* culture maintenance of *P. falciparum*

Chloroquine sensitive (NF54) and resistant (FCR-3) reference strains of *Plasmodium falciparu*m were continuously maintained *in vitro*, with an approximate parasitaemia of 5% in accordance with the method described by Trager and Jensen (1976) with slight modifications as per van Zyl, Seatlholo, and Viljoen (2010). Briefly, cryopreserved parasites were thawed in a series of sodium chloride solutions [1: 12%; 2: 1.6%; 3: 0.9%], centrifuged at 252 x g in a Thermo Scientific Heraeus™ Megafuge™40 Centrifuge for 5 minutes, and the pellet resuspended in complete RPMI-1640 culture medium at a 2% haematocrit (van Zyl, Seatlholo, and Viljoen (2010).

Spent media was replaced every second day and blood fed predominantly in the parasites' trophozoite/ schizont stage. Cultures were incubated in controlled atmospheric conditions (5%

carbon dioxide (CO_2), 3% oxygen (O_2)), and a 92% nitrogen (N_2)) at 37 °C. Staging and parasitaemia (%) were determined by light microscopy of Giemsa-stained thin blood smears of *Plasmodium* cultures (Trager and Jensen, 1976). Air dried thin blood smears were fixed with methanol, dried, stained with eosin stain for 10-20 seconds, rinsed with tap water and dried again, then counter-stained with Azure B for 30 seconds (Rapi-diff stain set, Clinical Sciences Diagnostics). The slide was then viewed with a 100X oil immersion objective lens at 1000x magnification on an Olympus® CX41 Phase Contrast Microscope (Tokyo, Japan).

3.4.1.2 Preparation of complete RPMI-1640 culture medium

Complete culture media for parasite culture maintenance, the antimalarial drug sensitivity assays and combination antimalarial assays were prepared as described by Traeger and Jenson (1976) and Duffy and Avery (2017). Incomplete RPMI-1640 culture medium (0.0104 g/mL MilliQ® H_2O) supplemented with 8 g glucose, 11.8 g HEPES, and 0.3 mM hypoxanthine was further supplemented with 10% (v/v) Albumax II™ and 5% (w/v) $NaHCO_3$ to prepare complete RPMI-1640 culture medium, that was sterile filtered (0.2 µm filters; GVS™) before being stored at 4 °C, for no longer than two weeks, until required.

3.4.1.3 Preparation of erythrocyte suspension

With the assistance of a phlebotomy technician, blood was drawn from healthy volunteers into anticoagulant blood tubes containing citrate phosphate dextrose-1 (Clearance certificate; Appendix A.1). The whole blood was stored at 4 °C until required at which time the tubes were centrifuged at 252 x g for 5 minutes in a Thermo Scientific Heraeus™ Megafuge™40 Centrifuge. The supernatant and buffy coat were discarded and the remaining blood washed in PBS (pH 7.4) at least three times. This was to ensure all white blood cells and plasma were removed leaving only red blood cells behind for culturing and experimentation. The washed red blood cells were hydrated in 1 mL of PBS, which was always removed prior to use, and stored at 4 °C until require for feeding and experimentation. Washed blood was typically used for a maximum of 14 days before being discarded and fresh blood drawn from a healthy volunteer.

3.4.2 Synchronisation of *P. falciparum* culture

Uniform ring stage cultures were achieved using the 5% D-sorbitol technique described by Lambros and Vanderburg (1979). Predominantly ring stage cultures (Figure 3.4) were centrifuged at 252 x g for 5 minutes on a Thermo Scientific Heraeus™ Megafuge™40 Centrifuge and the supernatant discarded.

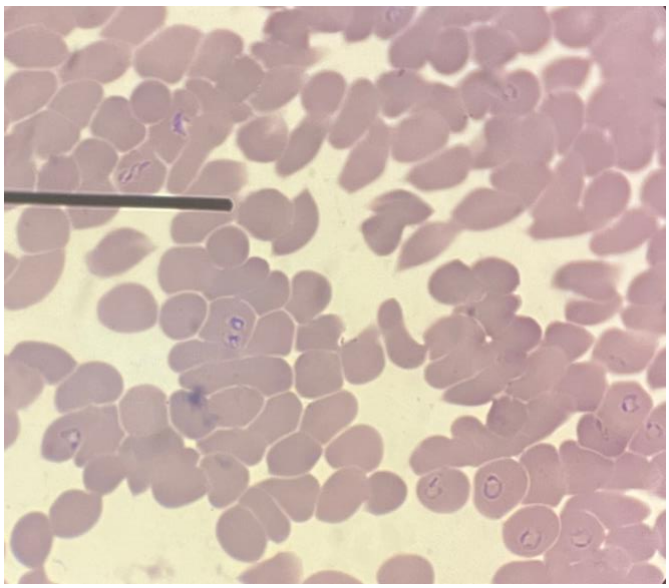


Figure 3.4: A Giemsa stained photomicrograph (1000X; *Olympus® CX41 Phase Contrast Microscope (Tokyo, Japan)*) of the NF54 *P. falciparum* strain ring stage culture.

The pellet was resuspended in a volume of 10 mL warmed D-sorbitol and incubated at 37 °C for 15 minutes. Thereafter, the suspension was centrifuged again at 252 x g for 5 minutes in a Thermo Scientific Heraeus™ Megafuge™40 Centrifuge and the discarded supernatant was replaced with warmed RPMI-1640 culture medium and the parasites maintained as described in Section 2.4.1.

3.4.3 *Plasmodium* lactate dehydrogenase (pLDH) assay

3.4.3.1 Preparation of reagents

Malstat™ solution was prepared by sterile filtering (0.2 µm filters; GVS™) 0.004 µL/mL Triton™ X-100, 0.18 mM calcium-L-lactate, and 0.5 mM 3-acetylpyridine adenine (APAD) into Tris base (6.6 g/L MilliQ® H_2O; pH = 9.1), covered in aluminium foil and stored at 4 °C for a maximum of seven days. Phenazine ethosulfate (PES; 0.3 mM) and nitroblue tetrazolium (NBT; 2 mM) were individually dissolved in MilliQ® H_2O and stored at 4 °C in the dark for a maximum of 6 months and 7 days, respectively. PES and NBT were combined in a 1:1 ratio before being added to the experimental plate.

3.4.3.2 Preparation of experimental plates

A suspension of sorbitol-synchronised ring stage parasites (Section 3.4.2) was adjusted to a 2% parasitaemia and 2% haematocrit by the addition of complete RPMI-1640 culture medium and freshly washed erythrocytes which were at least three days old (to ensure low levels of viable platelets and low LDH levels) (Section 3.4.1.3). The latter adjusted suspension (50 µL) was added to sterile 96-well microtitre plate along with appropriate untreated uninfected and infected

erythrocytes, before being incubated for 24 hours at 37 °C using the candle jar method to ensure a humidified environment with a 5% CO_2, 3% O_2, and a 92% N_2 (Trager and Jensen, 1976). After incubation, 12.5 µL extracts/pure compounds and positive controls were added in duplicate wells to the parasite suspension consisting of mid-trophozoites and the plates returned to the candle jar where they were incubated for a further 48 hours at 37 °C. Post 48 hours, the plates were placed in a sterile container and frozen at -70 °C for 1 hour, and thawed for a further 30 minutes at 37 °C to ensure both erythrocyte and parasite lysis for pLDH enzyme release.

3.4.3.3 Addition of pLDH reagents to experimental plates

Once thawed, 25 µL of the freeze-thawed parasite red blood cell lysate was added to new sterile 96-well microtitre plates, along with 100 µL of Malstat™, and 20 µL of NBT/PES (Section 3.4.3.1). The plates were incubated for 45 minutes at 37 °C. Thereafter, 50 µL acetic acid (5%) was added to stop the colour reaction and any bubbles were removed. The plate was read at 620 nm and the percentage parasite growth inhibition was calculated using Equation 3.9.

$$\% \text{ Parasite growth inhibition} = \left(\frac{OD_{TC} - OD_{CC}}{OD_{100\%} - OD_B}\right) \times 100 \qquad \textbf{Equation 3.9}$$

Where $OD_{100\%}$ is the optical density of the parasite suspension, OD_B is the optical density of the blank, OD_{TC} is the optical density of the test compound, and OD_{cc} is the optical density of the colour control.

3.5 Preliminary toxicity properties

Toxicity assays paramount in preliminary studies of both crude extracts and pure compounds, not only to determine their safety but to characterise possible toxicity they may exert.

3.5.1 Haemolysis Assay

To establish if the test compounds had a toxic effect on erythrocytes, the method described by Hayat *et al*. (2011) was used (Figure 3.5). Crude extracts and pure compounds were initially screened at 100 µg/mL and if shown to possess >60% haemolysis were further diluted in DMSO for IC_{50} value determination. Blood for the assay (Section 3.4.1.1.2) was no older than 3 days to ensure optimal cell membrane integrity. A 2% haematocrit of freshly washed erythrocyte suspension was prepared in complete RPMI-1640 culture media (Section 3.4.1.2) to which the crude extracts/pure compounds/controls were added to evaluate potential haemolytic properties. The percentage red blood cell haemolysis was calculated using Equation 3.10.

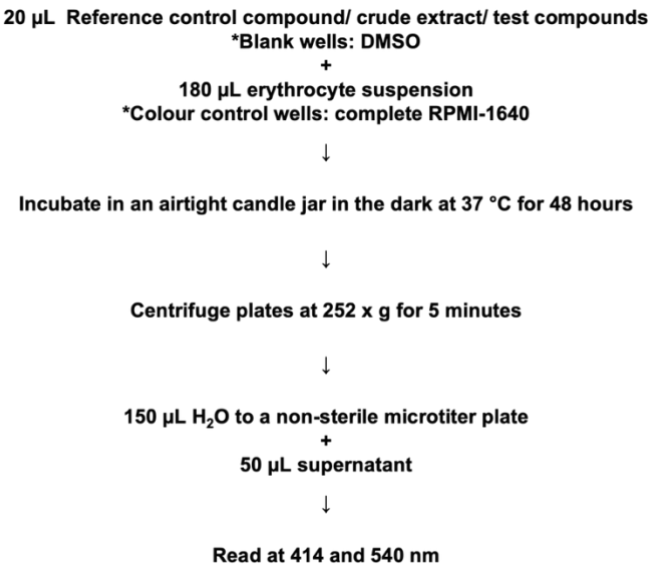


Figure 3.5: Red blood cell haemolysis assay methodology for a 96-well microtitre plate (Hayat et al., 2011).

$$\% \text{ Haemolysis} = \left(\frac{OD_{TC} - OD_{CC}}{OD_{100\%} - OD_B}\right) \times 100 \qquad \textbf{Equation 3.10}$$

Where $OD_{100\%}$ is the optical density of the 100% haemolysis control, OD_B is the optical density of the blank, OD_{TC} is the optical density of the test compound, and OD_{cc} is the optical density of the colour control.

3.5.2 *In vitro* cytotoxicity properties

To determine preliminary *in vitro* cytotoxicity effects of the crude extracts and isolated compounds, HepG2 cells derived from a human hepatocellular carcinoma (ATCC, 2024), along with K562 cells, a human lymphoblast cell line originally derived from a myeloid leukaemia (ATCC, 2023), were obtained from commercial companies.

3.5.2.1 Preparation of complete cell culture medium

Cell line specific complete cell culture medium was prepared for cell culture maintenance and the cytotoxicity tetrazolium assays. The incomplete culture medium consisted of prefiltered liquid DMEM, and Ham's F12 nutrient medium (Gibco®). Complete culture medium for cell culture maintenance and MTT assays was prepared by sterile filtering (0.2 µm filters; GVS™) heat inactivated FBS (v/v) into DMEM (4.5 g/L glucose) and Ham's F12 nutrient medium (1:1). The final complete culture medium was prepared a day prior to use specifically for cell line maintenance cytotoxicity tetrazolium assays as follows:

(a) K562 cell line: DMEM + Ham's F12 nutrient media (1:1) + 10% FBS.

(b) HepG2 line: DMEM + Ham's F12 nutrient medium (1:1) + 20% FBS + 1% sodium pyruvate + 1% MEM non-essential amino acids (100X).

3.5.2.2 *In vitro* maintenance of K562 and HepG2 cells

K562 and HepG2 cell lines were grown to 80% confluency in 25 cm^2 culture flasks in complete cell culture media. Cell morphology and growth was observed under a confocal microscope (20X magnification) and the medium changed every 48 to 72 hours or when the cells reach confluency. Upon 80% confluency, spent media was discarded and the cells washed with 3 mL PBS that was discarded. The cells were then trypsinised using 0.2% Trypsin-EDTA and incubated for 5-10 minutes at 37 °C in a 5% CO_2 humidified incubator until the cells detached. Culture flasks were gently tapped to detach any cells which remained further adherent. The trypsin-EDTA reaction was stopped by the addition of an equal volume of complete cell culture medium. The solution was decanted into a 50 mL centrifuge tube and centrifuged at 252 x g for 5 minutes on a Thermo Scientific Heraeus™ Megafuge™40 Centrifuge for 5 minutes. The supernatant was carefully discarded so not to disturb the cell pellet, which was then resuspended in 5 mL complete cell culture. The resuspended cells were counted by the Trypan blue exclusion assay for further experimental use (Section 3.5.2.3.1).

3.5.2.3 Cytotoxicity tetrazolium assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay is a colorimetric assay that gives a preliminary indication of cytotoxic or cytostatic activity. Nicotinamide adenine dinucleotide phosphate-dependent cellular oxidoreductase enzymes, under defined conditions, reflects the number of viable cells present. This is based in their ability to reduce the yellow tetrazolium MTT dye, into insoluble purple-formazan salts within living cells (Mosmann, 1983).

3.5.2.3.1 Cell counting via the trypan blue exclusion assay

The trypan blue exclusion assay was used to estimate the cell density of the cultures. The assay differentiates living cells from dead ones, by staining dead cells blue whereas the living cells remain unstained on the basis that their cell membranes remain intact and will therefore exclude the stain (Figure 3.6; Strober, 1997).

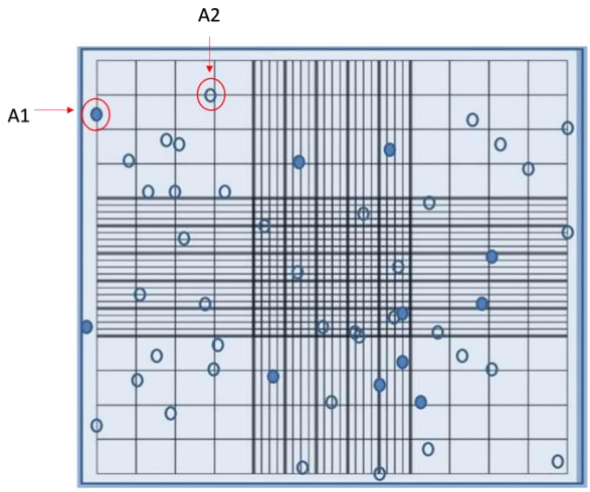


Figure 3.6: Haemocytometer quadrants, where - A1 indicates a dead cell and A2 indicates a living cell (LeGresley and McDermott, 2010).

A sample was prepared by combining 40 µL of trypan blue (0.4%, w/v) and 40 µL of cells suspended in complete cell culture media to obtain a 1:1 dilution (2 fold dilution). The cells that excluded the dye (live cells) were then counted using a Neubauer haemocytometer (Marienfeld, Lauda-Königshofen, Germany) under 10X eyepiece magnification with a compound light microscope (10X) (Olympus, Japan). In addition, the blue dead cells were counted to ensure there was an overall viability of 98% for the cells to be used in the experiment.

Cells were counted within 3-5 minutes of mixing with trypan blue to avoid errors in viability counts.

$$\text{Cell count} = \left(\frac{URQ+ULQ+LRQ+LLQ}{4}\right) \times 2 \times 10^4 \qquad \textbf{Equation 3.11}$$

Where URQ is the upper right quadrant on a haemocytometer, ULQ is the upper left quadrant, LRQ is the lower right quadrant, and LLQ is the lower left quadrant.

3.5.2.3.2 Preparation of reagents

The MTT solution was prepared by dissolving MTT in PBS (5 mg/mL) before being sterile filtered (0.2 µm filters; GVS™), covered in tinfoil, and stored at 4 °C until required.

3.5.2.3.3 Preparation of experimental plates

The cell suspensions (Section 2.4.1.3.2.1) were adjusted such that a density of $1x10^4$ cells/well in complete culture medium was seeded into a 96-well microtitre plate and incubated for 24 hours at 37 °C in a 5% CO_2 humidified incubator to allow for adherence.

3.5.2.3.4 Addition of test compounds and reagents to experimental plates

Following the attachment of cells to the well base surface, increasing concentrations of crude extracts (0.001 – 1000 µg/mL) were added to each well (Figure 3.7). Percentage cell viability was calculated using Equation 3.12.

$$\% \text{ Cell growth inhibition} = \left(\frac{(OD_{100\%} - OD_B) - (OD_{TC} - OD_{CC})}{OD_{100\%} - OD_B}\right) \times 100 \qquad \textbf{Equation 3.12}$$

Where $OD_{100\%}$ is the optical density of the 100% cell growth control, OD_B is the optical density of the blank, OD_{TC} is the optical density of the test compound, and OD_{cc} is the optical density of the colour control.

