PAN AFRICAN UNIVERSITY
INSTITUTE FOR BASIC SCIENCES, TECHNOLOGY AND INNOVATION

Effect of extracts of Artemisia afra collected from five different regions in Africa (Kenya, Burundi, Tanzania, South Africa and Senegal) on in vitro and in vivo cultures of Plasmodium Species

Ndeye Fatou Kane

A thesis submitted to Pan African University Institute of Science, Technology and Innovation in partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology of the Pan African University

2019

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

Signed …… Date……

Ndeye Fatou Kane (MB400-0002/2016)

This thesis has been submitted for examination with our approval as University Supervisors

Dr Mutinda C. Kyama
JKUAT, Department of Med. Lab Science
Signed…… Date ……

Dr Joseph K. Nganga
JKUAT, Department of Bio-Chemistry
Signed…… Date ……

Prof. Ahmed Hassanali
Kenyatta University Department of Chemistry
Signed…… Date ……..……

DR. Francis Kimani
Kenya Medical Research Institute (KEMRI)
Signed…… Date ……..……

Dedication

This work is dedicated to my lovely family who supported me all these years with their indefectible love and prayers specially my Mom and Daddy. Thanks for all your love and support throughout all these years.

Thanks to my fellow Senegalese from Kenya particularly Mr Said Diaw and his lovely wife Khady Sow for welcoming me in Kenya and being my second family.

I would like to dedicate this work also to all my supervisors and teachers; thanks for your precious advise and teaching and to all the technicians from the different lab I have been working for their help and assistance.

ACKNOWLEDGEMENT

I would like to thank all my supervisors for their assistance and availability: Dr. Kyama from JKUAT, Professor Ahmed Hassanali from KU, Francis Kimani from KEMRI, Dr. Joseph K. Nganga from JKUAT, and I would like to thanks also Dr. Mouhamadou Diallo from UCAD Pierre Lutgen from IFBV- BELHERB in Luxembourg.

This work was supported and funded by AU (African Union), through the Pan African University institute of Basic Sciences Technology and Innovation (PAUISTI). I would like to acknowledge all the facilities provided by Pauisti with their cutting edge laboratory where I did most of my work I'm very thankful.

Thanks for all the facilities provided by KEMRI/CBRD (Kenya Medical Research Institute/ Centre for Traditional Medicine and Drug Research). Thanks for Pierre Lutgen for Facilitate the obtention of the pant (*Artemisia afra*) through the network that is growthing and working with that plant in all over Africa and abroad. Thanks to all my supervisors for their assistance, advice, and guidance especially Dr. Kyama.

I also thank all the staff of PAUISTI especially the Director Prof. Gabriel Magoma and the coordinator of Molecular Biology and Biotechnology Prof. Naomi Maina.

Table of contents

Declaration ……ii

Dedication ……iii

Acknowledgment…….....iv

Table of content ……v

List of Tables ……x

List of Figures ……xi

Abbreviations and Acronyms ..……xii

Abstract……xiv

Chapter One: …….. ..1

1.0 General Introduction ……. ……..…….. ..1

1.1 Burden of Malaria ……2

1.2 Herbal medicinal plants with antimalarial effect: *Artemisia* species……2

1.3 Herbal medicinal plants with antimalarial effect: *Artemisia* species…….....3

1.4 Medicinal uses of *Artemisia afra* ……3

1.5 Statement of Problem…… 4

1.6 Justification ……5

1.8 Research questions ……5

1.7 Objectives ……6

1.7.1 General objective ……6

1.7.2 Specific objectives ……6

1.9 Scope and limitation ……6

Chapter 2: 7

2.0 Litterature review……..7

2.1 Epidemiology of Malaria 7

2.2 Causative agent of malaria……7

2.3 Vectors of malaria……8

2.4 Life cycle of human malaria parasite ……8

2.5 Blood stage (Morphology) …… 10
2.6 Vector Control ……10
2.6.1 Indoor residual Spraying…… 10
2.6.2 with insecticide end bed net …… 11
2.7 Diagnostic of malaria:…… ……11
2.8 Malaria treatment …… ……..12
2.9 Antimalarial drugs ……...…… 13
2.9.1 Classification ……..13
2.9.1.1 Classification According to anti-malarial activity…… 13
2.9.1.2 classification According to the structure ……14
2.10 Artemisinin and Artemisinin-based combination therapy (ACT**)**……..14
2.11Prophylactic drugs …… 15
2.12Vaccines against malaria ……..…15
2.13Resistance to Artemisinin ……16
2.14 Recrudescent of the artemisinin ……..……19
2.15 Artemisia afra genus ……..……19
2.16 Artemisia afra common names and geographical distribution……..21
2.17 Plant description …… 21
2.18 *Artemisia afra* common habitat ……..…22
2.19 Geographical variation…..……23
2.20 *Artemisia afra* a potential flagship for African medicinal plant……..23
2.21 Virtual screening……24
CHAPTER 3: ……..……27
3.0 Methodology ……27
3.1 Materials ……27
3.2 Study area and Source of *Artemisia afra* plants ……..……27
3.3 Swiss albinos Mice ……27
3.4 Extraction process of powdered leaves of Artemisia afra ……..27
3.4.1 Sample preparation ……27
3.4.2 Ethanolic, Hexane, DCM Extraction Process …… 27
3.4.3 Aqueous Extraction ……28

3.5 Phytochemical screening …… 28
3.5.1 Test for alkaloids ……28
3.5.2 Test for terpenoids ……28
3.5.3 Test for tannins ……28
3.5.4 Test for saponins ……29
3.5.5 Test for glycosides ……29
3.6 Determination of total phenolic and total flavonoid contents .. ……29
3.6.1 Total phenolic content ……29
3.6.2 Total flavonoid content ……29
3.7 Antioxidant activities (DPPH assay) ……30
3.8 *In vitro, in vivo* antimalarial essay and acute oral toxicity ……30
3.8.1 Parasite culturing and *In vitro* antimalarial essay ……30
3.8.2 Oral acute toxicity for Artemisia afra plant collected from Burundi ……31
3.8.2.1 Experimentations on animals: ……31
3.8.2.2 Tissue harvesting and processing: ……32
3.8.3 *In vivo* antimalarial test of Burundi extracts ……33
3.9 GCMS Analysis of *Artemisia afra* Plant Extracts collected from Burundi ……34
3.9.1 Sample Preparation ……34
3.9.2 Artemisinin standard ……34
3.9.3 GCMS Method ……34
3.10 Gene expression study for Fab_Z and Fab_I from *P. falciparum* after the exposition of the parasites to the crude extracts. ……35
3.10.1 RNA extraction Protocol: ……35
3.10.2 cDNA synthesis ……36
3.10.3FAB_Z FAB_I primers ……36
3.10.4 Conventional PCR for FAB_I and FAB_Z ……37
3.10.5 Housekeeping gene for *P. falciparum* ……37
3.10.6 Gel electrophoresis for PCR products ……38
3.10.7 Real-time PCR ……..38
3.11 Drug screening with Autodock vina in PyRx …… 40
3.11.1 Preparation of the library of small molecules …… 40
3.11.2 3D structure of *Plasmodium falciparum* Fab enzymes: Fab Z and Fab I……..40

3.11.3 Virtual screening with PyRx: Protocol..…… 41
A. Protocol for virtual screening:……...41
a. Preparation of input files for Docking:……..41
b. Virtual screening with Vina wizard……;……..41

3.12 Statistical analysis …… 42

CHAPTER 4: ……..43
4.0 **Results** ……..43
4.1 Phytochemical screening ……43
4.2 Total phenolic and flavonoids content ……44
4.2.1 Total phenolic ……44
4.2.2 Total Flavonoids content ……45
4.3 DPPH assay results ……47
4.4 Correlation of phytochemical contents with antioxidant activities ……48
4.5 In vivo acute toxicity results ……50
4.5.1 Effect of acute toxicity of *Artemisia afra* extracts on the weight and behaviour……50
4.5.2 Effect of acute toxicity of *Artemisia afra* extracts on the organ's weight of the mice…52
……49
4.5.3 Effect of acute toxicity of *Artemisia afra* extracts in the biochemical parameters compared to the control group …… ……54
4.5.4 Acute toxicity effect in mice tissues……..54
4.6 In vivo antimalarial assay for Burundi extracts ……57
4.7 GCMS results of *A. afra* Burundi extracts...……58
4.8 Gene expression results ……62
4.8.1 Gel electrophoresis for PCR product ……62
4.8.2 Real-time amplification ……63
4.8.3 Expression for Fab I and FabZ when the parasites are exposed to different extracts of A. afra …… ……63
4.9 drug screening results: ...……66

CHAPTER 5: ……...…….…… ……71

5.0 Discussion ……...…… ……71
5.1 Phytochemical screening, Total phenol, and total flavonoid: ……71
5.1.1 Phytochemical screening …… ……71
5.1.2 Total phenols and total flavonoids ……71
5.2 DDPH and Antimalarial assay: …..……72
5.3 Acute oral toxicity and in vivo antimalarial activity ……73
5.3.1 Acute oral toxicity ……..……73
5.3.2 Body and organs weight ..……74
5.3.3 Biochemical parameters (ALT and AST) ……75
5.3.4 Effects of Acute Administration of the Extract on Histology of the Kidneys and liver ……...75
5.4 In vivo antimalarial assay ..…..……75
5.5 GCMS of *Artemisia afra* extracts ……76
5.6 Gene Expression..……77
5.7 Affinity binding with FabI and FabZ ..……77
5.8 Conclusion: ...…….78
5.9 Recommendation ……...78
5.10 Ethical consideration ……79
References: ...……81