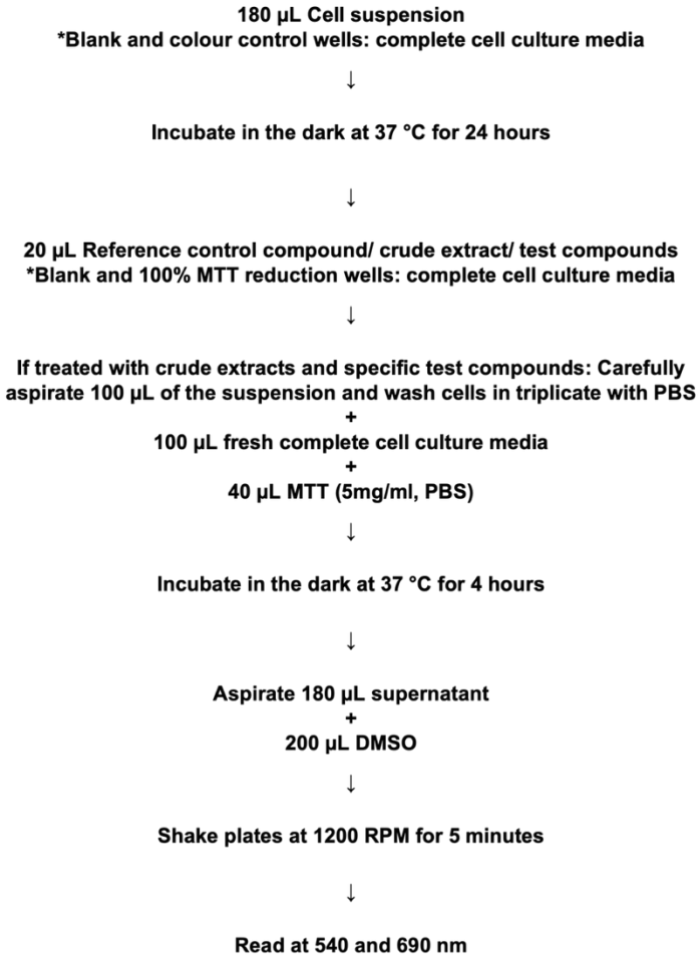


Figure 3.7: MTT assay methodology for a 96-well microtitre plate

3.5.3 *In silico* pharmacokinetic and toxicity properties

The absorption, distribution, metabolism, excretion, and toxicity (ADMET) in and through the human body are ADMET properties. The pharmacokinetic profile of a therapeutic molecule, represented by ADMET, is critical in determining pharmacodynamic activity in order to anticipate a drug candidates behaviour. Two online web tools: SwissADME (Daina *et al*., 2017) and Toxtree (Patlewicz *et al*., 2008) were employed. All compounds under investigation were converted into their canonical

Simplified Molecular Input Line Entry System (SMILES) structures and submitted to SwissADME and Toxtree. SwissADME was used to predict the physiochemical, pharmacokinetic, and drug-likeliness properties. Potential SMARTCyp-Cytochrome P450-Mediated Drug metabolism interactions of the compounds were predicted using Toxtree.

3.6 Data analysis and statistics

All assays were performed in triplicate, and the mean and standard deviation determined for each compound tested. Screening concentrations producing and effect greater than 60% were further investigated to determine their IC_{50} value as determined by non-linear regression (log sigmoid dose response, variable slope equation) using GraphPad Prism® version 5.0 (Intuitive software for science, San Diego, CA, USA). The students t-test was determined to compare inter- and intra-group differences using GraphPad Prism® version 5.0 (Intuitive software for science, San Diego, CA, USA). Differences were considered significant at $P<0.05$.

3.7 Ethics

Ethical clearance was obtained from the Human Research Ethics Committee at the University of the Witwatersrand. A waiver was granted to V. Govender to grow commercially available human HepG2 and K562 cells (W-CBP-220908-01, Appendix A.1), While waivers under Prof R. L. van Zyl were obtained to collect blood from human volunteers for *Plasmodium* parasites culture (M190579, (Appendix A.2)) and for biosafety clearance to culture *Plasmodium* parasites (20190404, (Appendix A.3)). Permission was also received.

CHAPTER 4 - RESULTS

The crude DCM:MeOH, acetone and aqueous extracts of *C. sinensis*, *S. aethiopicus*, *V. album*, and *W. salutaris* were screened for bioactive compounds to determine potential antiplasmodial, antioxidant, metal homeostatic, and cytotoxic activity. Major constituents potentially present in these crude extracts were also evaluated for antiplasmodial and cytotoxic activity. This chapter gives an overview of these findings.

4.1 Phytochemical analysis

4.1.1 Qualitative phytochemical analysis

The % extraction yield of crude extracts was the highest using aqueous solvents and generally decreased as solvent polarity decreased (aqueous > DCM:MeOH > acetone) (Table 4.1). Yield values recovered from plants materials during extraction ranged from 3.43% (*V. album* DCM:MeOH) to 33.99% (*C. sinensis* aqueous).

Table 4.1: Percentage yield for each solvent used during extraction processes for *C. sinensis*, *S. aethiopicus*, *V. album*, and *W. salutaris*.

Plant species	Plant part	Extract Yield (%)		
		DCM:MeOH	Acetone	Aqueous
C. sinensis	**Leaves**	5.81	1.57	33.99
S. aethiopicus	**Tubers**	4.40	5.30	22.23
V. album	**Leaves, Stems, & Berries**	3.43	4.07	11.51
W. salutaris	**Leaves**	6.43	7.30	19.47

Prepared crude extracts were subjected to phytochemical characterisation; qualitative, TPC, TFC, and HPLC-UV-Vis procedures were used to gauge a preliminary chemical profile of the extracts (Table 4.2). Qualitative phytochemical screening of the crude extracts of *C. sinensis*, *V. album*, *S. aethiopicus*, and *W. salutaris* are presented in Table 4.2.

Seven secondary metabolite families were investigated: alkaloids, anthraquinones, coumarins, flavonoids, phenols, tannins, and saponins. Of the seven families tested alkaloids, flavonoids, phenols and tannins appear to be the most abundantly present in the crude extracts, with saponins present to a lesser degree, and anthraquinones and coumarins were completely absent. The organic extract of *C. sinensis* appeared to have the highest amounts of secondary metabolites, closely followed by its aqueous extract, while the aqueous extract of *S. aethiopicus* had the least. Anthraquinones and coumarins were absent in all extracts.

Table 4.2: Qualitative phytochemical screening of crude extracts.

Crude Extracts	Secondary Metabolites						
	Alkaloids	Anthra-quionones	Coumarins	Flavonoids	Phenols	Tannins	Saponins
C. sinensis							
DCM:MeOH	+++	–	–	+++	+++	+++	+
acetone	+++	–	–	+++	+++	+++	–
aqueous	++	–	–	++	+++	+++	++
V. album							
DCM:MeOH	++	–	–	+	+++	+++	+
acetone	+++	–	–	+	+++	+++	–
aqueous	+++	–	–	+	+++	+++	+++
S. aethiopicus							
DCM:MeOH	–	–	–	+	+++	+++	+++
acetone	–	–	–	+	+++	+++	–
aqueous	–	–	–	+	+++	+++	–
W. salutaris							
DCM:MeOH	–	–	–	+++	+++	+++	+
acetone	–	–	–	+++	+++	+++	–
aqueous	–	–	–	+	+++	+++	++

(+): weakly present; (++): moderately; (+++): strongly present; (-): absent

4.1.2 Quantitative phytochemical analysis

4.1.2.1 Determination of TFC & TPC

At 100 µg/mL, the three *C. sinensis* crude extracts had the highest detectable amounts of phenolic constituents compared all the *S. aethiopicus*, *V. album*, and *W. salutaris* extracts, where its DCM:MeOH extract was the highest (34.82 ± 0.64 µg/GAE) while the *S. aethiopicus* aqueous extract had the lowest with 2.40 ± 1.18 (Figure 4.1). The *C. sinensis* DCM: MeOH and acetone extracts had the highest detectable amounts of flavonoid constituents (7.64 ± 1.12 and 4.71 ± 1.19 µg RE/g) while the *S. aethiopicus* aqueous extract had the least with no detectable constituents. TFC and TPC results are in line with the observations during initial qualitative analysis.

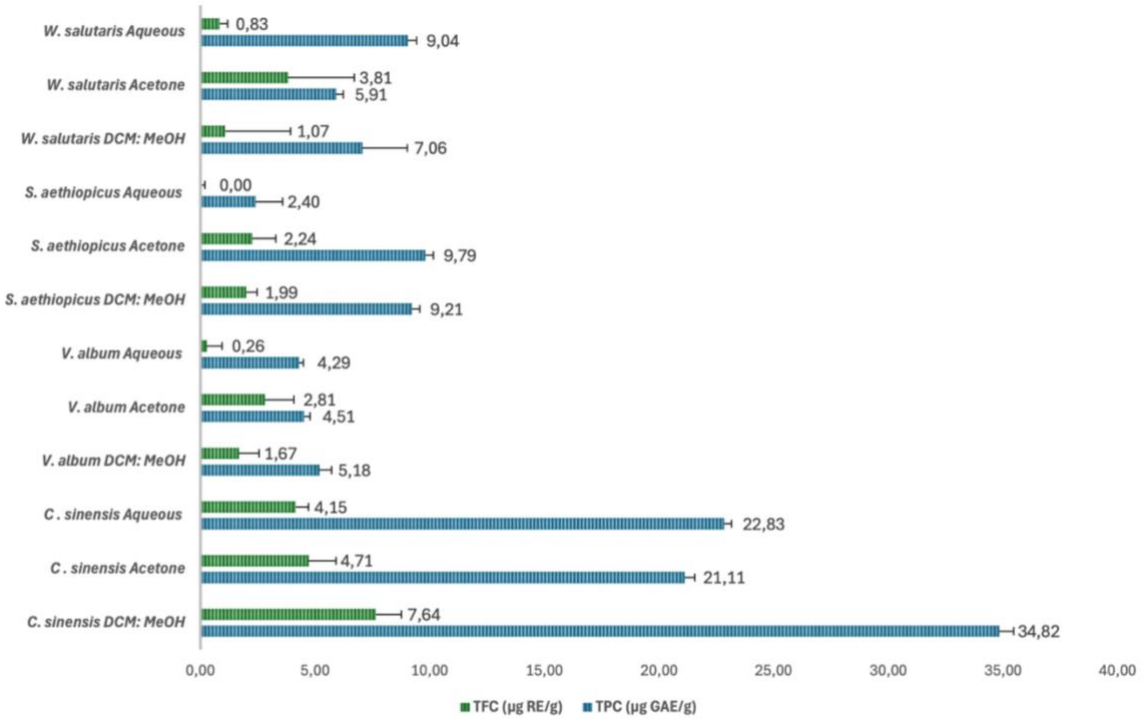


Figure 4.1: The TPC and TFC of aqueous and organic crude extracts.

4.1.2.1.2 Preliminary UV-Vis HPLC chemical profiling and quantitative estimation

Preliminary chemical profiling was done on all 12 crude extracts (Figure 4.2).

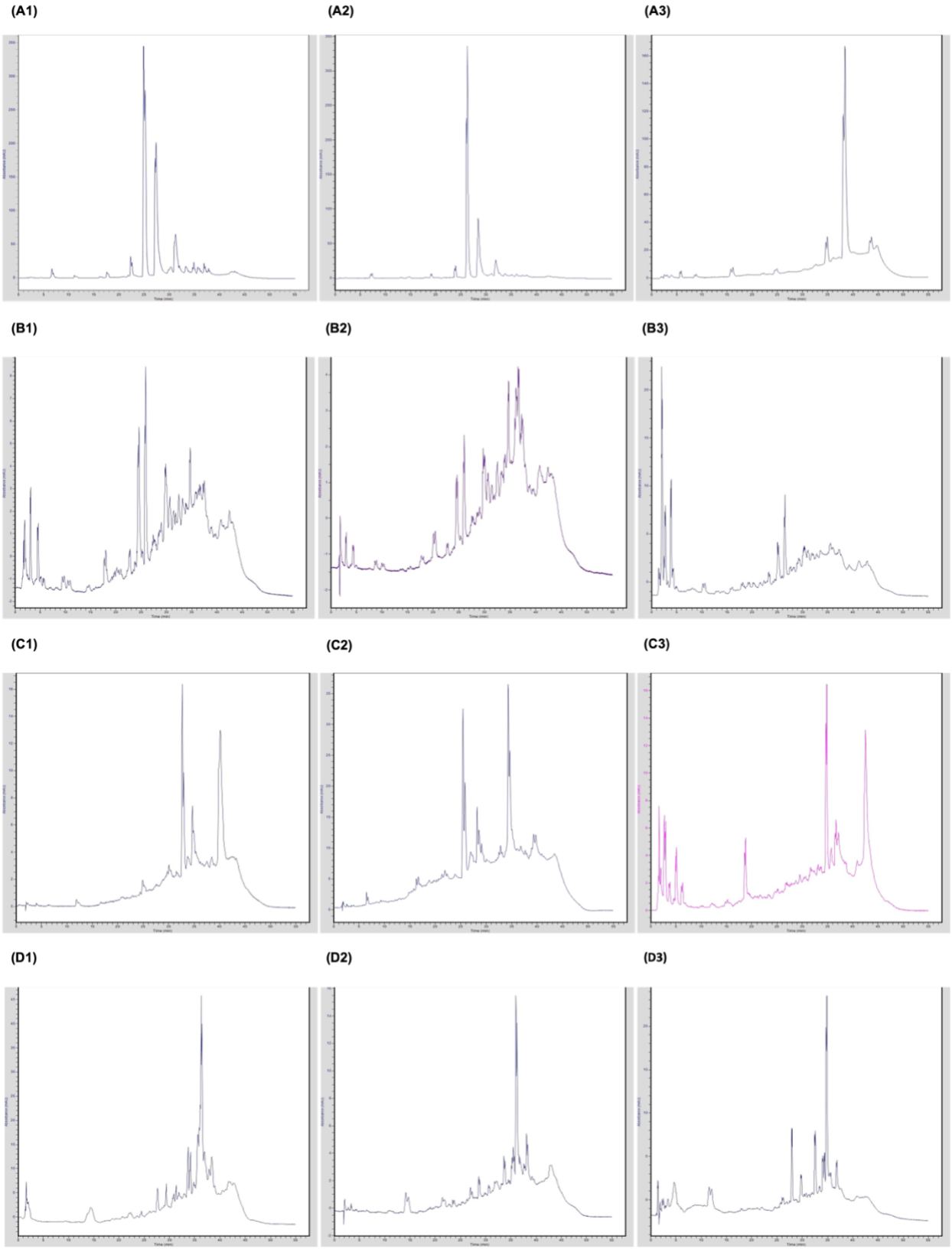
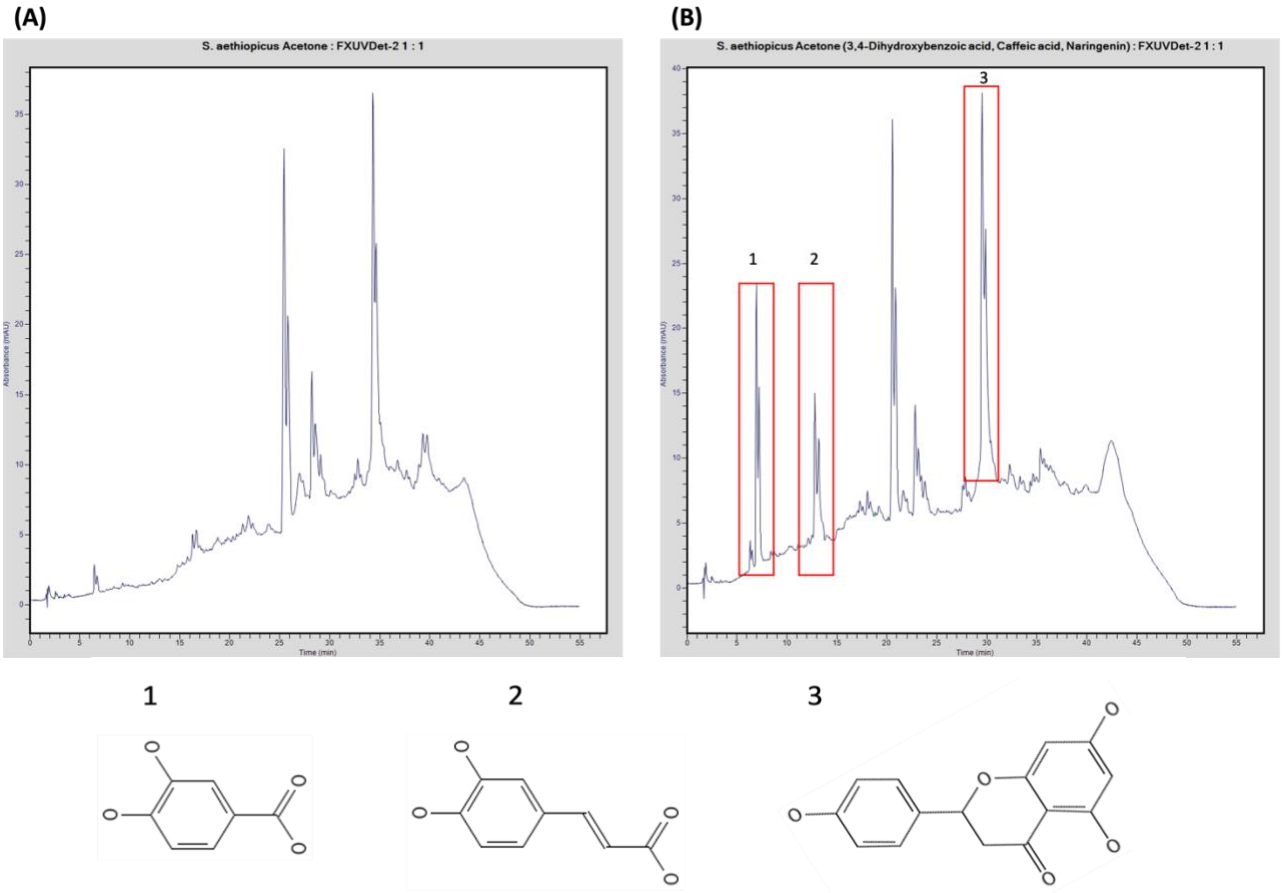


Figure 4.2: UV-Vis HPLC chromatograms of aqueous (A1, B1, C1, D1) , DCM:MeOH (A2, B2, C2, D2), and acetone (C1, C2, C3, C4) *C. sinensis* (A), *V. album* (B), *S. aethiopicus* (C), and *W. salutaris* (D) crude extracts.

Major peaks in the crude extracts ranged between 2 (A3) and 8 (B2). Following crude extract antiplasmodial IC_{50} value determination, the crude extract with the greatest antiplasmodial activity was selected for further HPLC quantitative estimation of predicted constituents (Appendix B). *S. aethiopicus* acetone, was selected for this purpose (Figure 4.3). The unspiked *S. aethiopicus* acetone extract indicated 3 major peaks. The spiked chromatogram peaks were found to have shifted an estimated 5 minutes earlier compared to the unspiked chromatogram, likely due to experiential conditions. Compounds 1, 2 and 3 eluted with increasing lipophilicity, with compound 3 being the most lipophilic.



Compound Number	Name	Retention time (min.)	Percentage present in extract (%)
1	3,4-Dihydroxybenzoic acid	P1: 7.99 P2: 8.33	P1: 0.03 P2: 0.03
2	Caffeic acid	P1: 13.85 P2: 14.17	P1: 0.02 P2: 0.02
3	Naringenin	P1: 34.15 P2: 34.72	P1: 0.21 P2: 0.27

Figure 4.3: HPLC-UV-Vis chromatograms showing absorbance over acquisition time of (A) *S. aethiopicus* acetone crude extract alone and (B) *S. aethiopicus* crude acetone extract spiked with 20 µg/mL of 3,4-dihydroxybenzoic acid (1), caffeic acid (2), and naringenin (3).

4.2 Antiplasmodial activity

The *in vitro* antiplasmodial activity was determined against the CQ-sensitive *P. falciparum* NF54 isolate, NF54, and a CQ-resistant *P. falciparum* FCR-3 isolate, using the methods described Section 3.4. The extracts and major constituents displayed variable activity in a dose dependent manner. Many however did not have promising activity (<60% *P. falciparum* inhibiting activity) and so further IC_{50} value investigations were not conducted for these compounds (Table 4.3).

Table 4.3: *In vitro* antiplasmodial and cytotoxic activity of selected aqueous and organic crude extracts expressed at 10 µg/mL on both CQ-sensitive and -resistant strains and HepG2.

Extract /Test compound	*P. falciparum* % growth		HepG2 % growth
	NF54	FCR-3	
***C. sinensis* aqueous**	101.96 ± 2.10	142.58 ± 3.15	105.76 ± 12.03
***C. sinensis* DCM:MeOH**	131.74 ± 2.60	91.78 ± 5.59	108.80 ± 1.89
C. sinensis acetone	118.02 ± 16.58	66.18 ± 0.20	105.81 ± .18
***S. aethiopicus* aqueous**	69.16 ± 17.72	40.71 ± 7.07	91.27 ± 3.90
***S. aethiopicus* DCM:MeOH**	16.48 ± 3.23	50.66 ± 6.18	98.87 ± 7.63
***S. aethiopicus* acetone**	14.83 ± 2.37	10.73 ± 9.03	125.70 ± 4.39
***V. album* aqueous**	49.29 ± 8.99	126.00 ± 12.06	109.05 ± 4.66
***V. album* DCM:MeOH**	156.17 ± 2.49	56.00 ± 8.06	116.56 ± 3.95
***V. album* acetone**	74.61 ± 2.94	25.34 ± 5.91	106.50 ± 4.52
***W. salutaris* aqueous**	126.95 ± 12.91	123.80 ± 2.92	111.21 ± .25
***W. salutaris* DCM:MeOH**	137.71 ± 1.77	34.76 ± 0.47	134.95 ± 7.99
***W. salutaris* acetone**	90.90 ± 11.61	66.87 ± 0.77	128.32 ± 4.46
Syringic acid	89.46 ± 5.04	76.93 ± 9.07	85.20 ± 2.03
(+)-Catechin hydrate	86.69 ± 1.27	81.92 ± 4.21	112.41 ± 2.11
Hesperetin	94.71 ± 3.30	99.11 ± 10.97	114.31 ± 0.76
Ferulic acid	79.76 ± 5.56	50.00 ± 18.58	95.65 ± 3.30
Kaempferol	79.17 ± 3.13	42.14 ± 1.65	78.35 ± 1.60
Polygodial	56.84 ± 4.43	50.00 ± 7.46	73.33 ± 2.58
Oleic acid	76.50 ± .61	100.62 ± 14.50	107.09 ± 3.00
Naringenin	117.62 ± 6.75	72.00 ± 7.10	108.54 ± 3.20
Caffeic acid	76.07 ± 2.18	67.89 ± 3.66	107.31 ± 9.07
Sinapic acid	84.95 ± 2.87	69.06 ± 2.14	100.70 ± 0.34
Ellagic acid	43.86 ± 8.90	47.44 ± 6.27	69.40 ± 3.49
(-)-Epicatechin	54.34 ± 6.72	93.75 ± 16.32	101.27 ± 2.36
4-Hydroxybenzoic acid	82.28 ± 5.28	77.64 ± 3.23	74.57 ± 4.18
(-)-EGC	88.86 ± 6.18	102.49 ± 0.48	106.50 ± 4.47
(-)-ECG	86.41 ± 4.12	91.99 ± 8.83	121.78 ± 3.98
3,4-Dihydroxybenzoic acid	74.03 ± 3.84	57.57 ± 4.39	74.41 ± 2.19
Quinine	0.20 ± 0.01	6.08 ± 4.03	95.94 ± 6.19
Dihydroartemisinin (DHA)	21.05 ± 3.79	0.00 ± 6.21	99.89 ± 5.74
CQ	179.47 ± 8.20	42.31 ± 3.79	96.44 ± 5.30
Verapamil	5.24 ± 4.91	62.12 ± 3.71	88.28 ± 5.93
Cisplatin	n.d.	n.d.	79.52 ± 3.18
Camptothecin	n.d.	n.d.	23.89 ± 3.79

Nine crude extracts and all 16 pure compounds inhibited >60% NF54 *P. falciparum* growth, while six crude extracts and all 16 pure compounds inhibited >60% FCR-3 *P. falciparum* growth and were subsequently selected for IC_{50} value determination. The IC_{50} values of selected crude extracts, pure compounds, and the positive control antimalarial drugs DHA, Q, and CQ, against both strains are summarised in Table 4.4.

Table 4.4: *In vitro* antiplasmodial and cytotoxic (IC_{50}) activity of selected aqueous and organic crude extracts.

Extract /Test compound	*P. falciparum* IC_{50} (µg/mL)		HepG2 IC_{50} (µg/mL)	Selectivity Index*
	NF54	FCR-3		
***C. sinensis* Aqueous**	87.00 ± 0.31	n.d.	n.d.	n.d.
***C. sinensis* DCM:MeOH**	28.66 ± 1.59	58.17 ± 7.24	n.d.	n.d.
***C. sinensis* Acetone**	21.82 ± 0.82	23.74 ± 2.25	n.d.	n.d.
***S. aethiopicus* Aqueous**	n.d.	n.d.	n.d.	n.d.
***S. aethiopicus* DCM:MeOH**	4.28 ± 0.67	4.39 ± 0.30	37.38 ± 4.82	8.46
***S. aethiopicus* Acetone**	2.35 ± 0.56	2.49 ± 0.02	35.70 ± 2.32	14.27
***V. album* Aqueous**	n.d.	n.d.	n.d.	n.d.
***V. album* DCM:MeOH**	49.15 ± 8.15	n.d.	n.d.	n.d.
***V. album* Acetone**	31.39 ± 1.48	n.d.	n.d.	n.d.
***W. salutaris* Aqueous**	n.d.	n.d.	n.d.	n.d.
***W. salutaris* DCM:MeOH**	12.51 ± 1.46	8.45 ± 1.70	45.56 ± 4.59	5.69
***W. salutaris* Acetone**	12.83 ± 2.59	8.14 ± 1.91	n.d.	n.d.
Syringic acid	37.42 ± 4.71	24.79 ± 2.82	n.d.	n.d.
(+)-Catechin hydrate	10.29 ± 2.21	9.77 ± 3.20	n.d.	n.d.
Hesperetin	22.64 ± 0.73	26.75 ± 3.27	n.d.	n.d.
Ferulic acid	39.66 ± 5.14	48.22 ± 5.69	n.d.	n.d.
Kaempferol	9.98 ± 0.70	22.30 ± 13.43	n.d.	n.d.
Polygodial	16.49 ± 2.21	24.58 ± 3.21	n.d.	n.d.
Oleic acid	25.18 ± 3.19	16.50 ± 1.45	n.d.	n.d.
Naringenin	35.17 ± 0.65	10.80 ± 2.87	n.d.	n.d.
Caffeic acid	59.11 ± 2.69	42.74 ± 1.41	n.d.	n.d.
Sinapic acid	24.97 ± 2.98	11.55 ± 1.58	n.d.	n.d.
Ellagic acid	0.46 ± 0.25	0.86 ± 0.54	n.d.	n.d.
(-)-Epicatechin	9.87 ± 2.52	13.32 ± 3.77	n.d.	n.d.
4-Hydroxybenzoic acid	16.03 ± 0.97	13.94 ± 1.27	n.d.	n.d.
(-)-EGC	18.55 ± 1.02	35.57 ± 8.92	n.d.	n.d.
(-)-ECG	3.69 ± 0.41	5.58 ± 2.61	n.d.	n.d.
3,4-Dihydroxybenzoic acid	8.12 ± 0.27	16.17 ± 1.26	n.d.	n.d.
Quinine	0.04 ± 0.01	0.03 ± 0.01	40.82 ± 2.81	1493.29
DHA	0.00092 ± 0.00	0.00027 ± 0.00	109.40 ± 3.93	410250.00
CQ	0.02 ± 0.00	0.14 ± 0.03	29.93 ± 1.85	208.79
Verapamil	7.80 ± 0.63	1.34 ± 0.31	50.40 ± 0.97	37.71
Cisplatin	n.d.	n.d.	15.56 ± 0.96	n.d.
Camptothecin	n.d.	n.d.	0.37 ± 0.05	n.d.

n.d. Not determined; * Selectivity Index is obtained from the ratio, HepG2 IC_{50}/NF54 IC_{50} for the NF54 strain and HepG2 IC_{50}/ FCR-3 IC_{50} for the FCR-3 strain, where a SI ≤10 was indicative of toxicity

All nine crude extracts and all 16 pure compounds inhibited the growth of intraerythrocytic *P. falciparum* in a dose-dependent manner. The *S. aethiopicus* acetone crude extract was the most active on both the NF54 and FCR-3 strains, yielding IC_{50} values of 2.35 ± 0.56 and 2.49 ± 0.02 µg/mL, respectively, followed by its DCM:MeOH extract, 4.28 ± 0.67 and 4.39 ± 0.30 µg/mL, respectively (Figures 4.4 A & B). The aqueous *C. sinensis* crude extract (IC_{50}: 87.00 ± 0.31 µg/mL) was the least active on the NF54 strain, while its DCM:MeOH extract (IC_{50}: 58.17 ± 7.24 µg/mL) was the least active on the FCR-3 strain.

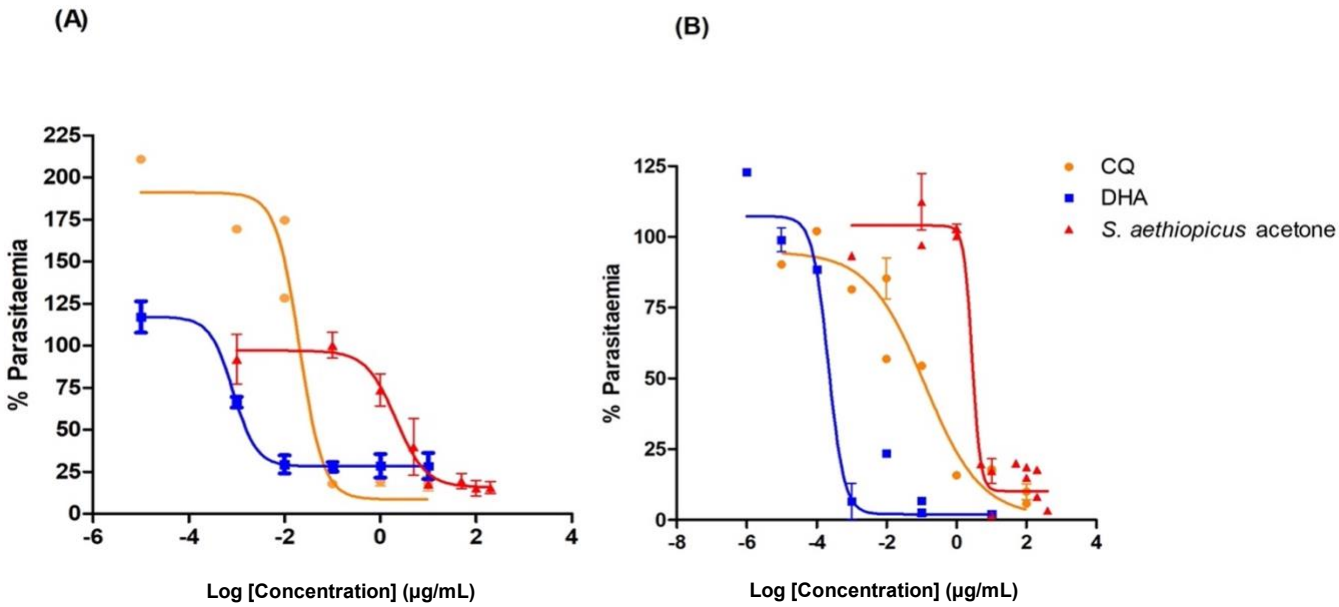


Figure 4.4: The log sigmoid dose response curve of the most active crude extract compared to CQ and DHA on (A) NF54 and (B) FCR-3 P. falciparum in vitro survival (n=3).

Overall the only significant differences in IC_{50} values of tested compounds on the NF54 strain compared to CQ were the *C. sinensis* aqueous crude extract (P<0.0001, 95% CI [-58.37, 14.76]), the *V. album* DCM:MeOH crude extract (P=0.0009, 95%CI [-85.70, 12.56]), syringic acid (P=0.0102, 95% CI [-70.11, 4.70]), ferulic acid (P=0.202, 95% [-76.21, 3.07]), and caffeic acid (P=0.0021, 95% CI [-74.35, 8.94]. On the FCR-3 strain, the only significant differences in IC_{50} values of tested compounds compared to CQ were the *C. sinensis* DCM:MeOH crude extract (P<0.0001, 95% CI [-70.48, - 45.58]), the *C. sinensis* acetone crude extract (P<0.0001, 95% CI [-36.05, -11]), syringic acid (P<0.0001, 95% CI [-37.10, -12.19]), hesperetin (P<0.0001, 95% CI [-39.06, -14.16], ferulic acid (P<0.0001, 95% CI [-60.53, -35.63]), kaempferol (P<0.0001, 95% CI [-45.54, -17.69]), polygodial (P<0.0001, 95% CI [-36.89, -11.98]), oleic acid (P=0.0013, 95% CI [-28.81, -3.90]), caffeic acid (P<0.0001, 95% CI [-55.05, -30.14]), (-)-epicatechin (P=0.0269, 95% CI [-25.63, -0.72]), 4-hydroxybenzoic acid (P=0.0154, 95% CI [-26.25, -1.34]), (-)-epigallocatechin (P<0.0001, 95% CI [-47.88, -22.97]), and (-)-epicatechin gallate (P=0.018, 95% CI [-28.48, -3.57]). Both crude extracts and pure compounds performed better on the NF54 strain compared to the FCR-3 strain, while syringic acid, ferulic acid, and caffeic acid performed worse on both strains.