List of Tables

Table 3.1: Experimentation on mice……32
Table 4.1: phytochemicals screening of *Artemisia afra* plant extracts from Burundi, Senegal, South Africa, Kenya, and Tanzania……43
Table 4.2: Total phenolic content in *Artemisia afra* extract from Burundi, Senegal, Tanzania, and South Africa. ……45
Table 4.3: Total Flavonoids content in *Artemisia afra* extract from Burundi, Senegal, Tanzania, and South Africa. ……46
Table 4.4: Correlations between total phenols, total flavonoids and DDPH ……48
Table 4.5: In vitro antimalarial activity; IC50 of the 20 extracts from the 5 samples of *Artemisia afra* plant collected from South Africa, Kenya, Tanzania, Burundi and Senegal (IC50 ± SD (ug/ml)) ……49
Table 4.6: Mean body weight of mice treated with 3 extracts as compared to the control during 14days. Results presented as the mean of the body weight plus standard Error of the mean (P-value are in bracket). ……52
Table 4.7: Mean weight organs of mice treated with DCM extract as compared to the control (P-value in bracket). ……53
Table 4.8: Mean weight organs of mice treated with HEX extract as compared to the control (P-value in bracket). ……53
Table 4.9: Mean weight organs of mice treated with ETH extract as compared to the control (P-value in bracket). ……53
Table 4.10: Effect of DCM, ETH, Hex. Extracts on AST biochemical parameter, mean of experimental groups compared to the control (Expressed in Mean ± SDE, P-value in bracket).54
Table 4.11: Effect of DCM, ETH, Hex. Extracts on ALT biochemical parameter, mean of experimental groups compared to the control (Expressed in Mean ±SDE, P-value in bracket).54
Table 4.12: Effect of DCM, ETOH and Hexanolic extracts of *Artemisia afra* on *P. Berghei* infected mice ……57
Table 4.13: ED50 from in vivo antimalarial assay for DCM, ETOH and Hexanolic extracts of *Artemisia afra* collected from Burundi……58
Table 4.14: Main compounds detected by GCMS from dichloromethane extract, hexane and ethanolic extract of *A. afra* and result for GCMS for artemisinin standard……60
Table 4.15: Fold change expression for FabI after treatment with *A. afra* extracts……..64
Table 4.16: Fold change expression for FabZ after treatment with *A. afra* extracts……65

Table 4.17: *Artemisia afra* compounds from GCMS and Docking energy with FabI and FabZ.67

List of Figures

Figure 1.1: Map of malaria transmission……1
Figure 2.1: the Life cycle of the human malaria parasite……9
Figure 2.2: Morphology of malaria parasites *Plasmodium falciparum* inside the infected erythrocyte. ……...10
Figure 2.3: Current global distribution of artemisinin-based combination therapies as the first-line treatment of uncomplicated falciparum malaria. ……..15
Figure 2.4: Increase in number of results on Scirus, a search engine for scientific articles, for *Artemisia afra*. ……...21
Figure 2.5: Artemisia afra plant……22
Figure 2.6 virtual screening workflow……..26
Figure 3.1: Plate setting for real-time PCR……39
Figure 3.2: PyRx virtual screening interface……41
Figure 4.1: Calibration curve for the standard Gallic acid……44
Figure 4.2: Calibration curve for Rutin standard……..46
Figure 4.3: DPPH radical scavenging capacity (EC50) of *Artemisia afra* extracts ……47
Figure 4.4: Mean body weight change in mice treated with 2500mg/kg; 2000mg/kg, 1000mg/kg DCM extracts one single dose. ……51
Figure 4.5: Mean body weight change in mice treated with 2500mg/kg; 2000mg/kg, 1000mg/kg Ethanolic extracts one single dose. ……..51
Figure 4.6: Mean body weight change in mice treated with 2500mg/kg; 2000mg/kg, 1000mg/kg Hexane extracts one single dose. ……51
Figure 4.7: Photomicrographs of liver sections of control mice (A) and mice treated with hexane extract (B), ethanolic extract (C) and dichloromethane extract (D) ……55
Figure 4.8: Photomicrographs of Kidney sections of control mice (A) and mice treated with hexane extract (B), ethanolic extract (C) and dichloromethane extract (D) ……56
Figure 4.9: Chromatogram of artemisinin standard, dichloromethane extract, hexane and ethanolic extract of *A. afra* ……..59
Figure 4.10: Gel electrophoresis of Actine FabI and FabZ ……62
Figure 4.11: Amplification curves for FabI, FabZ, and Actine……63
Figure 4.12: Gene expression for Fab I……65
Figure 4.13: Gene expression for Fab Z……...66

Figure 4.14: Interaction site for FabI (chain B) and hit N°1 (Hydroxyprogesterone) ……68
Figure 4.15: Interaction site for FabI (chain B) and hit N°2 (Aspidospermidin). ……69
Figure 4.16: Interaction site for FabZ (chain K, L, I, H) and hit N°1 (Hydroxyprogesterone).70
Figure 4.17: Interaction site for FabZ (chain K, L, I, J, G) and hit N°2 (Thiourea)…….70

Abbreviations and acronyms

ACTs	Artemisinin combine therapies
ALT	Alanine transaminase
ALAT	Alanine aminotransferase
AL	Artemether + lumefantrine
A. afra	Artemisia afra
ASAQ	Artesunate + amodiaquine
AS	Artesuanate
ASMQ	Artesunate + mefloquine
ASSP	Artesunate + sulfadoxine–pyrimethamine
AST	Aspartate transaminase
AS+SP	Artesunate-sulfadoxine-pyrimethamine
CDC	Centers for disease control and prevention
COPD	Chronic obstructive pulmonary disease
CPM	Count per minute
CYP3A4	Cytochrome P 3A4
DCM	Dichloromethane
DDT	DichloroDiphenylTriClhoroethane
DHA-PPQ	Dihydroartemisinin + piperaquine
DMSO	Dimethyl sulfoxide
DPPH	2,2'-diphenyl-1-picrylhydrazil (Free Radical Scavenging)
DVS	Dominant vector species
ED50	Median effective dose
EtOH	Ethanol
GAE	Gallic acid
GCMS	Gas chromatography mass spectrometry
GMS	Greater mekong subregion
G6PD	Glucose-6-Phosphate Dehydrogenase
HZ	Hemozoin
HRP-2	Histidine rich protein-2,
HEX	Hexane

IC50	Half maximal inhibitory concentration
iRBCs	Infected erythrocytes
ITN	Insect treated net
KEMRI	Kenya Medical Research Institute
LCMS	Liquide chromatography mass spectrometry
LD50	Median lethal doses
pACT	Plant based artemisia combination therapy
P. falciparum	Plasmodium falciparum
pLDH	*Plasmodium* associated lactate dehydrogenase
PTFE	Polytetrafluoroethylene
RDT	Rapid diagnostic test
SP	Sulfadoxine-pyrimethamine
uCi	Microcurie
uRBCs	Uninfected erythrocytes
WHO	World health organization

Abstract:

Malaria is one of the deadliest disease in the world affecting millions of individuals yearly. Artemisinin combination therapies (ACTs), which are the first line of defense against this disease for many years, show some inefficacy due to delay in parasite clearance. Many episodes of resistance against these drugs have been registered in many countries in Africa and in Asia. Currently, there is growing research interest in the use of full blend extract of medicinal plants like *Artemisia afra* or *Artemisia annua* as an alternative treatment. *Artemisia afra* is an indigenous species to Africa, and are traditionally used for decades by traditional healers to cure a lot of afflictions among them malaria. The main objective of this thesis is to compare the growth inhibition effects and molecular profiles of the parasite exposed to extracts of the *Artemisia afra* plants collected from five regions. *Artemisia afra* leaves were collected from five countries in Africa (South Africa, Tanzania, Burundi, Senegal, and Kenya), and compared for their level of phytochemicals content, antimalarial, and antioxidant activities. The plant extracts were first tested *in vitro* and the most active extracts were incubated during 2 days with the parasites to study the expression level of FabI and FabZ. A virtual screening was ran using PyRx with vina to determine the potential interactions between the Fab enzymes (FabI and FabZ) and the active compounds of the plant extracts. The result showed a big antimalarial property of the plant, however a different level of activity depending on the geographical localization. *A. afra* collected from Burundi was found to have the highest level of phenols and flavonoids. This plant also exhibited the highest antioxidant and antimalarial activities compared to the others. Acute toxicity test run in mice revealed an ED50 greater than 2500mg/kg body weight and no toxic sign was detected on the liver, the organs, and the tissues. The Fab I enzyme, which plays important rule during the liver stage of the malaria infection was found to be downregulated in the W2 strain, after exposition of the parasites with the ethanolic and dichloromethane of the plant extract collected from Burundi. In the D6 strain the enzyme was downregulated with the hexane and ethanolic extracts of the plant. Compared to Fab I, Fab Z was found to be downregulated only on the D6 strain when exposed to the hexane and ethanolic extracts, both extracts downregulate Fab I and Fab Z in the two strains (D6, W2). Virtual screening showed interaction between the active compounds of the plant and the Fab enzymes. In conclusion the results confirm the high antimalarial effect of *Artemisia afra* and also its prophylactic effect with the inhibition of Fab I enzyme which is crucial during liver stage of the *plasmodium falciparum* life cycle. The difference in level of flavonoids and phenols

suggest that the agro-ecological zone play an important role in influencing the level of phytochemical. *Artemisia afra* active compounds detected during GCMS analysis was found to interact with the fab enzymes of the parasite, further study need to be done to confirm their *in vitro* activities of those active compounds.

CHAPTER ONE.

1.0 Introduction:

Malaria is one of the most dangerous infectious diseases in Africa. In 2017, there were an estimated 219 million cases of malaria in 90 countries. Malaria deaths reached 435 000, with 93% in Africa (WHO, 2017). There are approximately 156 named species of *Plasmodium* which infect various species of vertebrates, such as reptiles, birds, and various mammals (CDC, 2017). Five plasmodium species are considered human parasite, as they utilize humans almost exclusively as a natural intermediate host: *P. falciparum, P. vivax, P. ovale* and *P. malariae*, and *P. knowlesi, P. falciparum* and *P. vivax* pose the greatest threat because there are the most virulent. In 2017, *P. falciparum* accounted for 99.7% of estimated malaria cases in the WHO African Region, as well as in the majority of cases in the WHO regions of South-East Asia (62.8%), the Eastern Mediterranean (69%) and the Western Pacific (71.9%). *P. vivax* is the predominant parasite in the WHO Region of the Americas, representing 74.1% of malaria cases (WHO, 2017). Malaria occurs mostly in poor, tropical, and subtropical areas of the world. Africa is the most affected due to a combination of those three factors (figure 1.1). Local weather conditions often allow also transmission to occur year round. A very efficient mosquito (*Anopheles gambiae* complex) is responsible for the high transmission of the parasite (CDC, 2019).