Ellagic acid performed the best on both the NF54 and FCR-3 strains, while the predicted major constituents of the *S. aethiopicus* acetone crude extracts performed comparatively worse. 3,4-Dihydroxybenzoic acid had better activity on the NF54 strain (IC_{50}: 8.12 ± 0.27 µg/mL) while naringenin had better activity on the FCR-3 strain (IC_{50}: 10.80 ± 2.87 µg/mL). Caffeic acid the lowest activity of all three on both the NF54 and FCR-3 strains, yielding IC_{50} values of 59.11 ± 2.69 and 42.74 ± 1.41 µg/mL, respectively. Their IC_{50} values and those of all the *S. aethiopicus* acetone crude extract, CQ, and DHA are summarised in Table 4.5, along with the ratios of their IC_{50} values on the FCR-3 strain over their IC_{50} values on the NF54 strain.

Table 4.5: IC_{50} values of the best performing crude extract and its predicted major constituents.

Extract/compound	IC_{50} NF54 (µg/mL)	IC_{50} FCR-3 (µg/mL)	FCR-3/NF54 (Resistance Ratio)
***S. aethiopicus* acetone**	2.35 ± 0.56	2.49 ± 0.02	1.06
3,4-Dihydroxybenzoic acid	8.12 ± 0.27	16.17 ± 1.26	1.99
Caffeic acid	59.11 ± 2.69	42.74 ± 1.41	0.72
Naringenin	35.17 ± 0.65	10.80 ± 2.87	0.31
CQ	0.00092 ± 0.00	0.00027 ± 0.00	9.36
DHA	0.02 ± 0.00	0.14 ± 0.03	0.29

Crude extract with IC_{50} values <30 µg/mL are generally considered to have cytotoxic potential (Odira *et al*., 2022). Similarly, pure compound with IC_{50} values <100 µM may have cytotoxic potential (Černá, et al., 2024). According to these guidelines, the extracts *S. aethiopicus* DCM:MeOH, *S. aethiopicus acetone*, and *W. salutaris* DCM:MeOH, are unlikely to be cytotoxic against tested immortalised HepG2 cells (Table 4.4 and 4.5), while amongst the pure compounds, none are likely to have cytotoxic potential of note against immortalised HepG2 cells.

Compounds with higher SI values indicate more selectivity against *P. falciparum*, while compounds with SI values <2 suggests general cytotoxicity, rendering them unsuitable for further development as a drug candidate (Koch *et al*., 2005). Based on this criterion, *S. aethiopicus* DCM:MeOH, *S. aethiopicus* acetone, and *W. salutaris* DCM:MeOH, exhibited moderate *in vitro* antiplasmodial activity with SI values all >2, indicating selectivity against *P. falciparum* over HepG2 cells. Among the pure compounds, specific IC_{50} values would need to be determined to confirm selectivity, but it is likely that all the pure compounds are more selective towards *P. falciparum* on basis that none were selected for IC_{50} value determination on HepG2 cells due to lack of activity.

S. aethiopicus acetone was the most active crude extract on both *P. falciparum* strains, however comparison revealed no differences in effect of *S. aethiopicus* acetone on the NF54 *P. falciparum* stain compared to the FCR-3 strain (Figure 4.5). There was no difference between *S. aethiopicus* acetone and CQ and DHA within each strain.

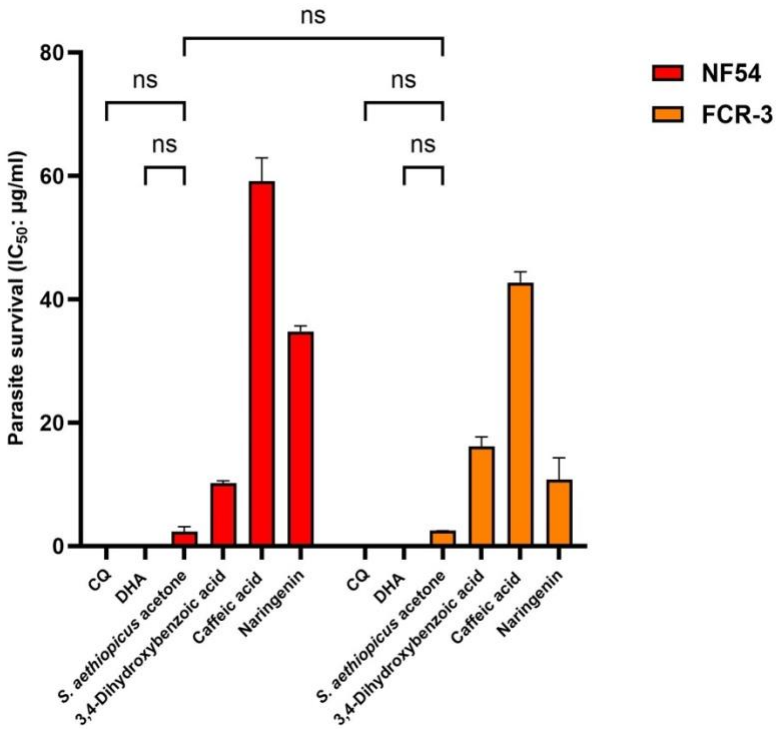


Figure 4.5: *The IC_{50} values of CQ, DHA, and* S. aethiopicus *acetone compared in NF54 P. falciparum compared to FCR-3 P. falciparum. (ns = not significant; P > 0.05).*

4.3 Oxidative Stress &, metal homeostasis & lipid peroxidation inhibiting activity

4.3.1 DPPH• scavenging activity

This assay indicated preliminary oxidative stress activity regarding the ability of the crude extracts to scavenge free radicals. During the screening process, all other crude extracts demonstrated <60% DPPH free radical scavenging activity and so were not considered for IC_{50} value determination (Appendix B.3.1). Except for, *C. sinensis* DCM:MeOH, *C. sinensis* acetone, *C. sinensis* aqueous, and *W. salutaris* acetone, which were subsequently further investigated for IC_{50} value determination (Figure 4.6 and Table 4.6). *C. sinensis* DCM:MeOH had the lowest IC_{50} value (9.97 ± 0.17 µg/mL) and was closest to the positive controls ascorbic acid and Trolox™ (2.21 ± 0.004 and 3.70 ± 0.37 µg/mL), while *W. salutaris* acetone had the highest IC_{50} value (55.24 ± 6.56 µg/mL).

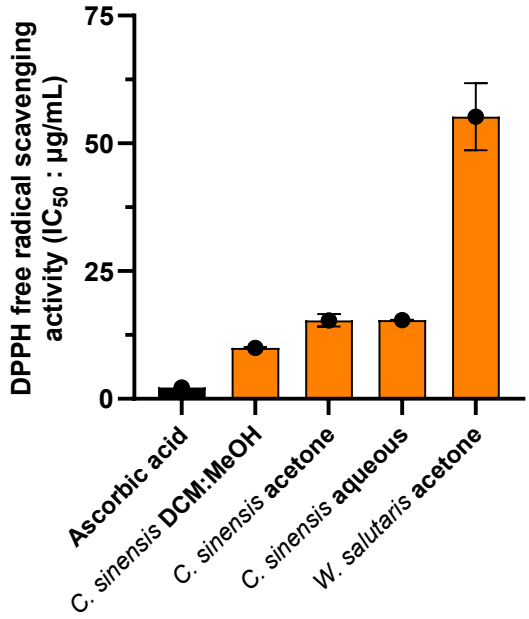


Figure 4.6: The % FRSCA of the most and least active crude extracts compared to ascorbic acid and Trolox™.

Table 4.6: The percentage DPPH free radical scavenging activity of selected crude extracts and controls.

Crude Extracts/Controls	DPPH free radical scavenging activity (IC_{50} : µg/mL)
***C. sinensis* aqueous**	15.40 ± 0.05
***C. sinensis* DCM:MeOH**	9.97 ± 0.17
***C. sinensis* acetone**	15.36 ± 1.22
***W. salutaris* acetone**	55.24 ± 6.56
Ascorbic acid	2.21 ± 0.0.004

4.3.2 Metal chelating and lipid peroxidation inhibiting activity

These assays indicated preliminary metal homeostatic activity regarding the ability of the crude extracts to chelate both iron and copper(II) ions, respectively. At the tested concentrations, the compounds demonstrated a low chelating ability of both iron and copper(II) ions, where all crude extracts demonstrated <60% iron and copper(II) chelation, between 7.24 ± 2.44% and 59.00 ± 11.03% and 0.00 and 14.83 ± 0.24%, respectively. As such, they were not considered for IC_{50} value determination (Appendix B.4.1 and B.4.2). The positive controls, EDTA for iron and TMB for copper(II), had IC_{50} values of 8.64 ± 0.38 and 3.30 ± 0.22 µg/mL, respectively.

The lipid peroxidation assay assessed if compounds were capable of inhibiting the lipid peroxidation process. At the tested concentrations, the crude extracts demonstrated a low ability to inhibit lipid peroxidation (<60%), between 0.00 and 57.12%, and so were not considered for IC_{50} value determination (Appendix B.5). The positive controls, ascorbic acid and Trolox™, had IC_{50} values of 12.24 ± 0.73 and 7.03 ± 0.47 µg/mL, respectively.

4.4 Toxicity

4.4.1 RBC haemolysis

Prior to the commencement of antiplasmodial activity testing, haemolytic activity of the crude extracts, pure compounds, and positive controls were assessed. Haemolysis ≥30% is considered to be haemolytic (Hayat *et al*., 2011). DHA had the highest degree of haemolysis (116.21 ± 0.21%) followed by polygodial (106.60 ± 0.60%), while syringic acid had the lowest rates of haemolysis (0.11 ± 0.37%). All crude extracts, other pure compounds, and positive controls demonstrated haemolytic effects ≤5% at 100 µg/mL (Appendix B.6). The positive control, Triton™ X-100, demonstrated 100% haemolysis at a 2% concentration.

4.4.2 MTT reducing activity

The reduction of the MTT tetrazolium compound to a soluble, purple formazan dye is controlled by NAD(P)H-dependent oxidoreductase enzymes in the mitochondria of viable cells (Mosmann, 1983). Compounds which inhibit this reduction gives a preliminary indication of whether that compound is toxic to cells. The effects of select crude extracts and pure compounds after screening, i.e. crude extracts and pure compounds which possessed ≥60% activity, were investigated using the K562 and HepG2 cell lines as liver and blood models are involved in the lifecycle of *P. falciparum* (Figure 1.1). All crude aqueous extracts appeared to not display toxicity towards the two cells lines at a concentration range of 0.1 – 100 µg/mL.

The *S. aethiopicus* DCM:MeOH and acetone, and *W. salutaris* DCM:MeOH and acetone crude extracts, displayed ≥60% MTT reducing activity and were subsequently selected for IC_{50} value determination on the K562 cell line, while *S. aethiopicus* DCM:MeOH and acetone extracts, and *W. salutaris* acetone extracts were selected on the HepG2 cell line. Of these, on the K562 cell line, *S. aethiopicus* acetone had the lowest IC_{50} value (8.72 ± 0.89 µg/mL) and *C. sinensis* DCM:MeOH the highest (46.02 ± 7.00 µg/mL), compared to the IC_{50} values camptothecin (0.40 ± 0.01 µg/mL) and cisplatin's (28.03 ± 0.48 µg/mL) IC_{50} values (Figure 4.7 B).

On the HepG2 cell line, *S. aethiopicus* acetone had the lowest (35.70 ± 2.32 µg/mL) and *W. salutaris* acetone the highest IC_{50} value (45.56 ± 4.59 µg/mL), compared to the IC_{50} values of camptothecin (0.37 ± 0.05 µg/mL) and cisplatin's (15.56 ± 0.96 µg/mL) IC_{50} values (Figure 4.7 A).

The IC_{50} values observed for selected crude extracts on both cell lines all ranged between 8.72 ± 0.89 and 46.02 ± 7.00 µg/mL, indicating low to moderate toxicity compared to standard positive controls camptothecin and cisplatin which had IC_{50} values of 0.04 ± 0.01 and 28.03 ± 0.41 µg/mL on the K562 cell line, and 0.37 ± 0.05 and 15.56 ± 0.96 µg/mL on the HepG2.

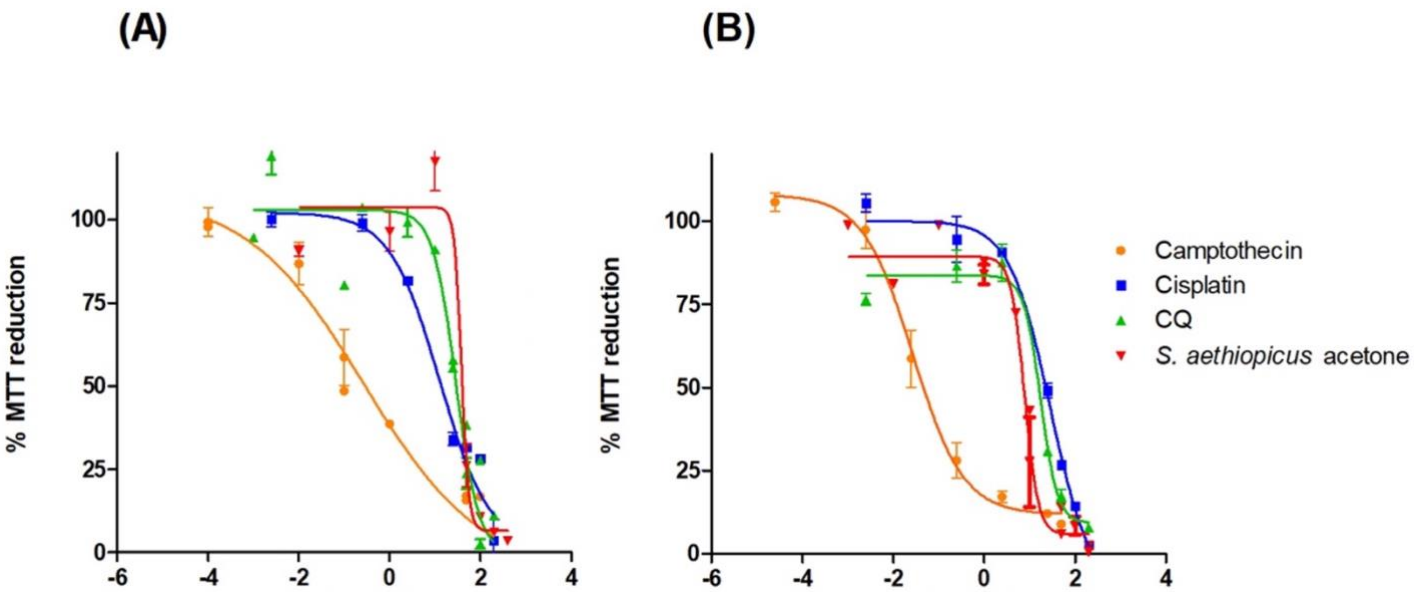


Figure 4.7: *The log sigmoid dose response curve of the % MTT reducing activity of the* S. aethiopicus *acetone crude extract compared to camptothecin, cisplatin, and CQ on HepG2 (A) K562 (B) cells (n=3).*

S. aethiopicus acetone was the most toxic crude extract on both cell lines, however comparison revealed a more substantial statistical difference in the effect of *S. aethiopicus* acetone on the HepG2 cell line compared to the K562 cell line (P<0.001, 95% CI [22.04, 31.93]) (Figure 4.8).

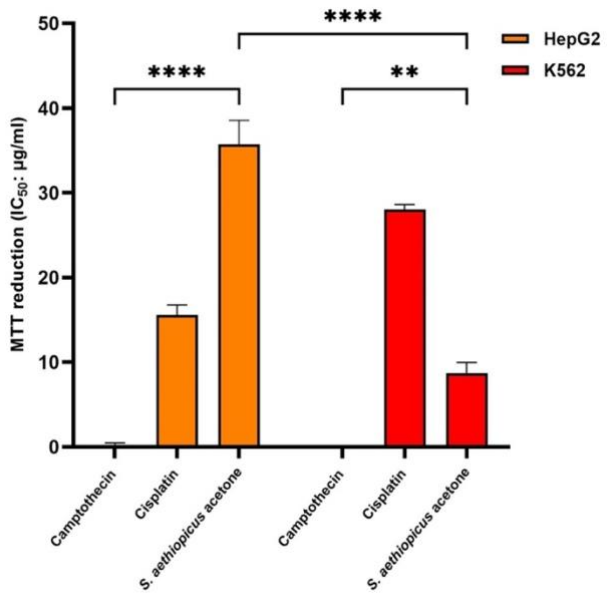


Figure 4.8: Camptothecin, cisplatin, and *S. aethiopicus* acetone IC_{50} values on HepG2 and K562 cells. (** = P ≤ 0.01, **** = P ≤ 0.0001).