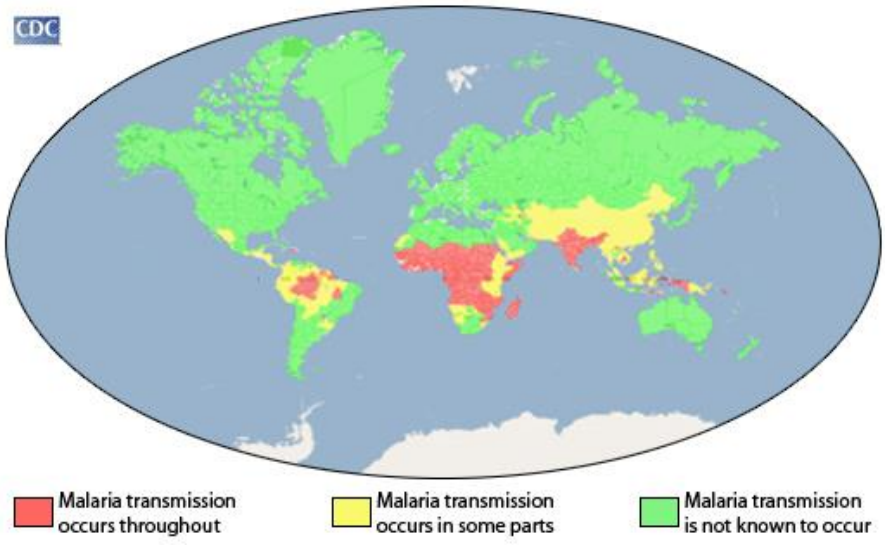


Figure1.1: Geographical distribution of Malaria

Sources: https://www.cdc.gov/malaria/malaria_worldwide/impact.html#

In many of the countries affected by malaria, it is a leading cause of illness and death. In areas with high transmission, the most vulnerable groups are young children, who have not developed immunity to malaria yet, and pregnant women, whose immunity has been decreased by pregnancy. The costs of malaria to individuals, families, communities, nations are enormous (WHO, 2017).

1.1 Burden of Malaria

According to the latest World malaria report, released in November 2018, there were 219 million cases of malaria in 2017, up from 217 million cases in 2016. The estimated number of malaria deaths stood at 435 00 0 in 2017, a similar number to the previous year.The WHO African Region continues to carry a disproportionately high share of the global malaria burden. In 2017, the region was home to 92% of malaria cases and 93% of malaria deaths. In 2017, 5 countries accounted for nearly half of all malaria cases worldwide: Nigeria (25%), the Democratic Republic of the Congo (11%), Mozambique (5%), India (4%) and Uganda (4%). Children under 5 years of age are the most vulnerable group affected by malaria; in 2017, they accounted for 61% (266 000) of all malaria deaths worldwide (WHO, 2019).

US$ 3.1 billion were made available for control and elimination programmes in 2017 including US$ 900 million (28%) from governments of malaria endemic countries. The United States of America remains the largest single international donor, contributing US$ 1.2 billion (39%) in 2017. To meet the 2030 targets of the global malaria strategy, malaria investments should reach at least US$6.6 billion annually by 2020 – more than double the amount available today (WHO, 2018).

1.2 Plant extracts as possible sources of antimalarial drugs

Natural products, including medicinal plants, have been used in the traditional treatment of malaria for thousands of years due to their efficacy, safety, lower cost, and availability. In fact, medicinal plants are the source of the two most successful antimalarial drugs, artemisinin and quinine. Studies into the antimalarial activity of natural products (extracts and compounds) derived from medicinal plants, animals, or micro-organisms therefore become quite expedient (https://www.hindawi.com/journals/ecam/si/585486/cfp/).

1.3 Herbal medicinal plants with antimalarial effect: *Artemisia* species

The large number of *Artemisia* species in the family of Asteraceae have been used as herbal medicines for millennia in different parts of the world. Some medicinal plants, like *A. annua* or *A. afra,* show high antimalarial effects. *A. annua* leaves has been used for many years by individuals in China to treat a different form of fever. Artemisinin extracted from *A. annua* is the major antimalarial component. Artemisinin or synthetic derivatives are used in combination therapy with other antimalarial drugs referred to as artemisinin combination therapy (ACTs) (James Gathany, www.wwarrn.org).

Although ACTs drugs have worked well in many parts of the world, there is a serious concern that malaria parasites are once again developing widespread resistance to this vital treatment. Thus there is a need to develop alternative therapies, as well as to put in place effective preventive measures (James Gathany, www.wwarrn.org).

A. annua extracts and powdered plant materials were found to be many times more potent than pure artemisinin. This is in line with the recent findings published by P Weathers, showing that *A. annua* leaf powder can be up to 40x as efficient as pure artemisinin (Weathers *et al.*, 2014). *A. afra* also was found to have a potent antimalarial effect and worked as effectively as *A. annua.* Over the last ten years, a series of small-scale clinical trials have shown the superiority of *Artemisia* plants over ACTs (P. Lutgen, 2016). Moreover, other *Artemisia* plants, such as *A. herba alba, A. sieberi, A. absinthium, A.ludoviciana, A. apiacea, A. maritima* have shown varying levels of antimalarial effects.

1.4 Medicinal uses of *Artemisia afra*

Artemisia afra is common species of the genus Artemisia in Africa with medicinal properties. It is the only Artemisia species indigenous to Africa and widely distributed from South Africa to Ethiopia (van der Walt, 2004). The traditional African medicinal uses of *A. afra* include the treatment of colds, coughs, influenza, sore throat, asthma, pneumonia, blocked nose, stomach ailments, headache, earache, poor appetite, heartburn, parasites, measles, gout, diabetes, colic, flatulence, constipation, malaria, and wounds (Van Wyk, 2008). This shows the vast range of diseases and conditions it is used for and thus its large medicinal potential.

An earlier *in vitro* study in Ethiopia with five medicinal plants found that *A. afra* had the highest antimalarial activity (IC50 7 mg/L) (Kassa *et al*., 1998). Recent clinical trials in the RD Congo have shown that *A. afra* is equivalent to *A. annua* in the cure of malaria (Munyangi and Idumbo,

2015). Another recent study by South African research team shows that among 8 medicinal plants *A. afra* has the lowest IC50 for impairing the development of late-stage gametocytes. This represents a very important finding, as not many plants have such a significant gametocytocidal effect (Moyo *et al.*, 2016).

Artemisia afra is rich in luteolin, which may partly account for its antimalarial properties. *A. afra* has the highest concentration of luteolin (1.9 mg/g) in all medicinal herbs (Dube, 2006), 20 times more than in *A. annua*. The author also found that luteolin present at high concentrations in the herb specimen studied stays stable after several processing operations. The IC50 value of luteolin displayed against *P. falciparum* was 2.9 µg/ml (P. Lutgen, Malaria World, 2015).

Artemisia afra plants do not contain artemisinin, so other constituents are responsible for their anti-malarial effect. Luteolin, one of the most important constituents of *A. afra*, is related to the inhibition of fatty acid biosynthesis of *P. falciparum*. These lipids are required for the detoxification by crystallization into hemozoin of heme resulting from haemoglobin digestion by the parasite. Apicomplexan parasites utilises a fatty acid synthesis pathway, independent of the human host and catalysed by specific enzymes like FabG, FabZ, and FabI. These enzymes are a potential target of new antimalarials (Tasdemir *et al*., 2006). The antimalarial activity might also be related to the inhibition of alpha-glucosidase and amylase by luteolin. (Kim *et al*., 2000). Among 40 flavonoids studied by these authors, luteolin was the most potent. These enzymes convert carbohydrates into glucose required for the development and multiplication of parasites. In addition to luteolin, other constituents may also contribute to the gross anti-malarial effects of *A. afra*, similar to those of *A. annua*.

1.5 Statement of the problem

Malaria is still raising concern with high rate of death each year. The use of artemisinin and its derivatives with other antimalarial drugs as ACTs are become problematic because of the emerge of parasite resistance (WHO, 2019) and an enhance of recrudescence of gametocyte carriage (Abdoulaye A. Djimde *et al.*, 2016). The spread of that resistance is a significant threat to humanity. The use of powdered or extracts of *Artemisia afra* can be a potential solution due to his high antimalarial potential (Weathers *et al.*, 2014). Also, the full blend of *Artemisia afra* is much less likely to lead to resistance development because it doesn't contain artemisinin. *In vitro* studies have shown that *Artemisia afra*, which is native to tropical Africa, has an

antimalarial effect comparable to that of *A. annua* (Jerome *et al*., 2019). This plant does not contain artemisinin, have not toxic effect, and can be stronger than *A. annua* against malaria. In this study, the antimalarial effects of five *A. afra* from different agro-ecological areas will be compared using both *in vitro* and *in vivo* modes. Also, the molecular effects of the parasites when exposed to the extracts of *A. afra* extracts will be studied.

1.6 Justification

Malaria is one of the most widely spread diseases in the world. In Africa, this disease affects millions of individuals. It is one of the most deadly tropical diseases. However, in many countries, resistance against ACTs is noticed. The spread of that resistance is a significant threat to humanity. Alternative solutions should be put in place to stop or mitigate this nascent resistance. The use of full phytochemical blends of *A. afra* as prophylactics and curatives remedy seems to be a promising way to address the problem. The different phytochemical constituents of that plant seem to work in synergy against the malaria parasite. Our approach is in line with the official WHO Traditional Medicine Strategy 2014 – 2023 which recognizes that still, 80% of the people in endemic malaria countries rely on herbal medicines to boost their immunity against malaria instead of using expensive imported drugs. The levels of prophylactic and curative efficacy of these plants need to be established, and ways of improving them need to be developed.