There was also less of a difference in effect between camptothecin and *S. aethiopicus* acetone on the HepG2 cell line (P=0.018, 95% CI [-39.87, -31.02] compared to the K562 cell line (P=0.028, 95% CI [-14.09, -3.26]. *S. aethiopicus* acetone is more toxic to K562 cells compared to HepG2 cells.

4.5 *In silico* studies

The pure compound with the highest antiplasmodial activity as well as those present in the crude extract with the best performing antiplasmodial activity were selected for *in silico* analysis to provide further information about their drugability, pharmacokinetic and toxicological profile. Such assessments offer important insight to help predict *in vivo* performance.

4.5.1 Drug Likeliness and ADME pharmacokinetics

Drug-likeliness was investigated to assess if predicted pure compounds possess favourable ADME properties. Orally active drugs should obey Lipinski's rule of five, where their molecular weights are not > 500 g/mol, hydrogen bond acceptors are not >10, hydrogen bond doners are not >5, LogP values <5, and number of rotatable bonds not <10 (Lipinski, 1997). A violation of two or more rules predict that it is unlikely a drug will be orally active in humans. The data generated indicates that all three pure compounds detected in *S. aethiopicus* acetone as well as ellagic acid satisfy the criteria of Lipinski's rule of five, attesting to their drug-likeliness (Table 4.7).

Table 4.7: *In silico* predictions of predicted major constituent drug-likeliness and toxicity (Rydberg *et al*., 2010a; Rydberg *et al*., 2010b).

Compound	Drug-Likeliness		Toxicity
	Lipinski	Bioavailability score	Toxic hazard
3,4-Dihydroxybenzoic acid	Yes; 0 violations	0.56	Low (Class I)
Caffeic acid	Yes; 0 violations	0.56	Low (Class I)
Naringenin	Yes; 0 violations	0.55	Intermediate (Class II)
Ellagic acid	Yes; 0 violations	0.55	High (Class III)
Quinine	Yes; 0 violations	0.55	High (Class III)
CQ	Yes; 0 violations	0.55	High (Class III)
DHA	Yes; 0 violations	0.55	High (Class III)

Additionally good drug candidates display passive gastrointestinal absorption but neither blood brain barrier (BBB) penetration nor cytochrome P450 interactions. Gastrointestinal absorption and BBB

permeation as calculated by the Brain Or Intestinal EstimateD (BOILED)-Egg permeation model; which indicated both passive gastrointestinal absorption with no BBB permeability for any of the pure compounds (Figure 4.9 and Table 4.8).

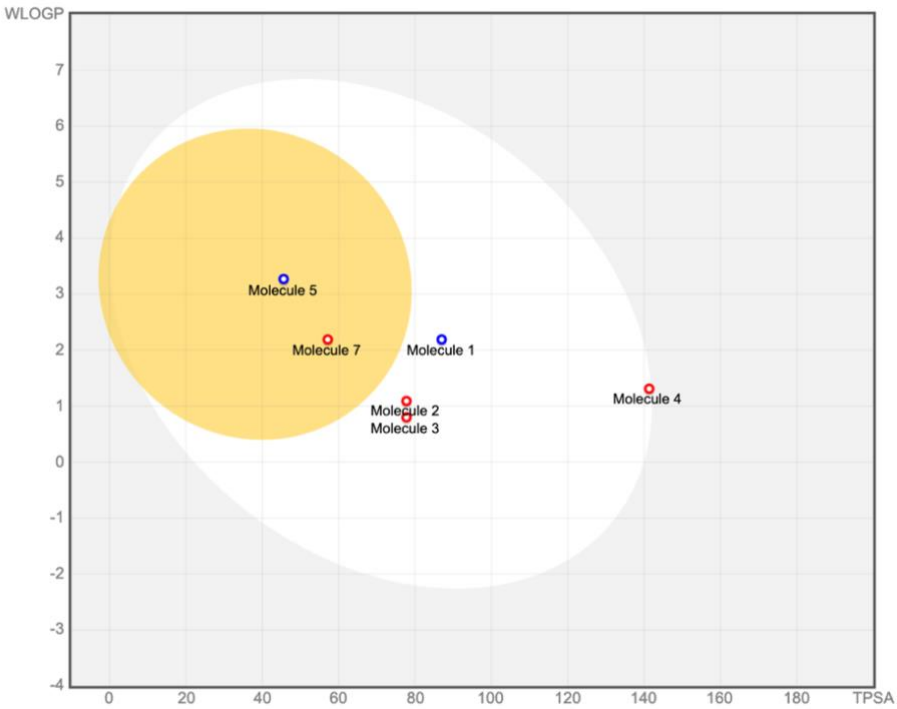


Figure 4.9: SWISS ADME prediction of bioavailability and distribution of select pure compounds and reference control compounds. Molecules 1, 2, 3, 4, 5, and 7 represent the compounds (1) naringenin, (2) caffeic acid, (3) 3,4-dihydroxybenzoic acid, (4) ellagic acid, (5) quinine, and (7) DHA . While molecule 6 (CQ) is out of range and does not appear on the graph. Points located in the white region are molecules predicted to be passively absorbed by the GI-tract while those found in the yellow are predicted to be able to passively penetrate through the BBB. Blue dots indicate molecules that are predicted to be extruded from the CNS by P-gp; red dots indicated molecules that are not**.**

Amongst the pure compounds, naringenin was the only non-P-gp substrate, suggesting reduced susceptibility to efflux. However, based on the both the BOILED-Egg model and bioavailability scores of the pure compounds (0.55-0.56), gastrointestinal (GI)-absorption may be incomplete compared to CQ, which although does not appear on the BOILED-Egg permeation model, has a bioavailability of 3.60.

Cytochrome P450, one of the most important drug-metabolising enzymes in the liver, has two main subtypes, CYP2D6 and CYP3A4. The inhibition of these two subtypes may lead to several adverse effects due to toxicity. With regards to metabolism, predication indicated that all four of the compounds of interest were non-inhibitors/non-substrates of CYP2D6, while only one (naringenin) was an inhibitor/substrate of CYP3A4 (Table 4.8). Additionally, both naringenin and ellagic acid were

predicted to be inhibitors/substrates of CYP1A2 (Table 4.8). Preliminary toxicity profiles of the compounds were also ascertained, where ellagic acid was predicted to be the most toxic compound, followed by naringenin, while 3,4-dihydroxybenzoic acid and caffeic acid were predicted to be low toxic hazards in comparison (Table 4.7).

Overall, ellagic acid, 3,4-dihydroxybenzoic acid, caffeic acid, and naringenin are predicted to have good aqueous and lipid solubility, no BBB permeability, good bioavailability scores, good absorption in GI-tract, and a good agreement with Lipinski's rule-of-five (Table 4.8).

Table 4.8: *In silico* predictions of predicted major constituent lipophilicity and pharmacokinetics.

Compound	Lipophilicity	Pharmacokinetics					
	Log P(O/W) MLOGP	GI	BBB	P-gp	CYP3A4	Add. CYP	Log Kp
3,4-Dihydroxybenzoic acid	0.40	High	No	No	No	No	-6.42
Caffeic acid	0.70	High	No	No	No	No	-6.58
Naringenin	0.71	High	No	Yes	Yes	CYP1A2	-6.17
Ellagic acid	0.14	High	No	No	No	CYP1A2	-7.36
Quinine	2.45	High	Yes	Yes	Yes	CYP2D6	-5.89
CQ	2.23	High	Yes	No	Yes	CYP2D6	-4.96
DHA	1.95	High	Yes	No	No	No	-5.90
Verapamil	3.16	High	No	Yes	Yes	CYP2D6	-6.04

4.5.2 Targets

Estimated molecular targets for constituent compounds (Figure 4.10 and Table 4.9) indicate a preferential inhibition of lyases for 3,4-dihydroxyebnzoic acid (80.0%), caffeic acid (66.7%), and naringenin (20.0%). CQ and DHA indicated a preferential inhibition of family A G protein coupled receptors (60.0%) and enzymes (33.3%) respectively. Furthermore, 3,4-dihydroxybenzoic acid had exhibited a 92.6% binding affinity for its top 5 lyase targets, caffeic acid exhibited a 72.9% binding probability for its top 5 lyase and oxidoreductase targets, while naringenin exhibited a 91.3% binding probability for its top 5 cytochrome P450, lyase, primary active transporter, and enzyme targets. Overall, naringenin may bind several more targets than either 3,4-dihydroxybenzoic acid and caffeic acid, as well as greater similarity with both CQ and DHA with regards to target overlap.

Of all the predicted drug targets in *H. sapiens* for these compounds, only multidrug resistance-associated protein 1 has also been found in *P. falciparum*. The minimal overlap between the *H. sapiens* targets in these compounds and *P. falciparum* targets, suggests mechanisms by which their antiplasmodial activities are exerted may be more specific to *P. falciparum*.

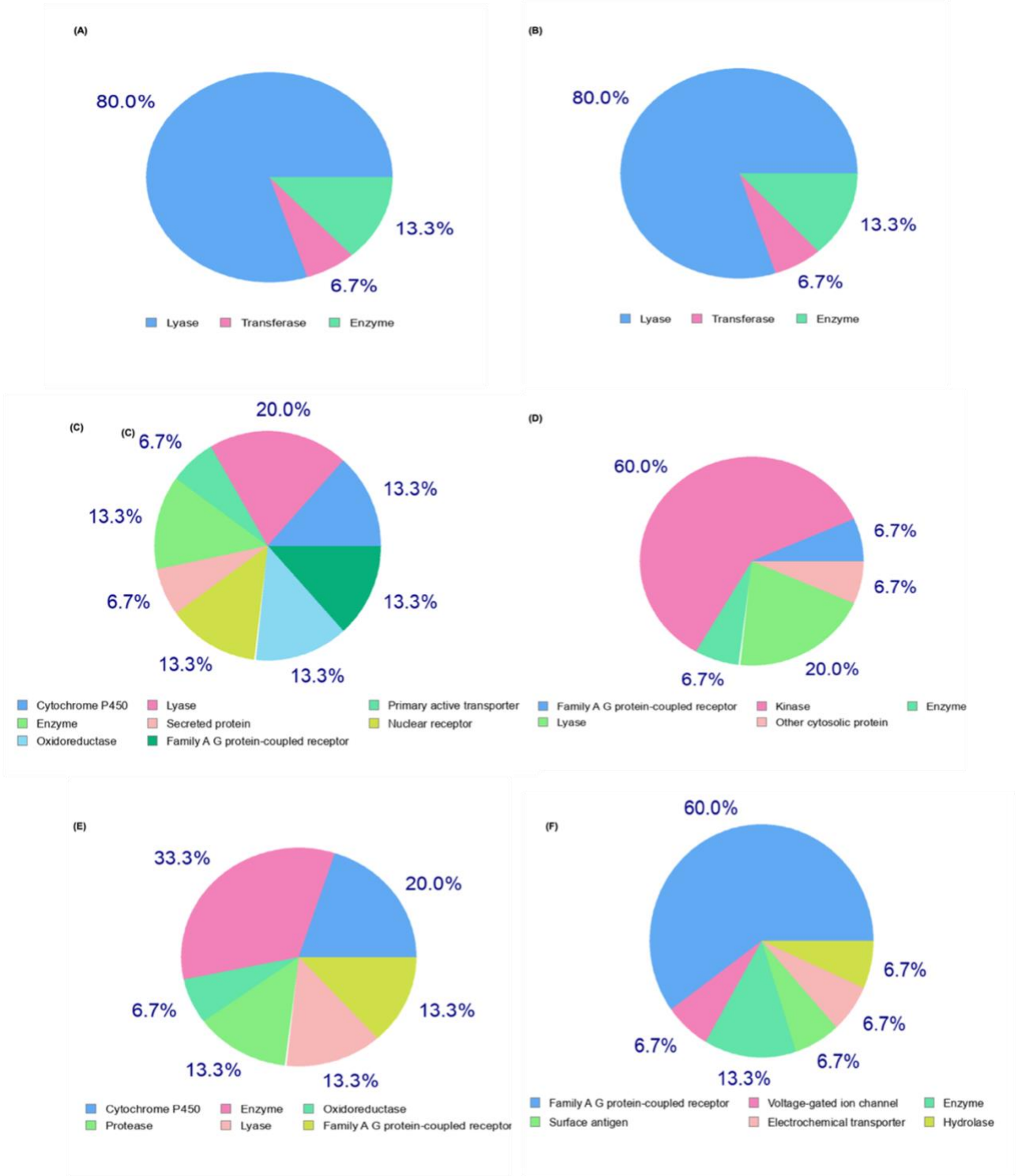


Figure 4.10: The top 15 molecular target class for *S. aethiopicus* acetone crude extracts identified constituents (A) 3,4-dihydroxybenzoic acid, (B) caffeic acid, (C) naringenin, and the standard antimalarials (E) DHA and (F) CQ. Potential targets can be employed as indicators to investigate bioactivity, possible side effects, and the possibility of repurposing therapeutically relevant compounds.

Table 4.9: The top 5 predicted pure compound and standard antimalarial drug targets in *Homo sapiens* and their overlap targets in *P. falciparum.*

Homo sapiens Target	Target class	Probability	*P. falciparum* target
3,4-Dihydroxybenzoic acid			
Carbonic anhydrase II	Lyase	0.93	No
Carbonic anhydrase VII	Lyase	0.93	No
Carbonic anhydrase I	Lyase	0.93	No
Carbonic anhydrase VI	Lyase	0.93	No
Carbonic anhydrase XII	Lyase	0.93	No
Caffeic acid			
Carbonic anhydrase II	Lyase	0.73	No
Arachidonate 5-lipoxygenase	Oxidoreductase	0.73	No
Carbonic anhydrase VII	Lyase	0.73	No
Carbonic anhydrase I	Lyase	0.73	No
Carbonic anhydrase VI	Lyase	0.73	No
Naringenin			
Cytochrome P450 19A1	Cytochrome P450	0.91	No
Carbonic anhydrase VII	Lyase	0.91	No
Multidrug resistance-associated protein 1	Primary active transporter	0.91	PF3D7 0112200
Estradiol 17-beta-dehydrogenase	Enzyme	0.91	No
Carbonic anhydrase XII	Lyase	0.91	No
Ellagic acid			
G-protein coupled receptor 35	Family A G protein-coupled receptor	1.00	No
Receptor protein-tyrosine kinase erbB-2	Kinase	1.00	No
Aldose reductase	Enzyme	1.00	No
Cyclin-dependent kinase 4/cyclin D1	Kinase	1.00	No
Platelet-derived growth factor receptor beta	Kinase	1.00	No
DHA			
Cytochrome P450 1A2	Cytochrome P450	0.10	No
Acyl coenzyme A: cholesterol acyltransferase	Enzyme	0.10	No
Carboxylesterase 2	Enzyme	0.10	No
Cyclooxygenase-2	Oxidoreductase	0.10	No
Matrix metalloproteinase 9	Protease	0.10	No
CQ			
Histamine H3 receptor	Family A G protein-coupled receptor	0.66	No
HERG	Voltage-gated ion channel	0.66	No
Histamine N-methyltransferase (by homology)	Enzyme	0.52	No
Quinone reductase 2	Enzyme	0.48	No
Prion protein	Surface antigen	0.48	No

CHAPTER 5 - DISCUSSION

Natural compounds that were discovered in plant species with activity against *P. falciparum* have significantly contributed to the development of novel drugs over the past century (Newman and Cragg, 2012). While traditional drug discovery concerns itself with a one-drug, one-target model; plant crude extracts are frequently characterised as having several biologically active compounds and targets (Sinha *et al*., 2023). Crude extracts may contain a mixture of several natural compounds with antiplasmodial potential and can in theory outperform single natural compounds or standard antimalarial drugs, which are single active compounds. In this study, the antiplasmodial activities of aqueous and organic *C. sinensis*, *V. album*, *S. aethiopicus*, and *W. salutaris* crude extracts, as well as several pure compounds which these species are reported to produce as secondary metabolites, were evaluated. Their efficacy was assessed against both a CQ-sensitive (NF54) and resistant (FCR-3) strain of *P. falciparum*. Prior research into the same species or genera has demonstrated varying degrees of antiplasmodial potential against both strains, thereby confirming the use of these medicinal plants in an antimalarial context (Audomkasok *et al*., 2014; Nyaba, 2017; Igoli *et al*., 2021; Akkawi *et al*., 2023).