In this study, *Artemisia afra* from five countries will be used to test their antimalarial effect the rationale behind using this same species is they may have various level of antimalarial activities or different phytochemical concentration due to their different geographical areas. This study will shed some light about difference in their activities. The plant is also claim to have prophylactic effect so the inhibition test with the fab enzymes especially with FabI will confirm their prophylactic properties

1.7 Research questions

1 *Do A. afra* plants collected from different agro-ecological area have the same or different levels of phytochemical compositions?
2 *Do A. afra* plants collected from different agro-ecological area have the same or different antimalarial activities?

3 The *in vitro* antimalarial activity of *A. afra* plant is it the same compared to the *in vivo* activity?

4 Antimalarial activity of *A. afra* is it because of one compound or several compounds working in synergy?

1.8 Objectives

1.8.1 General objective:

To compare the growth inhibition effects and molecular profiles of the malaria parasites exposed to *Artemisia afra* extracts collected from five different regions.

1.8.2 Specific objectives

1 To determine and compare *in vitro* antimalarial activities and phytochemical profiles of extracts of *A. afra* plants collected from 5 different agro-ecological areas.

2 To determine the acute toxicity and the *in vivo* anti-plasmodium activities of the most active extracts of *A. afra* on *P. berghei*.

3 To identify the active compounds of *A. afra* extracts using GC-MS.

4 To assess gene expression levels for Fab_Z, and Fab_I associated with *Plasmodium* cultures exposed to the most active *A. afra* extracts.

5 To determine potential interactions between active compounds from *A. afra* and Fab_proteins from the *Plasmodium falciparum* (W2 AND D6) using the software PyRx with AUTODOCK vina.

1.9 Scope and Limitation

This study will help to determine if *Artemisia afra* plants still have the same activity when it is growing in different agro-ecological areas. Will also help to know the difference in the level of phytochemical of that plant. Because of the time and resource limitations, (i) Five samples only will be collected from five different countries. (ii) No clinical trial will be run to assess toxicity in human, and (iii) full blend will be tested in *vitro* and *in vivo* but not active isolated compounds.

CHAPTER TWO.

2.0 LITERATURE REVIEW.

2.1 Epidemiology of malaria

According to the World Health Organization (WHO) Malaria Report 2011, a total of 106 countries in the world are at risk of transmission of malaria infection. A total of 216 million estimated malaria cases occurred in 2010, 81% of which were reported in the African Region, followed by South-East Asia (13%) and Eastern Mediterranean Region (5%). The total number of malaria deaths was estimated to be 655.000 in 2010; 91% of whom occurred in the African Region, 6% in South-East Asia and 3% in the Eastern Mediterranean Region. However, between 2005 and 2010, malaria cases decreased from 244 million to 216 million; moreover, malaria mortality rates showed a global reduction of 26% between 2000 and 2010 (WHO, 2011).

In 2012, there were an estimated 207 million cases worldwide, a decrease compared to 2011 causing an estimated 627 000 deaths, mainly among children under five years of age in Africa. Ninety per cent of all deaths occur in Sub Sahara Africa (WHO, 2013).

In 2016, there were 216 million cases of malaria in 91 countries, 5 million more than the 211 million cases reported in 2015. The disease continues to claim a significant number of lives: in 2016, 445 000 people died from malaria globally, compared to 446 000 estimated deaths in 2015. According to the latest *World malaria report*, released in November 2018, there were 219 million cases of malaria in 2017. The estimated number of malaria deaths stood at 435 000 in 2017 a slight decrease compared to 2016 (WHO, 2019). Children under 5 are particularly susceptible to infection, illness and death; more than two thirds (70%) of all malaria deaths occur in this age group. The number of under-5 malaria deaths has declined from 440 000 in 2010 to 266 000 in 2017. However, malaria remains a major killer of children under five years old, taking the life of a child every two minutes (WHO, 2018). Fifteen countries – all but one in sub-Saharan Africa carry 80% of the global malaria burden.

2.2 Causative agent of malaria

Malaria is caused by the Plasmodium parasite. The parasite can be spread to humans through the bites of infected mosquitoes.

The plasmodium parasite is spread by female Anopheles mosquitoes, which are known as "night-biting" mosquitoes because they most commonly bite between dusk and dawn. If a mosquito bites a person already infected with malaria, it can also become infected and spread the parasite on to other people. However, malaria can't be spread directly from person to person. Malaria can also be spread through blood transfusions and the sharing of needles, but this is very rare(https://www.nhs.uk/conditions/malaria/causes/).

2.3 Vectors of malaria

Malaria is transmitted exclusively through the bites of *Anopheles* mosquitoes. There are 512 *Anopheles* species recognised worldwide, and 50 only provisionally designated and awaiting description (http://mosquito-taxonomic-inventory.info). *Anopheles* mosquitoes breed in water, and each species has its own breeding preference. Forty-one of the 512 *Anopheles* species are defined by experts "Dominant Vector Species" (DVS). DVS are the most important malaria vector worldwide, providing the majority of human malaria cases. Characteristics of dominant vector species are their propensity for humans feeding, longevity, abundance and elevate vectorial capacity (Takken *et al*., 2003). Africa has the most effective and efficient DVS of human malaria, the *Anopheles gambiae complex*; thus some areas account the highest entomological inoculation rates and the highest malaria prevalence worldwide (Hay *et al*., 2009; Coluzzi M, 1999; Guerra *et al*., 2008).

Environmental factors play an essential role in vector distribution and malaria biodiversity. Climate seasonality, rainfall patterns, temperature, humidity, presence of vegetation and surface water all are directly related to the malaria transmission cycle. Also, human activities such as agriculture, irrigation, deforestation, urbanisation, population movements, dam/road constructions and wars are also connected to transmission levels and malaria epidemiology (Machault *et al*., 2011).

2.4 Life cycle of human malaria parasite

Infection in humans begins with the bite of an infected female Anopheles mosquito (Figure1.2). The parasites undergo a series of morphological transformations during their life cycle. The malaria parasite life cycle involves two hosts: mosquito (in red) and human (in blue). During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host❶. In the human host, sporozoites infect liver cells❷ where they continue maturation and mature into schizonts❸ before they are released into the bloodstream, where another stage called the intra-erythrocytic stage is formed. The schizonts will rupture and

release merozoites[4] (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later). After this initial replication in the liver (exo-erythrocytic schizogony[A]), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony[B]). Merozoites infect red blood cells[5]. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites[6]. Some parasites differentiate into sexual erythrocytic stages (gametocytes)[7]. Blood stage parasites are responsible for the clinical manifestations of the disease.

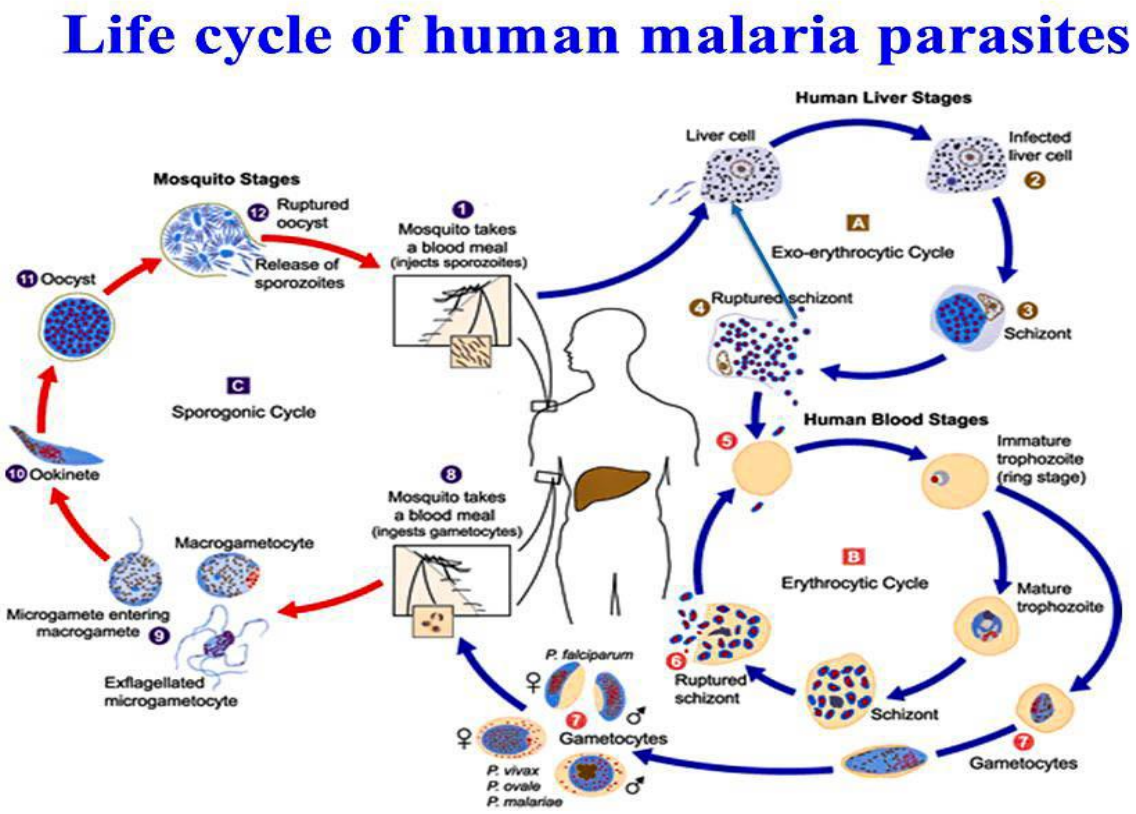


Figure 2.1: the Life cycle of the human malaria parasite

Sources: https://www.cdc.gov/dpdx/malaria/index.html

The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal[8]. The parasites' multiplication in the mosquito is known as the sporogonic cycle[C]. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes[9]. The zygotes, in turn, become motile and elongated (ookinetes)[10] which invade the midgut wall of the mosquito where they develop into oocysts[11]. The oocysts grow, rupture, and release sporozoites[12], which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle (CDC, 2017).

Inside of the erythrocyte, the malaria parasite changes into a form called the "ring stage," at which the parasite degrades haemoglobin for its biosynthetic requirements (Rathore, 2006; Pagola *et al*., 2000). Hemozoin formed during this life cycle is considered an important target in the search for new antimalarial drugs (Sullivan, 2000).

2.5 Blood stage (Morphology):

During the erythrocytic cycle, we can observe different stages of the parasites inside the erythrocyte (Figure 3) visible with a microscope (x100 magnificent) after a thin blood smear stained by GIEMSA. Trophozoite stage (1) following by a ring stage (2) whose growing and turning into schizonts (3) who after maturation burst and liberate the merozoites. Some of the merozoites develop to become gametocytes (4) which are the sexual blood stages while the other merozoites infect new red blood cells, and the cycle starts again.

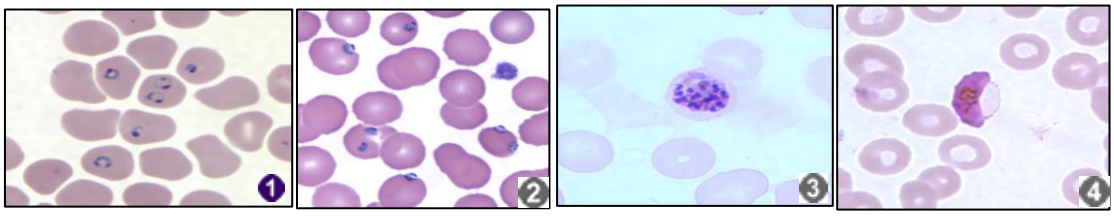


Figure 2.2: Morphology of malaria parasites *Plasmodium falciparum* inside the infected erythrocyte.

Sources: https://www.cdc.gov/dpdx/malaria/index.html

2.6 Vector Control

2.6.1 Indoor residual spraying

The control of vectors passes by the use of insecticides that are spraying on the interior walls of homes in malaria-affected areas. After feeding, many mosquito species rest on a nearby surface while digesting the bloodmeal, so if the walls of dwellings have been coated with insecticides, the resting mosquitos will be killed before they can bite another victim, transferring the malaria parasite (Lucy *et al*., 2016).

The World Health Organization (WHO, 2006) currently advises the use of 12 different insecticides in Indoor residual spraying operations. These include DDT and a series of alternative insecticides (such as the pyrethroids permethrin and deltamethrin) to both, combat malaria in areas where mosquitoes are DDT-resistant, and to slow the evolution of resistance.

One problem with all forms of Indoor Residual Spraying is insecticide resistance via the evolution of mosquitos.

2.6.2 Insecticide and bed net

Mosquito nets help keep mosquitoes away from people, and thus significantly reduce the infection and transmission of malaria. The nets are not a perfect barrier, so they are often treated with an insecticide designed to kill the mosquito before it has time to search for a way past the net (http://www.eac.int/health/index).
Insecticide-treated nets (ITN) are estimated to be twice as effective as untreated nets and offer a protection greater than 70%. For maximum effectiveness, the nets should be re-impregnated with insecticide every six months.

New technologies like Olyset or DawaPlus allow for production of long-lasting insecticidal mosquito nets (LLINs), which release insecticide for approximately 5 years (http://www.eac.int/health/index).
ITNs have the advantage to kill mosquitoes that contact the net and simultaneously protecting people sleeping under the net. This has the effect of killing the most dangerous mosquitoes. Some protection is also provided to others, including people sleeping in the same room but not under the net (http://www.eac.int/health/index).

2.7 Diagnostic of malaria

Microscopy (morphologic analysis) continues to be the "gold standard" for malaria diagnosis. Parasites may be visualized on both thick and thin blood smears stained with Giemsa (https://www.cdc.gov/dpdx/malaria/index.html) and quantify (CDC, 2017). There are other technics to diagnose malaria species in the case where microscopists fail to identify them due to parasite overlaps or changes in their morphology because of drug treatment or improper storage of samples. The *Plasmodium* species can be determined by using confirmatory molecular diagnostic tests. PCR testing is most useful for definitively identifying the species of the malaria parasite and detecting mixed infections. Detection and identification of *Plasmodium* to the species level is done with a real-time PCR assay as described by Rougemont *et al*. 2004 for a duel duplex assay (e.g., *P. falciparum* and *P. vivax* in one reaction, and *P. malariae* and *P. ovale* in a parallel reaction, using species-specific TaqMan probes) and also with conventional nested PCR assay in case of infection by more than one species is

suspected (Snounou *et al*, 1993). In addition to microscopy and molecular methods, there are methods for detecting malaria parasites based on antigens or enzymatic activities associated with the parasites. These methods are often packaged as individual test kits called rapid diagnostic tests or RDTs. These methods include, among others:

- Detection of an antigen (histidine rich protein-2, HRP-2) associated with malaria parasites (*P. falciparum*)
- Detection of a *Plasmodium* specific aldolase
- Detection of a *Plasmodium* associated lactate dehydrogenase (pLDH) either through its enzymatic activity or by immunoassay (CDC, 2017).

3.11 Malaria treatment:

3.11.4 Treatment for Uncomplicated malaria

Patients with suspected malaria should have parasitological confirmation of diagnosis with either microscopy or rapid diagnostic test (RDT) before antimalarial treatment is started. WHO recommends artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated malaria caused by the *P. falciparum* parasite. *P. vivax* infections should be treated with an ACT or chloroquine in areas without chloroquine-resistant *P. vivax*. In areas where chloroquine-resistant *P. vivax* has been identified, infections should be treated with an ACT. In low transmission areas, a single low dose of primaquine as a gametocytocide should be added to the antimalarial treatment to reduce transmission of the infection. The frequency of the administration of primaquine should be guided by the patient's glucose-6-phosphate dehydrogenase (G6PD) enzyme activity. ACTs are the most effective antimalarial medicines available today. WHO currently recommends 5 ACTs for use against *P. falciparum* malaria. The choice of ACT should be based on the results of therapeutic efficacy studies against local strains of *P. falciparum* malaria (WHO, 2018).

3.11.5 Treatment for complicated malaria

Severe malaria should be treated with injectable artesunate (intramuscular or intravenous) for at least 24 hours and followed by a complete 3-day course of an ACT once the patient can tolerate oral medicines. This is to ensure full cure and prevent the development of resistance to the artemisinin derivatives. In recent years, access to ACTs has expanded substantially. By the

end of 2016, ACTs had been adopted as first-line treatment policy in 80 countries. In recent years, parasite resistance to artemisinin has been detected in many countries.

It has been shown that amino acid (AA) mutations at the *P. falciparum* Kelch13 (Pfk13) gene provide resistance to ART. Artemisinin resistance was reported first in western Cambodia, (Dondorp *et al.*, 2009; Noedl *et al*., 2008; Elizabeth et al., 2014) where failure rates for artemisinin-based combination therapies are rapidly increasing (WHO, 2014) and where resistance to previous first-line antimalarial drugs also first emerged. Artemisinin resistance has since spread, emerged independently, or both in other areas of mainland Southeast Asia (Hein *et al*., 2012; Phyo *et al*., 2012), but also in some countries in Africa. Because of the growing threat of resistance to that vital and first-line treatment for malaria, there is an increasing interest in Africa in the use of medicinal plants as an alternative treatment.

2.9 Antimalarial drugs

2.9.1 Classification

Anti-malarial drugs can be classified according to antimalarial activity and according to the structure.

2.9.1.1. Classification according to anti-malarial activity:

a. **Tissue schizonticides for causal prophylaxis:**

These drugs act on the primary tissue forms of the plasmodia which, after growth within the liver, initiate the erythrocytic stage. By blocking this stage, further development of the infection can be theoretically prevented. Pyrimethamine and Primaquine have this activity. However, since it is impossible to predict the disease before clinical symptoms begin, this mode of therapy is more theoretical than practical.

b. **Tissue schizonticides for preventing relapse:**

These drugs act on the hypnozoites of *P. vivax* and *P. ovale* in the liver that cause a relapse of symptoms on reactivation. Primaquine is the prototype drug; pyrimethamine also has such activity.

c. **Blood schizonticides:**

These drugs act on the blood forms of the parasite and thereby terminate clinical attacks of malaria. These are the most important drugs in anti-malarial chemotherapy. These include chloroquine, quinine, mefloquine, halofantrine, pyrimethamine, sulfadoxine, sulfones, tetracyclines etc.

d. **Gametocytocides:**

These drugs destroy the sexual forms of the parasite in the blood and thereby prevent transmission of the infection to the mosquito. Chloroquine and quinine have gametocytocidal activity against *P. vivax* and *P. malariae*, but not against *P. falciparum*. Primaquine has gametocytocidal action against all plasmodia, including *P. falciparum*.

e. **Sporontocides:**

These drugs prevent the development of oocysts in the mosquito and thus ablate the transmission. Primaquine and chloroguanide have this action. Therefore in effect, treatment of malaria would include a blood schizonticide, a gametocytocide and a tissue schizonticide (in case of *P. vivax* and *P. ovale*). A combination of chloroquine and primaquine is thus needed in all cases of malaria (https://www.malariasite.com/malaria-drugs/) (White NJ, 1996).