Antiplasmodial activity of plant extracts can be classified as good (IC_{50} < 5 µg/mL), moderate (IC_{50} = 5−30 µg/mL), and inactive (IC_{50} > 30 µg/mL) (Abiodun *et al*., 2011). A pure compound can be considered good if it has an IC_{50} value ≤1 µg/mL and a selectivity index that is at least ten times more active on the parasite than it is on human cell lines (Fidock *et al*., 2004). Under this classification, both the *S. aethiopicus* DCM:MeOH and acetone crude extracts had good antiplasmodial potential on both strains, followed by the *W. salutaris* DCM:MeOH and acetone crude extracts, and the *C. sinensis* acetone crude extract had moderate activity on both strains. The *C. sinensis* DCM:MeOH crude extract had moderate activity on the NF54 strain but was inactive on the FCR-3 strain along with both the *V. album* DCM:MeOH and acetone crude extracts. Ellagic acid was the only pure compound with good antiplasmodial activity on both the NF54 and FCR-3 strains followed in order by (-)-ECG > 3,4-dihydroxybenzoic acid > (-)-epicatechin > kaempferol > (+)-catechin hydrate > 4-hydroxybenzoic acid > polygodial > (-)-EGC > hesperetin > sinapic acid > oleic acid > naringenin > syringic acid > ferulic acid > caffeic acid on the NF54 strain; and (-)-ECG > (+)-catechin hydrate > naringenin > sinapic acid > (-)-epicatechin > 4-hydroxybenzoic acid > 3,4-dihydroxybenzoic acid > oleic acid > kaempferol > polygodial > hesperetin > syringic acid > (-)-EGC > caffeic acid > ferulic acid on the FCR-3 strain (Table 4.4).

The collective antiplasmodial potential of any given crude extract is attributed to a combination of its constituents present, their concentrations, and their interactions with each other (Sinha *et al*., 2023). Additionally, structural characteristics enable natural product constituents to act on multiple and/ or

novel targets compared to synthetic antimalarial compounds (Tajuddeen and Van Heerden, 2019). Qualitative analysis of the *S. aethiopicus* acetone crude extract, the best performing crude extract, revealed an array of constituent classes where alkaloids, flavonoids, and phenols predominated. These chemical classes, have all demonstrated *in vitro* and *in vivo* antiplasmodial potential (Amoa Onguéné *et al*., 2013; Ntie-Kang *et al*., 2014; Abubakar *et al*., 2020; García *et al*., 2024). Both the NF54 and FCR-3 strains exposed to the *S. aethiopicus* acetone crude extract showed significant growth inhibition, 2.35 ± 0.56 µg/mL and 2.49 ± 0.02 µg/mL respectively, comparable to the effects produced by CQ and artemisinin ($P<0.05$) (Figure 4.8). Similarly, *S. aethiopicus* crude extracts have demonstrated comparable activity against *P. falciparum* (Igoli *et al*. 2021). Additionally, studies investigating the chemistry of similar *S. aethiopicus* crude extracts, have observed the presence of compounds not assessed in this study but which possess antiplasmodial potential (Holzapfel *et al*., 2002; Viljoen *et al*., 2002; Igoli *et al*., 2021). For example, siphonochilone, a sesquiterpenoid has been postulated to be the major compound present in *S. aethiopicus* (Holzapfel *et al*., 2002; Viljoen *et al*., 2002), while 3-diacetoxy-7-(3″,4″-dihydroxy-5″-methoxyphenyl)-1-(4′-hydroxy-3′-methoxyphenyl)-5-heptene, a diarylheptanoid, and the flavanols 3,4′,7-trimethyl kaempferol and 3,7-dimethoxyquercetin, have been detected in *S. aethiopicus* crude extracts with antiplasmodial potential (Igoli *et al*., 2021). Igoli *et al*. (2021) also tested the antiplasmodial activity of a diarylheptanoid isolated form *S. aethiopicus* in *P. falciparum* which inhibited parasite growth by 69-74%, similar to CQ, without producing cytotoxic effects on HEK 293 cells or significant haemolytic effects. Antiplasmodial activity of similar linear diarylheptanoids have been observed *in vitro*, e.g. curcumin for example, has been reported to inhibit histone acetylation, generate reactive oxygen species, and stop *P. falciparum* microtubule formation, mechanisms, which similar to *S. aethiopicus* diarylheptanoids may exert (Cui *et al*., 2007; Chakrabarti *et al*., 2013; Saxena *et al*., 2016). This highlights that the antiplasmodial effects observed in the *S. aethiopicus* acetone crude extract is likely attributable to the action of one of its bioactive metabolites or the combined action of several of them not directly tested in this study. This is further corroborated by the quantitative TFC and TPC tests performed which revealed only a moderate amount of flavonoid and phenolic compounds in *S. aethiopicus* crude extracts, making it unlikely that constituents from these classes were responsible for the observed antiplasmodial activity. Studies which investigated the toxicity of *S. aethiopicus* crude extracts and its pure compounds, demonstrated low cytotoxic potential against several models of mammalian cells (Steenkamp *et al*., 2005; Lategan *et al*., 2009; Igoli *et al*., 2021; Fuentes-Ríos *et al*., 2024). Indicating, in agreement with this study, that *S. aethiopicus* crude extracts are more selective for *P. falciparum* parasites.

Quantitative analysis, detected 3,4-dihydroxybenzoic acid, naringenin, and caffeic acid in the crude extract (Figure 4.3), which all demonstrated some degree of growth inhibitory activity against both *P. falciparum* strains. Of these 3,4-dihdryoxybenzoic acid had the best activity on the NF54 strain (IC_{50}: 8.12 ± 0.27 µg/mL), naringenin on the FCR-3 strain (IC_{50}: 10.80 ± 2.87 µg/mL), while caffeic

acid performed comparatively worse on both, 59.11 ± 2.69 µg/mL and 42.74 ± 1.41 µg/mL, respectively. Ellagic acid, which has previously been reported in *C. sinensis* crude extracts but not *V. album*; *W. salutaris* or *S. aethiopicus* crude extracts (Yang and Tomás-Barberán, 2018), performed best of all on both *P. falciparum* strains, 0.46 ± 0.25 µg/mL and 0.86 ± 0.54 µg/mL, respectively, with no significant difference between them ($P > 0.05$). 3,4-Dihydroxybenzoic acid, and ellagic acid have previously demonstrated a strong potential for *P. falciparum* growth inhibition with limited toxicity both (Pradines *et al*., 2002; Degotte *et al*., 2021). Similar antiplasmodial activity has been observed in the other pure compounds assessed in this study but not all, where current information on the topic is lacking. Compounds were selected for testing based on known likelihood of presence in crude extracts, cost and accessibility, so it should be noted that antiplasmodial activity observed in the crude may also be due compounds which might not be currently known or were not tested due to cost or inaccessibility.

Ellagic acid is a polyphenolic compound found in several plant species and has been reported to possess diverse biological activities (Reddy *et al*., 2007; Soh *et al*., 2009; Aldulaimi *et al*. 2017; Ríos *et al*., 2018). Present freely or in complex compounds (ellagitannins), its constituted by a hydrophilic moiety, 4 hydroxyls, and a lipophilic moiety, it contains 2 hydrocarbon rings that render it the capability to act as both an acceptor and donor of electrons. This property enables ellagic acid to act as a preventative and potent antioxidant, with the capability of cellular membrane and entering the parasite. Consequently, the same structure diminishes its oral bioavailability (Reddy *et al*., 2007; Soh *et al*., 2009; Ríos *et al*., 2018). In the context of malaria, ellagic acid has frequently been observed to possess a high degree of activity against the parasite (Banzouzi *et al*., 2002; Reddy *et al*., 2007; Soh *et al*., 2009). Ellagic acid has also indicated curative and prophylactic effects *in vivo*, Soh *et al*. (2009) demonstrated that mice infected with *P. vinckei petteri* and treated with ellagic acid at 1, 50, and 100 mg/kg/day intraperitoneally in a 4-day suppressive test, achieved a full inhibition of parasite growth, with no recrudescence over the next 60 days. Ellagic acid demonstrated antiplasmodial activity through various mechanisms and this is still a matter of discussion. One argument postulated that ellagic acid functions similarly to the quinolines by preventing the formation of haemozoin (Ríos *et al*., 2018; Herraiz *et al*., 2019). Its flat molecular structure endows it with a dispersed electron density which allows the formation of a π-π complex, while others proposed the inhibition of enzymes responsible for the digestion of haemoglobin, like plasmepsin, or via iron chelation by forming coordination bonds with iron present in haem (Dell'Agli *et al*, 2003). Ellagic acid may even function by altering *P. falciparum's* food vacuole pH by inhibiting the regulation of proton pumps by acidification of the membrane or as a useful bioflavonoid for alleviating severe malaria pathogenesis by reducing cytokine storms and oxidative stress (Ríos *et al*., 2018). A key mechanism is ellagic acid's ability to dissociate at physiological pH, creating a proton gradient that disrupts membrane potential and calcium signalling (Vattem and Shetty, 2005). In *P. falciparum* species, calcium signalling is crucial both to parasite survival and invasion of new host cells (Brochet and

Billker, 2016). The disruption of membrane potential and calcium signalling can impair the function of essential ion pumps, sequester critical ions, and inactivate membrane proteins involved in nutrient uptake (Vattem and Shetty, 2005).

Compounds with antimalarial potential must show good activity against resistant strains of *P. falciparum*. This is because random non-synonymous mutations occur at a frequency of around 1-5 per 10^9 for blood-stage parasites (Duffy *et al*., 2021). In severe malaria, parasitaemia can be as high as 1.9 x 10^6 parasite/µL, increasing the statistical risk of mutant selection (Duffy *et al*., 2021). Similarly, gene amplifications that improve survival will be more often selected at higher parasitaemia under drug pressure (Duffy *et al*., 2021). Although drug resistance usually manifests as a loss of efficacy during the erythrocytic cycle, ART-R manifests as delayed parasite clearance and represents a serious threat to malaria control, particularly in Africa (Björkman *et al*., 2024; Dhorda *et al*., 2024; Ishengoma *et al*., 2024; Rosenthal *et al*., 2024). In this study, ellagic acid demonstrated an IC_{50} value of 0.46 ± 0.25 µg/mL against the NF54 strain and 0.86 ± 0.54 µg/mL against the FCR-3 strain. Previous studies have also reported on ellagic acid's activity on several drug-resistant *P. falciparum* strains, including F32, Dd2, FcB1, W2, and FcM29 (Soh *et al*., 2009; Turrini *et al.*, 2019) These findings suggest that drug resistant *P. falciparum* strains may have greater susceptibility towards ellagic acid. Prior research has also demonstrated ellagic acid's synergistic interactions when combined with CQ, atovaquone, mefloquine, artesunate, and artemisinin *in vitro* (Soh *et al*., 2009; Beshbishy *et al*., 2019; Ishola *et al*., 2024). Ellagic acid may therefore potentially work well as a partner drug in the context of ACTs to clear remaining parasites post artemisinin administration. Ultimately, ellagic acid demonstrates a broad range of activity and high selectivity to both sensitive and resistant *P. falciparum* strains, but its low bioavailability is major hinderance to its practical clinical use as antimalarial. As bioavailability depends on several factors such as the quantity absorbed by the intestinal epithelium, drug solubility, gastrointestinal pH, and gastrointestinal transit; ellagic acid derivates with more favourable pharmacokinetics may give an increased bioavailability. Alternatively, ellagic acid could be reformulated in a dosage form of nanoparticles or liposomes to aid in overcoming its low bioavailability.

Naringenin and caffeic acid, like the other polyphenols assessed in this study, possess broad biological activity (Gupta *et al*., 2014; Liu *et al*., 2016; Mulvihill *et al*., 2016; Nyane *et al*., 2017; Langland *et al*., 2018; Merlani *et al*., 2019; Salehi et al., 2019; Shen *et al*., 2020; Zaremba-Czogalla *et al*., 2020). While reports on the antiplasmodial potential of naringenin is scarce, this study observed greater potential on the resistant *P. falciparum* strain ($p<0.05$), further evidenced by a low resistance ratio (0.31). Greater activity towards a resistant *P. falciparum* strain makes naringenin a good potential candidate to assess in combination with CQ. Reports on the antiplasmodial potential of caffeic acid indicate limited antiplasmodial potential as observed in this study (Alson *et al*., 2018; Sidoryk *et al*., 2023). However, Sidoryk *et al*. (2023) observed that although caffeic acid displayed

limited antiplasmodial (>200 µM), minor changes to the structure of caffeic acid's chemical backbone produced derivatives which displayed significantly better antiplasmodial activity against *Plasmodium* strains compared to caffeic acid. Furthermore, several of the caffeic acid derivates tested in study were more effective against CQ-resistant strains, which is particularly desirable given the propensity of *P. falciparum* to develop resistance (Chinappi *et al*., 2010; Wicht *et al*., 2020). Assessing if structural changes potentiate the antiplasmodial potential of compounds with limited activity may also therefore open another avenue of exploration.

The *W. salutaris* DCM:MeOH and acetone crude extracts performed second only to the *S. aethiopicus* extracts on both the NF54 and FCR-3 strains, 12.51 ± 1.46 and 8.45 ± 1.70 µg/mL, and 12.83 ± 2.59 and 8.14 ± 1.91 µg/mL, respectively, likewise under screening conditions its aqueous crude extracts did not perform well enough to warrant IC_{50} value determination. Similar antiplasmodial potential of *W. salutaris* against *P. falciparum* has been observed but studies are scarce. Nyaba *et al*. (2018) observed that a DCM stem bark extract and the pure compound, isomukaadial acetate, isolated from the extract had antiplasmodial potential against a *P. falciparum* CQ-sensitive strain, IC_{50} values of 0.01 µg/mL and 0.44 µg/mL, respectively. The differences in antiplasmodial potential is likely due to the fact that the crude extracts in this study were made using leaf *W. salutaris* plant material as opposed to stem bark, indicating differences in either the constituents present or their concentrations. Although a previous study which tested differenced in *W. salutaris* leaf and stem bark crude extracts found no significant difference, so the variation could be as a result of still unknown constituents (Zschocke *et al*., 2000). Additional *in vivo* studies on *P. berghei* in Sprague-Dawley rats, demonstrated that *W. salutaris* reduced the parasitaemia percentage compared to CQ, and indicated protective effects on survival (Nyaba *et al*., 2018). Several studies have also evaluated the antiplasmodial potential of *W. ugandensis* and *W. stuhlmannii,* which share a similar chemical profile to *W. salutaris* (Muthaura *et al*., 2007; Nanyingi *et al*., 2010; Were *et al*., 2010). Polygodial, the pure compound predicted to be present in the *W. salutaris* crude extracts had moderate activity of 16.49 ± 2.21 and 24.58 ± 3.21 µg/mL on the NF54 and FCR-3 strains respectively, which were higher than the observed activity in both the DCM:MeOH and acetone crude extract. The antiplasmodial activity of *W. salutaris* DCM:MeOH and acetone crude extracts is potentiated in part by another or multiple other constituents.

The *C. sinensis* crude extracts had limited antiplasmodial potential where its acetone extract performed the best on both strains compared to the aqueous crude extracts which was only tested on the NF54 strain but performed comparatively worse. The pure compounds predicted to be present in the extracts performed significantly better compared to their crude extracts, (-)-ECG performed the best, followed by (+)-catechin hydrate, and (-)-EGC which performed worst of all. It should however be noted that the acetone crude extract performed better compared to the (-)-EGC on the FCR-3 strain. This follows what has previously been observed in other *in vitro* studies, where *C.*

sinensis crude extracts appear to have limited antiplasmodial potential compared to its pure compounds (Dormeyer *et al*., 2006; Sannella *et al*., 2007; Hellmann *et al*., 2010; Patil *et al*., 2011; Thipubon *et al*., 2015). A potential explanation for difference in activity between extracts and pure compounds might be the difference in their antioxidant potential. *Plasmodium* is highly susceptible to oxidative stress. Therefore, elevation of oxidative stress subsequently leads to a redox imbalance ultimately affecting *Plasmodium* survival during the intraerythrocytic stages (Goyal *et al*., 2012). Extracts which contain several constituents which confer greater antioxidant activity compared to pure compounds, may as a result decrease antiplasmodial potential of extracts. The *in vivo* activity of *C. sinensis* extracts and pure compounds are still unclear. According to some studies, they have limited potential to attenuate parasitaemia while others show a curative effect (Oderinde *et al*., 2019; Akinwunmi *et al*., 2021). To date, no consensus has been reach on this matter.

The DCM:MeOH and acetone *V. album* crude extracts had the least antiplasmodial potential, where only the DCM:MeOH and acetone extracts was considered to be test on the NF54 strain (Table 4.4 and 4.5). *In vitro* and *in vivo* studies of the effects of *V. album* on *P. falciparum* is a neglected area, with the majority focused on its anticancer potential (Adzhiakhmetova *et al*., 2024; Yosri *et al*., 2024).