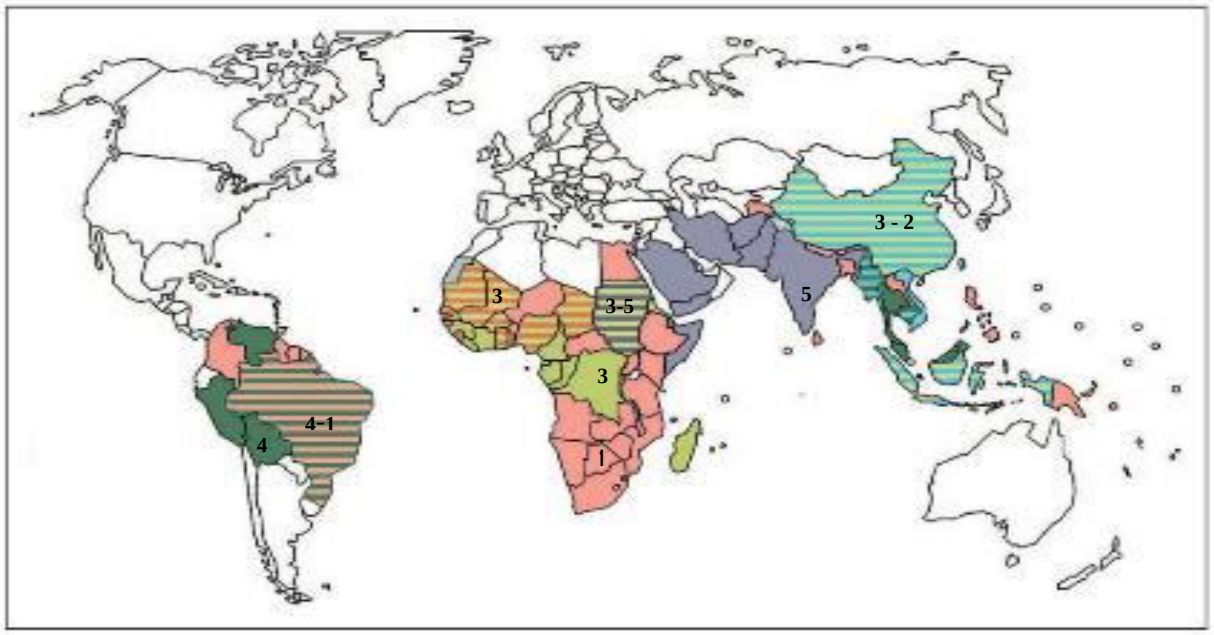
2.9.1.2. Classification according to the structure:

a. **Aryl amino alcohols:** Quinine, quinidine (cinchona alkaloids), mefloquine, halofantrine.
b. **4-aminoquinolines:** Chloroquine, amodiaquine.
c. **Folate synthesis inhibitors:** Type 1 – competitive inhibitors of dihydropteroate synthase – sulphones, sulphonamides; Type 2 – inhibit dihydrofolate reductase – biguanides like proguanil and chloroproguanil; diaminopyrimidine like pyrimethamine
d. **8-aminoquinolines:** Primaquine, WR238, 605
e. **Antimicrobials:** Tetracycline, doxycycline, clindamycin, azithromycin, fluoroquinolones
f. **Peroxides:** Artemisinin (Qinghaosu) derivatives and analogues – artemether, arteether, artesunate, artelinic acid
g. **Naphthoquinones:** Atovaquone
h. **Iron chelating agents:** Desferrioxamine (https://www.malariasite.com/malaria-drugs/) (White , 1996)..

2.10 Artemisinin and Artemisinin-based combination therapy (ACT)

The two most widely used artemisinin derivatives are artesunate and artemether, others are artetheer and dihydroartemisinin**.** While they are widely used in Southeast Asia, they are not licensed in much of the so-called "Western World", including Australia. It is now being combined with mefloquine for the treatment of falciparum malaria. In Africa, artemisinin combination therapy is being recommended (WHO, 2001). There are 5 ACTS: **1**-Artemether–

lumefantrine; **2**-dihydroartemisinin-piperaquine; **3**-artesunate-amodiaquine; **4** artesunate-mefloquine; **5**-artesunate– sulphadoxine/pyrimethamine



1-Artemether–lumefantrine; **2**-dihydroartemisinin-piperaquine; **3**-artesunate-amodiaquine; **4** artesunate-mefloquine; **5**-artesunate– sulphadoxine/pyrimethamine

Figure 2.3: Current global distribution of artemisinin-based combination therapies as the first-line treatment of uncomplicated falciparum malaria.
[http://www.who.int/malaria/publications/atoz/9789241500470/en/index.html].

2.11 Prophylactic drugs

Antimalarial medicines can also be used to prevent malaria. For travellers, malaria can be prevented through chemoprophylaxis, which suppresses the blood stage of malaria infections, thereby preventing malaria. For pregnant women living in moderate-to-high transmission areas, WHO recommends intermittent preventive treatment with sulfadoxine-pyrimethamine, at each scheduled antenatal visit after the first trimester. Similarly, for infants living in high-transmission areas of Africa, three doses of intermittent preventive treatment with sulfadoxine-pyrimethamine are recommended, delivered alongside routine vaccinations (WHO, 2018).

For travellers in areas with Chloroquine Sensitive *P. falciparum:* Chloroquine, to be started one week before exposure continued during exposure and for four weeks after that.

For travelers in areas with chloroquine-resistant *P. falciparum* (High degree, widespread): Chloroquine Plus Proguanil as above or Mefloquine, to be started 2-3 weeks before, continued during exposure and for 4 weeks thereafter or Doxycycline, to be started 2 days before, continued during exposure and for 4 weeks thereafter, or Atovaquone plus Proguanil, to be started 2 days before, continued during exposure and for 7 days thereafter (https://www.malariasite.com/prophylaxis/).

2.12 Vaccines against malaria

RTS, S/AS01 (RTS, S) – also known as Mosquirix – is an injectable vaccine that provides partial protection against malaria in young children. The vaccine is being evaluated in sub-Saharan Africa as a complementary malaria control tool that potentially could be added to (and not replace) the core package of WHO-recommended preventive, diagnostic and treatment measures (WHO, 2019).

In July 2015, the vaccine received a positive opinion by the European Medicines Agency, a stringent medicines regulatory authority. In October 2015, two WHO advisory groups recommended pilot implementation of RTS, S/AS01 in a limited number of African countries. WHO adopted these recommendations and is strongly supportive of the need to proceed with the pilot programme as the next step for the world's first malaria vaccine.

In November 2016, WHO announced that the RTS, S vaccine would be rolled out in pilot projects in selected areas in 3 countries in sub-Saharan Africa: Ghana, Kenya and Malawi. Funding has been secured for the initial phase of the programme and vaccinations are due to begin in early 2019. These pilot projects could pave the way for wider deployment of the vaccine if safety and effectiveness are considered acceptable (WHO, 2018).

2.13 Resistance to Artemisinin

Artemisinin is derived from a Chinese herbal plant called *A. annua* and covers a group of products. The two most widely used artemisinin derivatives are artesunate and artemether, others are arteether and dihydroartemisinin. Artemisinin and derivatives are used as the first line of defense to treat malaria this drug remain very efficient, but we are facing an emergency of resistance against this drug, which constitutes a huge threat.

2.13.1 Definition of artemisinin resistance

Artemisinin resistance is defined as delayed parasite clearance following treatment with an artesunate monotherapy (WHO; 2015) or with artemisinin-based combination therapy (ACT); this represents partial/relative resistance.

2.13.2 Possible consequences of artemisinin resistance

Artemisinin is used in combination with other medicines to reduce the parasite load quickly. The effects of partial/relative resistance could include:

1) The development of total artemisinin resistance;

2) Failure to rapidly clear parasites, which could compromise the use of artemisinin for the treatment of severe malaria; and

3) Slow parasite clearance in patients treated with an ACT, which could cause more parasites to be exposed to the partner medicine alone once the artemisinin component has been rapidly eliminated following the 3-day treatment course (WHO; 2017).

2.13.3 Prevalence of K13 mutations

It has been established that ACTs resistance is mostly related to mutations in the kelch13 propeller region of the parasite. Mutations have meanwhile raised to 90% (Timothy *et al*., 2016).

To date, more than 200 non-synonymous mutations in the K13 gene have been reported. Distinct alleles with K13 mutations originating from multiple independent emergence events have been observed in South-East Asia. The KARMA project has reported frequent C580Y, R539T, Y493H and I543T mutations in the eastern GMS (Greater Mekong Subregion) (Cambodia, Lao PDR and Viet Nam) and frequent F446L, N458Y, P574L and R561H mutations in the western GMS (China, Myanmar and Thailand). The P553L allele has been found in the two areas. There is evidence that selective sweeps have occurred throughout the GMS. Currently, the K13 C580Y mutation can be found in several genetic backgrounds (haplotypes) throughout the GMS. The prevalence of one specific K13 C580Y haplotype is increasing, replacing other haplotypes in an area that includes sites in western Cambodia, north-eastern Thailand and southern Lao PDR. This indicates a selective sweep in this part of the GMS. However, the frequencies of different K13 C580Y haplotypes vary by region, and no single haplotype is dominant throughout the GMS.

In Africa, non-synonymous K13 mutations are still rare and highly diverse. A very low (1.8%) prevalence (5/283, 1.8%) of non-synonymous K13 mutations has been reported in Angola, Burkina Faso, Cameroon, Central African Republic, Comoros, Congo, Cote d'Ivoire, Democratic Republic of the Congo, Equatorial Guinea, Ethiopia, Gabon, Gambia, Kenya, Liberia, Madagascar, Malawi, Mali, Mozambique, Niger, Nigeria, Rwanda, Senegal, Sierra Leone, Togo, Uganda and Zambia (WHO; 2017).

2.13.4 Resistance to artemisinin in South East Asia

ACTS is the first line of defence against malaria, but many episodes of resistance have been detected around some part in the world in South-Eastern Asia; Africa and in South America.

Artemisinin resistance likely emerged at the border between Thailand and Myanmar in 2001, but it was only clearly identified in 2008; Since 2009, available data have shown that parasite clearance times are consistently delayed in a significant proportion of patients treated with ACTs; delayed clearance has been observed with all three first-line ACTs (artemether-lumefantrine, artesunate-mefloquine and dihydroartemisinin-piperaquine) (WHO; 2017).

A K13 F446I propeller polymorphism is significantly associated with delayed parasite clearance. Surveys indicate that there is a high prevalence of the K13 F446I mutation along the China–Myanmar and India–Myanmar borders. This mutant appears to be associated with an intermediate rate of delayed clearance, and additional in vitro research is ongoing to validate its role in artemisinin resistance (WHO; 2017).

In Thailand; the efficacy of artemether-lumefantrine was evaluated in two provinces in 2012, revealing treatment failure rates close to or greater than 10%. In Viet Nam: A study in 2015 in Binh Phuoc province reported a high treatment failure rate (> 10%) following treatment with dihydroartemisinin-piperaquine. Investigation has confirmed the emergence of piperaquine resistance. In Cambogia: Artemisinin resistance was first identified in clinical studies in 2006; however, the retrospective analysis of molecular markers has indicated that artemisinin resistance likely emerged prior to 2001 and the widespread deployment of ACTs in Cambodia; In 2017, artesunate-amodiaquine studies reported significant treatment failure rates, which indicates that five ACTs are failing in Cambodia (WHO; 2017).

There are currently five ACTs recommended by WHO: artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine- pyrimethamine (AS+SP) and dihydroartemisinin-piperaquine. A sixth ACT, artesunate-pyronaridine, was given a positive

scientific opinion by the European Medicines Agency (EMA) under article 58 and is being considered for recommendation by WHO. By default, AS+SP is considered to have a high failure rate in the region because of high treatment failure rates with sulfadoxine-pyrimethamine and/or because quadruple and quintuple Pfdhfr and Pfdhps mutations (which are usually fixed) have been reported in the region (WHO, 2017).

2.13.5 Resistance to artemisinin in Africa

The most frequent allele observed in Africa is A578S. This allele is associated with clinical or in vitro resistance to artemisinin. A high number of mutations, including some related to delayed clearance in the GMS (in particular K13 C580Y), have been reported in Africa. However, many of these mutations reported in Africa have not expanded in the African parasite populations. A recent case report (Lu *et al.*; 2017) warned of the emergence of artemisinin resistance in Equatorial Guinea, yet further investigations are needed before the K13 mutation found (M579I) can be confirmed as an artemisinin resistance marker ; due to many confounding factors, a single case with incomplete data is not sufficient to establish the presence of resistance.

Artemisinin resistance in *Plasmodium falciparum* poses a threat to the control and elimination of malaria. Mapping the geographic extent of resistance is essential for planning containment and elimination strategies.

2.14 Recrudescent of artemisinin:

Recrudescence with oral artemisinin monotherapy had been observed in Vietnam in 2001 (Giao *et al.*, 2001). A recent paper from Mali is alarming. What worries the authors of the 2016 study from Mali is not only that similar results were found in a study in 2002-2004, but the fact that baseline gametocyte carriage was significantly higher 6 years after the deployment of ACTs in this setting. If artemisinin derivatives enhance recrudescence and gametocyte carriage, this is indeed alarming. It would mean that ACTs would enhance malaria transmission in the long run (Abdoulaye *et al.*, 2016).

Artesunate a derivate of artemisinin does not clear mature gametocytes during oral artesunate treatment and does not prevent the appearance of new gametocytes. This confirms, to a large extent the randomised, double-blind, large-scale clinical trials run in Maniema-Congo end of last year (Lutgen, 2016). It is well known that artemisinin drugs are gametocytocidal for immature, but not mature gametocytes.

2.15 Artemisia afra genus

Many plants from the *Artemisia* genus are used throughout different cultures as traditional medicine. The genus' name is derived from the Greek goddess Artemis, who gave Artemisia plants to Chiron the Centaur (Wright, 2002). *Artemisia* is one of the largest genera in the family of the Asteraceae and also one of the most widely distributed (El-Sahhar, 2010). There are a total of over 300 species, with the majority located in China (150 species), ex-USSR (174 species), and Japan (50 species) (Wright, 2002). The number of species in Europe totals 57 species (Stach *et al.*, 2007). *Artemisia ssp*. are often aromatic herbs or low shrubs (Wright, 2002). *Artemisia* species serve a variety of uses such as ornamental decoration, flavouring, perfume, and of course, as medicinal plants (Wright, 2002). The most well-known Artemisia species is *Artemisia annua*, has made headlines a few years ago as a completely novel treatment for malaria.

A. annua came under the spotlight during the Vietnam War. Viêt-Cong who operated in swamps and rain forests lost more soldiers by mosquito bites than by American bullets. Ho Chi Min turned to China for help. Researchers at the Chinese Institute of Material Medicine had found a region of China that reported no malaria cases, and when they investigated, they discovered that its people drank a decoction of *A. annua* at the first symptoms of malaria. And actually, wild Artemisia has been used for millenniums in several regions of China and is still used to treat fevers and malaria. It was easy to acquire tons of this dried herb for Viêt-Cong. Taken as an infusion, it worked wonders (Puotinen, 2003).

Artemisia afra is also one of the most important and widely used herbs in that species mostly in traditional medicine. In recent years, it has gained significant attention from the scientific community. Studies have been conducted either to verify or substantiate the traditional use of this herb. Further, its use is also being investigated in modern diseases like diabetes, cardiovascular diseases, cancer, respiratory diseases etc. (Patil *et al*., 2011).

The significance of genus *Artemisia* is seen in its number of hits, which is 89,080. The total number of hits appeared for "*Artemisia afra*" (*A. afra*) were 885 of which, 5 had no dates. Figure 5 is the graph of 162 publications that appeared in Journal Sources classified and plotted on a yearly basis from Jan. 1922 to Nov. 2011 for "*A. afra"* (Patil *et al*., 2011).

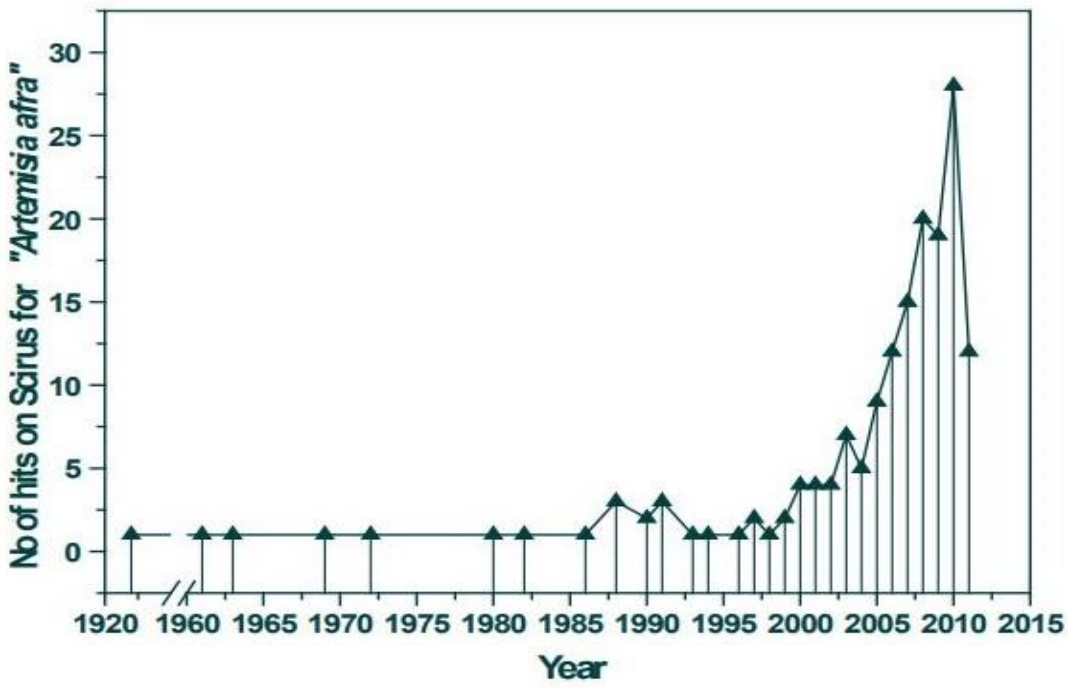


Figure 2.4: Increase in number of results on Scirus, a search engine for scientific articles, for *Artemisia afra*. [omicsonline.org/2153-0645/2153-0645-2-105.php]

2.16 Artemisia afra common names and geographical distribution

Artemisia afra is known by many names like "African wormwood" in English "Umhlonyane" in Xhosa, "Mhlonyane" in Zulu, "Lanyana" in Sotho, "Lengana" in Tswana, "Wilde als" in Africaans, "Koddoo-adi" & "Chugughee" in Ethiopia (Burits *et al*., 2001; Nibert *et al*., 2010; van der Walt, 2004; Mesfin *et al*., 2009). It is also known by other names viz., ALS, Wild wormwood, Fivi, Lusanje, Luyanga, Iliongana (FAO, 1993).

A. afra is a herb growing in the high land areas of Eastern and Southern Africa altitudes ranging between 1500 and 3000m where the soils range from volcanic ash, loamy sands, to sandy or calcareous clay loams of volcanic or granitic origin (FAO, 1993; Iwu, 1993). The plant grows in the South and Eastern regions of the continent and has been located in Ethiopia, Kenya, Tanzania, Zaire, Zambia, Zimbabwe, Angola and the Republic of South Africa (Watt *et al*., 1932; Iwu, 1993). In South Africa, it usually grows in rocky mountainous areas along forest margins, and stream sides and its natural distribution extends from the Northern and Eastern Transvaal to the Western Cape, except the Northern Cape (Mukinda, 2005). It is also predominantly found in Asia, Europe and North America (Tan *et al*., 1998; Mucciarelli *et al*., 2002; Patil *et al*., 2019).

2.17 Plant description

Artemisia afra is a medium-sized multi-stemmed, clump-forming woody perennial shrub, which grows up to 2 meters in height with a leafy, hairy ridged stem (Van, *et al*., 1997; Van *et al.*, 2004). Its soft leaves are finely-divided (like a fern), are silver-grey due to the presence of fine hairs reaching in length up to 80 mm and width up to 40 mm arranged alternately, oval in shape (Van *et al.*, 2000; Hutchings *et al*., 1996; Dyson, 1998). The plant has an easily identifiable aromatic odour and smells pungent and sweet after bruising (Liu *et al*., 2009).