Polyphenols, are a broad class of plant metabolites to which all the pure compounds assessed in this study belong. Phenolic compounds are broadly defined by their molecular structure, with one or more aromatic rings, without nitrogen, originated from a plants shikimate and/or acetate metabolic pathways (Subhan *et al*., 2024). When a hydroxyl group is associated to the aromatic ring, and subsequently repeated, the compound is referred to as a polyphenol and can be broadly divided into flavonoid and non-flavonoid phenolic acid polyphenols Subhan *et al*., 2024). Flavonoids and phenolic acids are some of the most abundant and frequently occurring classes of polyphenols (Ahn-Jarvis *et al*., 2019; Subhan *et al*., 2024). Flavonoid skeletons consists of two phenyl rings A and B and a heterocyclic pyrone ring C (Ahn-Jarvis *et al*., 2019; Subhan *et al*., 2024). Variations in ring C substitutions result in several classes of flavonoids, including flavanols (or catechins), flavones, flavonols, flavaonones, and anthocyanidins (Ahn-Jarvis *et al*., 2019; Ahn-Jarvis *et al*., 2019). Phenolic acid skeletons consist of a benzene ring with a carboxyl group attached, where two classes, benzoic acid derivatives (C6-C1) and cinnamic acid derivatives (C6-C3), are generally distinguished. Compounds which belong to these classes are renowned for their multifaceted biological potential (Al-Hazzani and Alshatwi, 2011; Monga *et al*., 2012; Sonoda *et al*., 2014; Devi *et al*., 2015; Wang *et al*., 2015b), particularly their antioxidant and anti-inflammatory activity (Yilma *et al*., 2013). Therefore, although several of the pure compounds assessed in this study lack the superior antiplasmodial potential observed in ellagic acid, they along with the *C. sinensis* and *V. album* crude extracts which also lacked good antiplasmodial potential, may have potential use to treat malaria in a secondary capacity. For example, oxidative stress is key feature of malaria infections as a result of the reactive oxygen species generated by parasite dissemination throughout the intraerythrocytic cycle and the

host immune system activity, leading to cell membrane injury of both infected and uninfected erythrocytes (Schwarzer *et al*., 2015; Tripathy and Roy, 2015; Omoregie and Pal, 2016; Vasconcellos *et al*., 2016). Likewise, inflammation is also a key factor in the malaria disease process (Obeagu *et al*., 2024). Therefore, crude extract and pure compounds which mediate inflammation processes may have a positive effect on treating the disease, irrespective of any direct antiplasmodial potential. *S. aethiopicus* organic extracts have demonstrated inhibitory activity of COX enzyme prostaglandin synthesis (McGaw *et al*., 1997; Lindsey *et al*., 1998). Prostaglandins are essential drivers and modulators of inflammation, a process which can have a major influence on the pathogenesis of malaria (Obeagu, 2024). This anti-inflammatory activity of *S. aethiopicus* can further contribute antimalarial activity by aiding in the host immune response to control and eliminate parasites (Lindsey *et al*., 1998; Zschocke *et al*., 2000; Obeagu, 2024). Likewise, multiple studies have reported on the *in vitro* and *in vivo* anti-inflammatory activity of ellagic acid, naringenin, and caffeic acid (Mulvihill *et al*., 2010; Yoshida *et al*., 2013; Jin *et al*., 2017; Jia *et al*., 2018). All polyphenol pure compounds in addition to any antiplasmodial potential they may possess are able to inhibit oxidative stress, reactive oxygen species formation, lipid peroxidation, and inflammation (Al-Numair *et al*., 2015; Suchal *et al*., 2016; Somsak *et al*., 2018; Chanda and Juvekar, 2019). Additionally, they may have further potential in combination with standard antimalarial drugs, for example several crude extracts and pure compounds have been found to synergise CQ irrespective of any intrinsic *in vitro* or *in vivo* antiplasmodial potential (Iwalokun, 2008; Lehane and Saliba, 2008; Hafid *et al*. 2014; Abdulah *et al*., 2017; Somsak *et al*., 2018; Ibraheem *et al*., 2019; Ibraheem *et al*., 2020). However, as with ellagic acid, many of these compounds have several pharmacokinetic limitations which require address before they could realistically be used clinically (Choi *et al*., 2022). For example may have limited absorption through the oral route due to low solubility in water and significant first-pass metabolism which would restrict optimal systemic concentrations (Joshi *et al*., 2018). They can serve as substrates for P-glycoprotein, be rapidly metabolised in the liver, and have short half-lives, which all serve to limit their therapeutic potential (Joshi *et al*., 2018).

Proposed antiplasmodial mechanisms of polyphenols involve the damaging structures or disrupting processes critical to parasite survival and proliferation and include antioxidant activity, intercalation with parasite DNA, suppression of parasite fatty acids and protein production, increased erythrocyte oxidation, immunomodulation, parasite invasion inhibition, inhibition of parasite falcipain-2 and plasmepsin II enzymes, inhibition of hat shock proteins, or other mechanisms not yet elucidated (MacRae *et al*., 2013; Jin *et al*., 2014; MacRae *et al*., 2014; Hamilton *et al*., 2017; Zininga and Shonhai, 2019; Sauer *et al*., 2023)

The *S. aethiopicus* acetone extract in this study had superior *in vitro P. falciparum* growth inhibitory activity compared to the other eleven aqueous and organic extracts, and showed no significant

difference in growth inhibitory activity compared to CQ and artemisinin against both the CQ-sensitive and resistant strains ($P<0.05$).

CHAPTER 6 – CONCLUSION, RECOMMENDATIONS, & LIMITATIONS

6.1. Conclusion

The primary aim of this study was to investigate the antimalarial potential and toxicity of aqueous and organic crude extracts of the plant species *C. sinensis*, *V. album*, S. *aethiopicus*, and *W. salutaris*. The antimalarial potential and toxicity of sixteen pure compounds which have previously been reported in these at least one of these plant species were also considered. Secondary to this, the chemical profiles of the crude extracts and the pharmacokinetic characteristics of their pure compounds were clarified.

The results indicated that of the fifteen crude extracts assed for antimalarial activity on the CQ-sensitive NF54 strain and CQ-resistance FCR-3 strains, the *S. aethiopicus* acetone crude extract had the greatest antiplasmodial potential on both stains, followed by the *W. salutaris* DCM:MeOH and acetone crude extracts. Likewise, ellagic had the greatest antiplasmodial activity on both strains compared to the other 15 pure compounds followed by (-)-ECG, The *C. sinensis* and *V. album* crude extracts and caffeic acid lowest antiplasmodial activity comparatively, and are unlikely to be useful in treating *P. falciparum* infections in a direct capacity. However, their vast antioxidant and anti-inflammatory activity may have potential use in treating malaria pathogenesis. All twelve of the crude extracts and pure compounds had limited cytotoxic activity on erythrocytes, K562, or HepG2 models. The results seems to indicate that ellagic acid, *S. aethiopicus* acetone crude extract, (-)-ECG and, *W. salutaris* DCM:MeOH and acetone crude extracts are more selective for *P. falciparum* parasites than mammalian cells. However, these findings are only preliminary.

6.2. Recommendations

The accumulated preliminary data for the *S. aethiopicus* acetone crude extracts encourages further study that should focus on establishing a more complete chemical profile via activity-guided fractionation, focusing on compounds which are likely to exert the antiplasmodial activity observed in the *S. aethiopicus* acetone crude extract in this study. Additionally, determining the causal relationships between such compounds and *P. falciparum* targets as well as validating their activity *in vivo* will be necessary to determine the clinical potential in humans. Further verification of cytotoxicity via fluorometric quantification of RNA and DNA is also warranted. Finally, the potential synergistic and antagonistic interactions between crude extracts and pure compounds in combination with standard antimalarial drugs in important in the context of treating malaria where drug combinations are first-line treatment. Overall, S. *aethiopicus* and *W. salutaris*, as well as ellagic acid and (-)-ECG have good potential for use in developing novel antimalarials and antimalarial combinations in the future.

6.3. Limitations

The study was primarily limited due to temporal and financial restraints. As a result, the chemical profiles of the crude extracts were unable to be fully characterised which prevented further experimentation with regards to the pure compounds assessed for antiplasmodial potential. Likewise, combinations to assess synergistic and antagonistic interactions with CQ were not conducted. Another major limitation of study is the inherent nature of *in vitro* experimentation. Although *in vitro* models offer valuable insights into biological processes, they cannot accurately reflect or replicate the complexity of *in vivo* activity, which should be confirmed in addition to preliminary *in vitro* tests.

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Appendix A. Ethics

A.1: Human cell waiver

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Innovation)

TO: Ms V Govender
School: Therapeutic Sciences
Department: Pharmacy and Pharmacology
Division: Pharmacology
Medical School
University
E-mail: 1645329@students.wits.ac.za

CC: Supervisor: Professor R van Zyl <Robyn.vanZyl@wits.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Mr Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 08/09/2022

REF: *R14/49*

PROTOCOL NO: W-CBP-220908-01 (This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)

PROJECT TITLE: *The chemotherapeutic and P-gp inhibitory effects of selected phytomedicines*

Please find attached the Ethics Waiver Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/ClearScan/Waiver.wps

A.2: Ethic clearance (blood from human volunteers): approved under supervisor, Prof R. van Zyl HREC approval.

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Professor R van Zyl

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M190579

NAME: (Principal Investigator)	Professor R van Zyl
DEPARTMENT:	School of Therapeutic Sciences Department of Pharmacy and Pharmacology Medical School University
PROJECT TITLE:	The chemotherapeutic properties of novel synthetic and natural compounds
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	Renewal of M140669
SUPERVISOR:	Not applicable
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2019/06/10

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. I **agree to submit a yearly progress report.** When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **May** and will therefore reports and re-certification will be due early in the month of **May** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature — Date: 01/07/2019

PLEASE QUOTE THE CLEARANCE CERTIFICATE NUMBER IN ALL ENQUIRIES

A.3: Biosafety clearance: approved under supervisor, Prof R. van Zyl HREC approval.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE (IBC)
(R 14/16)

CLEARANCE CERTIFICATE **PROTOCOL NUMBER: 20190404**

BRIEF DESCRIPTION OF APPLICATION:

The chemotherapeutic properties of novel synthetic and natural compounds

APPLICANT: Prof Robyn Van Zyl

SUPERVISOR: N/A

SCHOOL/DEPARTMENT: Pharmacology

DATE CONSIDERED: 24 February 2025

EXPIRY DATE: 07 March 2030

DECISION OF COMMITTEE: Approved unconditionally

1. This clearance certificate expires on 07 March 2030 and may be renewed on application
2. An annual report must be provided on the anniversary date of this certificate, for as long as the project continues
3. Notification of any proposed modifications must be submitted on the attached form
4. Unreported changes to the application may invalidate the clearance given by the IBC

DATE: 07 March 2025

CHAIRPERSON: 
(Professor J Larkin)

DECLARATION OF APPLICANT:

To be completed and emailed to mmatshepo.taunyane@wits.ac.za and HREC-Medical.ResearchOffice@wits.ac.za.

1. I have read, understood and accepted the approval conditions above
2. I agree to submit a yearly progress report to the Committee and to submit an interim report on the form provided, in the event of any significant unforeseen event, *e.g.* suspension of a drug trial, temporary closure or relocation of my laboratory, etc
3. I note that the University Safety Officer, or his/her representative, may at any reasonable time inspect my laboratory or trial site to ensure compliance with current Health and Safety legislation. I undertake to offer my full co-operation in any such inspection.
4. I have read, understood and will comply with the *recommended standard operating procedures for the handling of biohazardous materials* posted at http://web.wits.ac.za/Academic/Research/Biosafety.htm
5. I declare (delete as appropriate) that:
    a. I have all the approvals required by statute or regulation and by the funding agencies supporting this work, or
    b. that I will not begin work until such approvals are obtained

Signed: V. Govender **Date:** 23/03/2025

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B. Results

B.1: TFC and TPC standard curves of controls

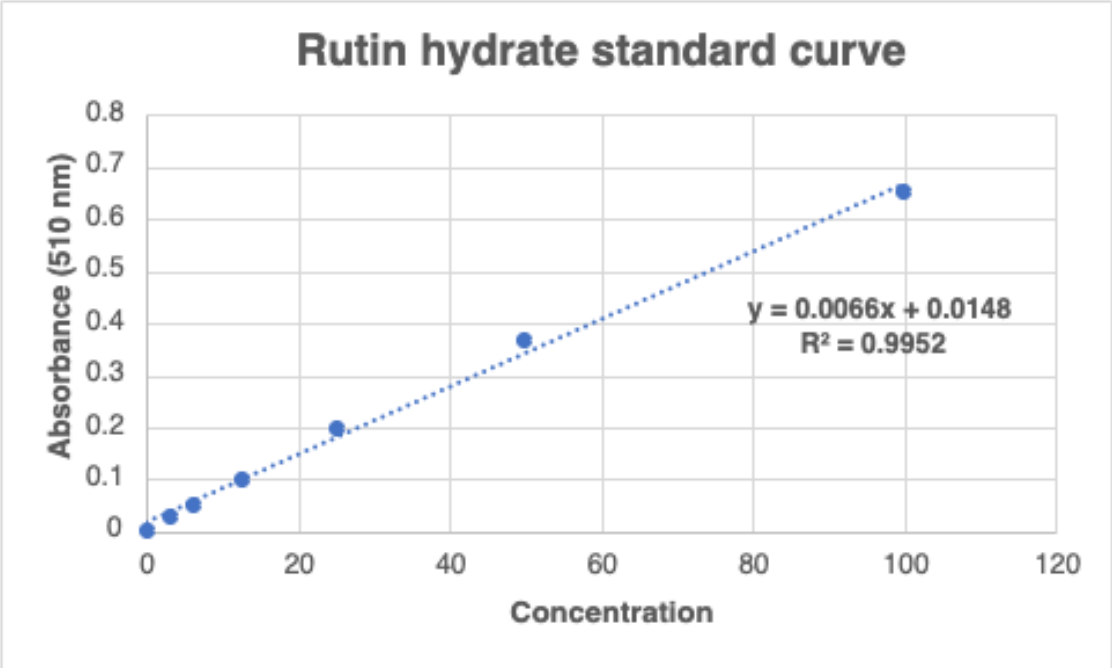

Figure B.1.1: The linear regression curve of rutin hydrate used to determine the TFC of the crude extracts.

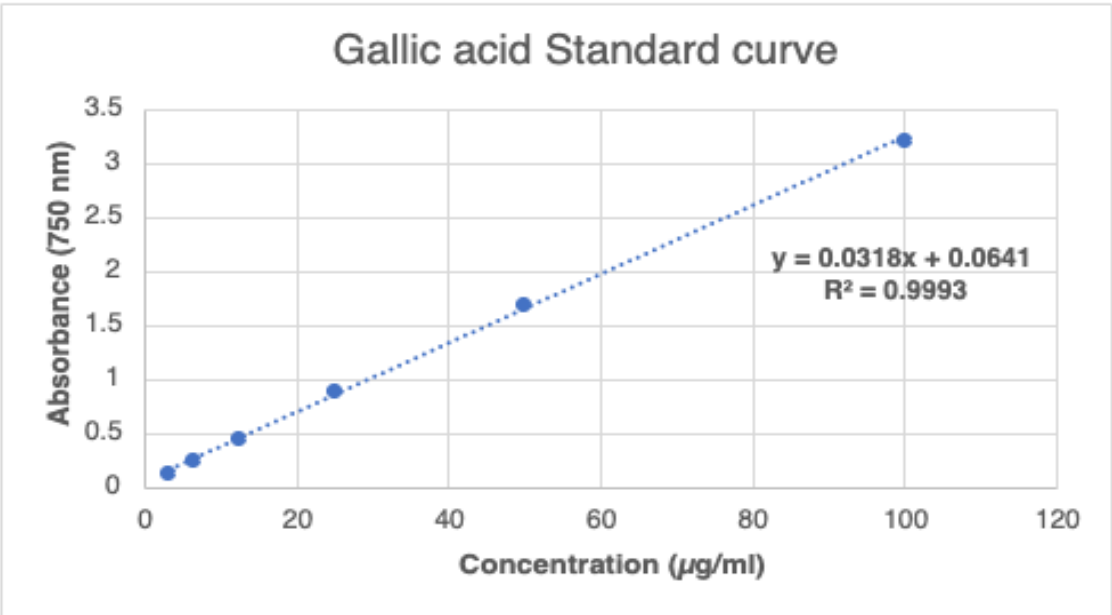

Figure B.1.2: The linear regression curve of gallic acid used to determine the TPC of the crude extracts.

B.2: HPLC pure compound chromatograms

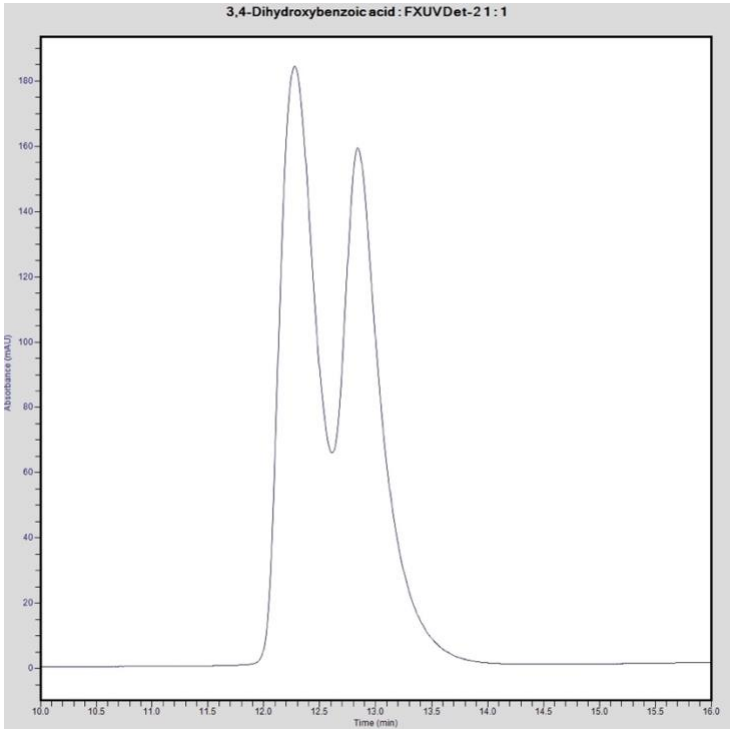

Figure B.2.1: Chromatogram of 3,4-dihydroxybenzoic acid.

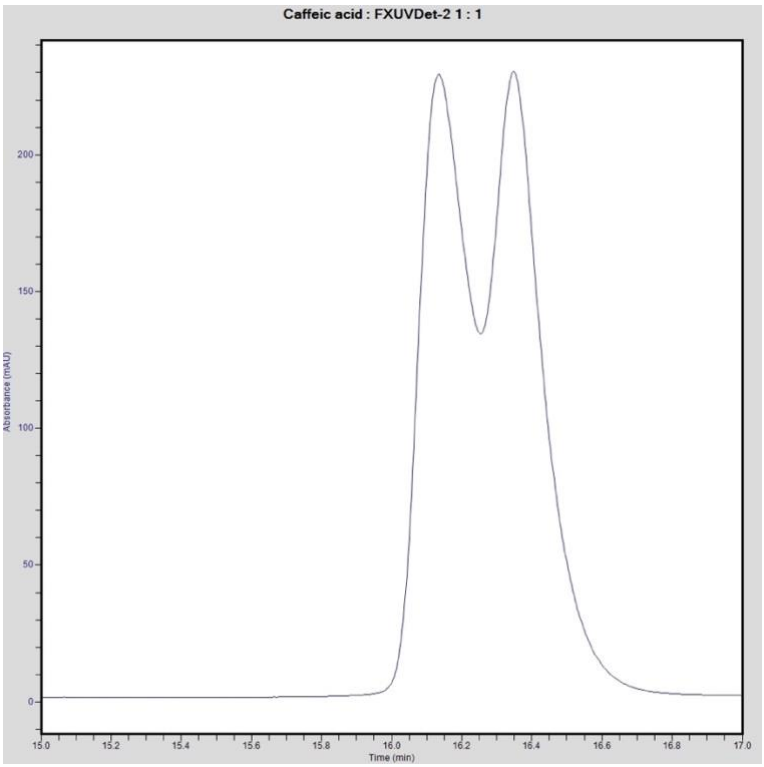

Figure B.2.2: Chromatogram of caffeic acid.

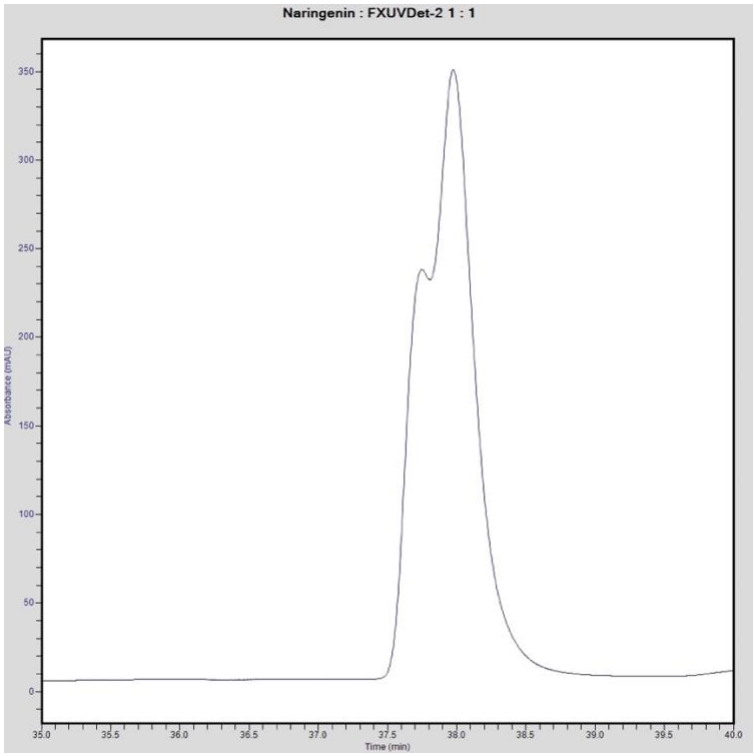

Figure B.2.3: Chromatogram of naringenin**.**

B.3: Antioxidant activity

Table B.3.1: The antioxidant activity of crude extracts compared to Trolox™ and ascorbic acid at 100 µg/mL.

Crude Extract/Controls	% FRSCA
***C. sinensis* aqueous**	94.23 ± 0.95
***C. sinensis* DCM:MeOH**	94.87 ± 0.58
***C. sinensis* acetone**	88.28 ± 12.57
***V. album* aqueous**	56.36 ± 0.43
***V. album* DCM:MeOH**	53.29 ± 1.61
***V. album* acetone**	48.10 ± 8.42
***S. aethiopicus* aqueous**	17.80 ± 3.31
***S. aethiopicus* DCM:MeOH**	73.41 ± 4.18
***S. aethiopicus* acetone**	16.27 ± 1.76
***W. salutaris* aqueous**	90.49 ± 1.50
***W. salutaris* DCM:MeOH**	0.00 ± 4.49
***W. salutaris* acetone**	63.51 ± 5.11
Trolox™	97.36 ± 0.75
Ascorbic acid	98.05 ± 0.26

Table B.3.2: The antioxidant activity of crude extracts compared to Trolox™ and ascorbic acid.

Crude Extract/Controls	IC_{50} (µg/mL)
***C. sinensis* aqueous**	15.49 ± 0.50
***C. sinensis* DCM:MeOH**	9.97 ± 0.17
***C. sinensis* acetone**	15.36 ± 1.22
***W. salutaris* aqueous**	55.24 ± 6.56
Trolox™	3.70 ± 0.37
Ascorbic acid	2.21 ± 0.004

B.4: Metal chelation

Table B.4.1: The Fe(II) chelating activity of crude extracts compared to EDTA at 100 µg/mL.

Crude Extract/Controls	% Cu (II) chelation
***C. sinensis* aqueous**	46.95 ± 2.19
***C. sinensis* DCM:MeOH**	59.29 ± 2.03
***C. sinensis* acetone**	43.41 ± 2.10
***V. album* aqueous**	41.59 ± 4.35
***V. album* DCM:MeOH**	29.87 ± 4.64
***V. album* acetone**	7.24 ± 2.44
***S. aethiopicus* aqueous**	46.37 ± 5.08
***S. aethiopicus* DCM:MeOH**	8.23 ± 2.13
***S. aethiopicus* acetone**	27.68 ± 6.15
***W. salutaris* aqueous**	14.26 ± 4.58
***W. salutaris* DCM:MeOH**	46.34 ± 12.29
***W. salutaris* acetone**	34.09 ± 1.95
EDTA	97.68 ± 0.98

Table B.4.2: The Cu(II) chelating activity of crude extracts compared to TMB at 100 µg/mL.

Crude Extract/Controls	% Cu (II) chelation
***C. sinensis* aqueous**	10.99 ± 2.36
***C. sinensis* DCM:MeOH**	14.83 ± 0.24
***C. sinensis* acetone**	0.00 ± 10.52
***V. album* aqueous**	2.92 ± 2.49
***V. album* DCM:MeOH**	0.00 ± 1.50
***V. album* acetone**	0.00 ± 1.15
***S. aethiopicus* aqueous**	0.00 ± 0.79
***S. aethiopicus* DCM:MeOH**	0.00 ± 0.22
***S. aethiopicus* acetone**	0.83 ± 1.54
***W. salutaris* aqueous**	1.99 ± 2.91
***W. salutaris* DCM:MeOH**	0.00 ± 3.52
***W. salutaris* acetone**	0.00 ± 3.25
TMB	81.96 ± 8.41

B.5: Lipid peroxidation

Table B.5: The lipid peroxidation activity of crude extracts compared to Trolox™ and ascorbic acid at100 µg/mL.

Crude Extract/Controls	% Lipid peroxidation inhibition
***C. sinensis* aqueous**	43.42 ± 5.64
***C. sinensis* DCM:MeOH**	16.59 ± 0.81
***C. sinensis* acetone**	34.77 ± 0.81
***V. album* aqueous**	0.00 ± 0.00
***V. album* DCM:MeOH**	0.00 ± 0.00
***V. album* acetone**	0.00 ± 0.00
***S. aethiopicus* aqueous**	0.00 ± 0.00
***S. aethiopicus* DCM:MeOH**	0.00 ± 4.28
***S. aethiopicus* acetone**	53.14 ± 11.31
***W. salutaris* aqueous**	28.69 ± 2.08
***W. salutaris* DCM:MeOH**	57.12 ± 11.07
***W. salutaris* acetone**	0.00 ± 12.37
Trolox™	68.47 ± 4.06
Ascorbic acid	65.65 ± 8.87

B.6:Haemolysis

Table B.6: The haemolytic activity of crude extracts and pure compounds compared to Triton™ X-100 at 100 µg/mL.

Crude Extract/Pure compounds/Controls	% Haemolysis
***C. sinensis* aqueous**	0.88 ± 0.16
***C. sinensis* DCM:MeOH**	0.91 ± 0.06
***C. sinensis* acetone**	0.89 ± 0.07
***V. album* aqueous**	0.35 ± 0.07
***V. album* DCM:MeOH**	0.65 ± 0.23
***V. album* acetone**	0.57 ± 0.18
***S. aethiopicus* aqueous**	0.44 ± 0.20
***S. aethiopicus* DCM:MeOH**	0.28 ± 0.26
***S. aethiopicus* acetone**	0.41 ± 0.30
***W. salutaris* aqueous**	1.03 ± 0.09
***W. salutaris* DCM:MeOH**	0.84 ± 0.09
***W. salutaris* acetone**	1.13 ± 0.14
Syringic acid	0.11 ± 0.37
(+)-Catechin hydrate	0.00 ± 0.11
Hesperetin	0.23 ± 0.14
Ferulic acid	0.77 ± 0.19
Kaempferol	4.85 ± 0.32
Polygodial	106.60 ± 2.78
Oleic acid	0.00 ± 0.52
Naringenin	0.49 ± 0.20
Caffeic acid	4.13 ± 0.29
Sinapic acid	0.13 ± 0.55
Ellagic acid	0.77 ± 0.13
(-)-Epicatechin	1.95 ± 1.33
4-Hydroxybenzoic acid	1.20 ± 0.30
(-)-Epigallocatechin	0.17 ± 0.04
(-)-Epicatechin gallate	0.74 ± 0.84
3,4-Dihydroxybenzoic acid	0.18 ± 0.09
DHA	116.21 ± 3.93
CQ	2.14 ± 1.12
Quinine	1.90 ± 1.38
Camptothecin	0.95 ± 0.32
Cisplatin	0.21 ± 0.03
Verapamil	0.20 ± 0.01
Ascorbic acid	2.62 ± 2.72
DMSO	2.08 ± 3.07

B.7: Cytotoxicity

Table B.7.1: The % MTT reduction activity of crude extracts on K562 cells compared to cisplatin, chloroquine, and quinine at 100 µg/mL.

Crude Extract/Pure compounds/Controls	%MTT reduction
***C. sinensis* aqueous**	64.47 ± 16.50
***C. sinensis* DCM:MeOH**	15.94 ± 3.00
***C. sinensis* acetone**	20.05 ± 1.76
***V. album* aqueous**	62.10 ± 19.62
***V. album* DCM:MeOH**	53.48 ± 19.01
***V. album* acetone**	38.94 ± 9.38
***S. aethiopicus* aqueous**	54.21 ± 21.19
***S. aethiopicus* DCM:MeOH**	3.51 ± 1.01
***S. aethiopicus* acetone**	4.67 ± 1.43
***W. salutaris* aqueous**	23.06 ± 14.29
***W. salutaris* DCM:MeOH**	5.94 ± 2.78
***W. salutaris* acetone**	1.72 ± 0.96
Cisplatin	10.97 ± 4.94
CQ	9.77 ± 1.14
Quinine	16.17 ± 12.13

Table B.7.2: The % MTT reduction activity of crude extracts on K562 cells compared to cisplatin, camptothecin, chloroquine, and quinine

Crude Extract/Controls	IC_{50} (µg/mL)
***C. sinensis* DCM:MeOH**	46.02 ± 7.00
***C. sinensis* acetone**	39.11 ± 6.25
***S. aethiopicus* DCM:MeOH**	25.87 ± 4.42
***S. aethiopicus* acetone**	10.25 ± 2.29
***W. salutaris* DCM:MeOH**	31.50 ± 1.28
***W. salutaris* acetone**	43.53 ± 5.31
Cisplatin	28.03 ± 0.40
Camptothecin	0.07 ± 0.05
CQ	17.14 ± 0.61
Quinine	26.46 ± 1.10

B.8: ADMET characteristics of standards and pure compounds

Table B.8.1: SwissADME and Toxtree in silico predictions of predicted major constituent drug-likeliness and toxicity.

Compound	Drug-Likeliness		Toxicity
	Lipinski	Bioavailability score	Toxic hazard
L-Ascorbic acid	Yes; 0 violations	0.56	High (Class III)
Gallic acid	Yes; 0 violations	0.56	Low (Class I)
Rutin hydrate	No; 3 violations: Mw >500, N or O ≥10, NH or OH ≥5	0.17	Intermediate (Class II)
Trolox ™	Yes; 0 violations	3.05	Intermediate (Class II)
EDTA	No; 1 violations: N or O ≥10	2.35	High (Class III)
TMB	No data generated by Swiss ADME	No data generated by Swiss ADME	High (Class III)
Cisplatin	No data generated by Swiss ADME	No data generated by Swiss ADME	High (Class III)
Camptothecin	Yes; 0 violations	0.55	High (Class III)
Triton™ X-100	Yes; 0 violations	0.55	High (Class III)
Syringic acid	Yes; 0 violations	0.56	Low (Class I)
(+)-Catechin hydrate	Yes; 1 violation: NH or OH > 5	0.55	High (Class III)
Hesperetin	Yes; 0 violations	0.55	High (Class III)
Ferulic acid	Yes; 0 violations	0.55	Low (Class I)
Kaempferol	Yes; 0 violations	0.85	Intermediate (Class II)
Polygodial	Yes; 0 violations	0.55	Intermediate (Class II)
Oleic acid	Yes; 1 violations: MLOGP > 4.15	0.85	Low (Class I)
Sinapic acid	Yes; 0 violations	0.56	High (Class III)
(-)-Epicatechin	Yes; 0 violations	0.55	Intermediate (Class II)
4-Hydroxybenzoic acid	Yes; 0 violations	0.82	Low (Class I)
(-)-Epigallocatechin	Yes; 1 violation: NH or OH > 5	0.55	Intermediate (Class II)
(-)-Epicatechin gallate	Yes; 1 violation: NH or OH > 5	0.55	Intermediate (Class II)
Verapamil hydrochloride	yes; 0 violations	0.55	n.d.

Table B.8.2: SwissADME *in silico* predictions of predicted major constituent lipophilicity and pharmacokinetics

Compound	Lipophilicity	Pharmacokinetics					
	Log P(O/W) MLOGP	GI	BBB	P-gp	CYP3A4	Add. CYP	Log Kp
L-Ascorbic acid	-2.6	High	No	No	No	No	8.54
Gallic acid	-0.16	Low	No	No	Yes	No	-6.84
Rutin hydrate	-3.89	High	No	Yes	No	No	-10.26
Trolox™	2.45	Low	Yes	Yes	Yes	CYP2D6	-5.89
EDTA	-0.1	High	No	Yes	No	No	-10.75
TMB	1.81	Low	Yes	No	No	No	-5.81
Cisplatin	-3.3	n.d.	No	Yes	No	No	-13.42
Camptothecin	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.
Triton™ X-100	n.d.	High	n.d.	n.d.	n.d.	n.d.	n.d.
Syringic acid	1.64	High	No	Yes	Yes	CYP1A2, CYP2C9	-7.19
(+)-Catechin hydrate	1.95	High	Yes	No	No	No	-5.9
Hesperetin	0.41	High	No	Yes	Yes	CYP1A2	-6.30
Ferulic acid	0.49	High	No	No	No	No	-6.77
Kaempferol	-0.56	High	No	No	No	No	-8.26
Polygodial	0.41	High	No	Yes	Yes	CYP1A2	-6.3
Oleic acid	1.00	High	Yes	No	No	No	-6.41
Sinapic acid	0.73	High	No	No	No	No	0.73
(-)-Epicatechin	0.24	High	No	Yes	No	No	0.24
4-Hydroxybenzoic acid	0.99	High	Yes	No	No	No	0.99
(-)-Epigallocatechin	-0.29	High	No	No	No	No	-0.29
(-)-Epicatechin gallate	0.05	Low	No	No	No	No	0.05
Verapamil hydrochloride	3.16	High	No	Yes	Yes	CYP2D6	3.16