Figure 2.5: Artemisia afra plant

2.18 Artemisia afra common habitat

A. afra is very drought resistant and hardy (Van *et al.*, 2008; Patil et al., 2019) common to arid soils (Greenham, 2000), open to the sunny situation with light, well-drained soil (Greenham, 2000), needing water occasionally (Van *et al.*, 2008). The optimal temperature and annual

rainfall as described in FAO *Artemisia afra* Data Sheet are 22-33°C and 550-750 mm, respectively (FAO, 1993).

2.19 Geographical variation

The main components of the volatile secondary metabolites in *A. afra* varied enormously in plants from different geographical regions. The principal constituent in Ethiopian oil (Worku *et al*., 1996) was artemisyl acetate (24.4–32.1%) while it was 1, 8-cineole (67.4%) in Kenyan oil (Mwangi *et al*., 1995). In Zimbabwean oil, α- and β-thujone (52%) was the major constituent (Graven *et al*., 1992) while α-thujone (54.2%) was in South African oil (Libbey *et al*., 1989; Patil et al., 2019).

2.20 *Artemisia afra* a potential flagship for African medicinal plant

Research run in Zimbabwe, South Africa, and Ethiopia proves the big antimalarial effect of *A. afra*. When compared with other medicinal plants, *A. afra* is always the strongest against malaria. (Moges *et al.,* 1998; Kraft *et al.,* 2003; Clarkson *et al.,* 2004*;* Gathirwa *et al.,* 2007)

A. afra tea also has proven to be very efficacy against malaria. The fact that the beverage of *A. afra* is as effective as that of *A. annua* shows that artemisinin is not essential to treat malaria and that other active ingredients present in numerous species of *Artemisia* play a role.

A freshly prepared infusion probably contains all the constituents of the plant, which have an antimalarial effect. A true polytherapy which explains why the cure rate in all trials in a dozen African countries is >95%, much higher than for any ACTs (artemisinin combined therapy) which are not more than the combination of two monotherapies without synergy but with resistances (www.iwerliewen.org/).

The debate on *in vitro* versus *in vivo* is linked to the debate on efficacy versus effectiveness. Ideally, one can only talk about efficacy in laboratory conditions because the experiment can be fully controlled. Measuring plasma levels of drug alone is not sufficient for efficacy because of many other factors such as food, immune status, environment, and genetics etc. influence drug efficacy. Therefore effectiveness describes the real-life situation, but the problem is that mathematically we cannot compute real-life situations, and this is why most studies calculate and report efficacy by having treatment group versus control group data compared. Even then, inter-human and intra-human variations cannot be controlled entirely (Elfawal., 2012).

When consumed consciously and systematically, many wild plants are essential for human health because of their constituents. The effect of essential oils of plants on microorganisms *in vivo* cannot be predicted from *in vitro* assays as concluded by the paper hereafter (Sevil, 2007).

Even more, *in vitro* trials may show the absence of an effect which is well present *in vivo*. The University of Leiden found that *Artemisia afra* aqueous infusion has no in vitro antimalarial effect, but numerous clinical trials in Africa show that it has a strong effect (Mouton *et al.,* 2013).

In this study, a virtual screening was run with the compounds found in *Artemisia afra* after a GCMS and the fab enzymes (FabI and Fab Z) from the malaria parasite (W2 and D6).

2.14 Virtual screening.

Docking simulations and virtual screening are being routinely used in drug design, enabling rapid identification of hits and lead compounds (Morris *et al*., 2008; Huang *et al*., 2010). The goal of docking simulations is to determine the binding mode (bound conformation) and the strength of binding (binding affinity) between a ligand, which is typically assumed to be a small molecule, and a macromolecular receptor, such as a protein (Morris *et al*., 2008; Huang *et al*., 2010; Petrenko *et al*., 2009). Given a resolved or modelled structure of a target receptor, virtual screening involves performing docking simulations for a large number of candidate compounds in order to identify putative leads (Ripphausen *et al*., 2011; Petrenko *et al*., 2009; Kellenberger et al. 2004). These candidates can subsequently be characterised and validated by empirical binding and activity assays, and by assessing their toxicity, pharmacokinetics and other properties for further drug development (Fang *et al*., 2001; Barrett *et al*., 2008).

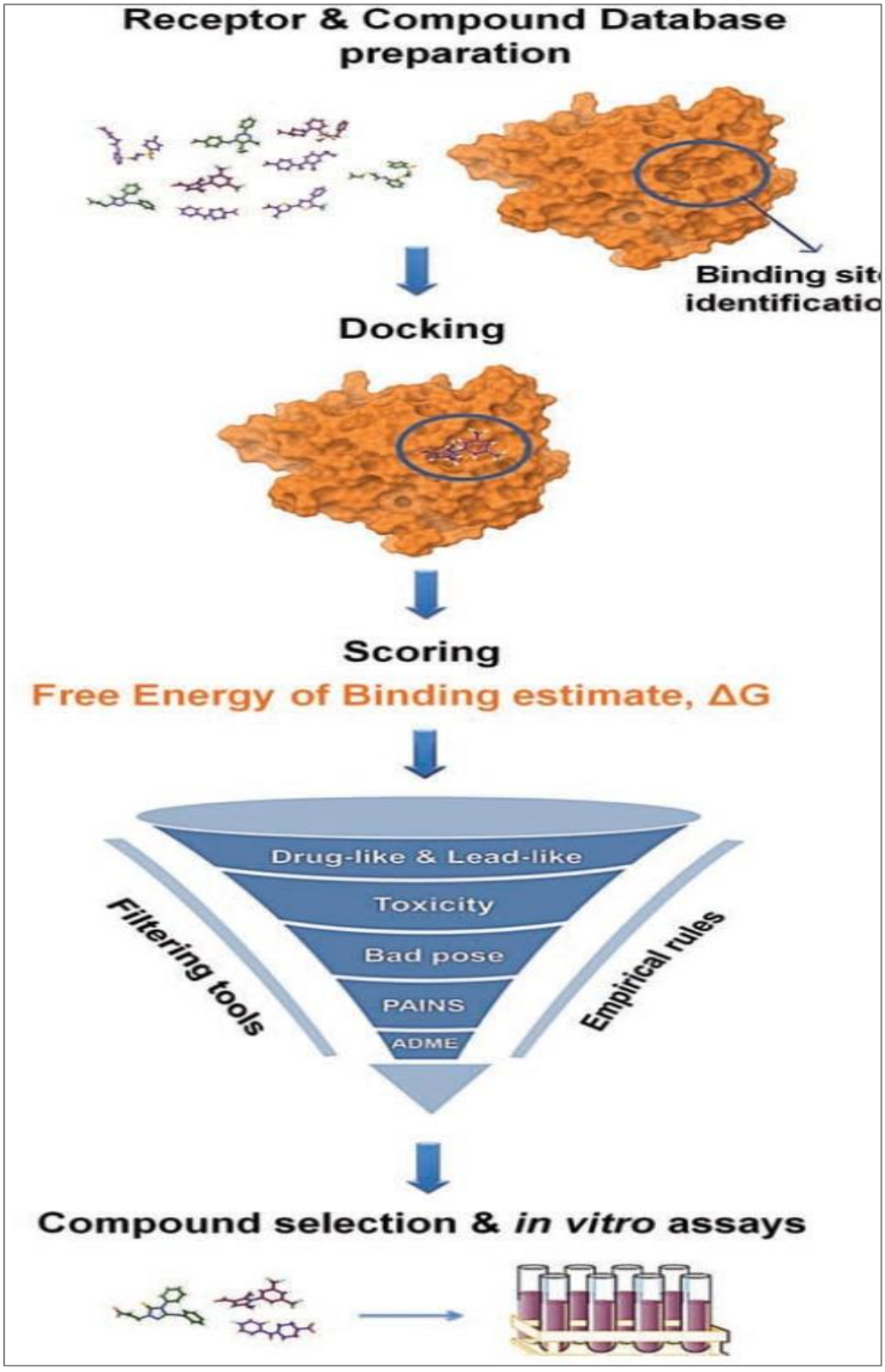


Figure 2.6: Virtual screening workflow

(Evanthia *et al.*, 2014)

Following library and receptor preparation, each compound in the library is virtually docked into the target binding site with a docking program. Docking aims to predict the ligand-protein complex structure by exploring the conformational space of the ligands within the binding site of the protein. A scoring function is then utilized to approximate the free energy of binding between the protein and the ligand in each docking pose. Docking and scoring produce ranked compounds, which are then post-processed by examining calculated binding scores, validity of generated pose, undesirable chemical moieties, metabolic liabilities, desired physicochemical properties, lead-likeness, and chemical diversity. Post-processing results in a small number of selected compounds, which proceed to experimental assaying (Lavecchia *et al*., 2013; Reddy *et al*., 2007; Cheng *et al*. 2012; Köppen *et al*., 2009)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

W2 and D6 lab strains from KEMRI,. The equipments used are the following: Centrifuge; plates; Autoclave, fridge to store samples; Eppendorf tubes (1.5 ml, 2 ml); GC-MS (liquid chromatography mass spectrometry) for identification of second metabolite compounds from the our plants extract; Microscope to calculate parasiteamia; rotary evaporator, freeze dried, uv spectrophotometer, real time qPCR machine etc.

3.2 *Artemisia afra* plants

Five samples of leaves of *A. afra* was collected each from different sources from five different countries in Africa: in Stellenbosch, Western Cape, at South Africa, in Tanga region at Rushoto district, East coast of Tanzania, in Kenya at Multiplant conservation Garden in GiGiri, in Senegal at Sebikotane (offspring collected from South Africa then growing in Togo then in Senegal), and in Burundi at Rumonge South Burundi. The leaves were harvested before blooming and dried under shade before being sent to Nairobi in paper bags, then kept at room temperature until extraction.

3.3 Mice

Fifty six Swiss albinos were used in the *in vivo* antimalarial test and thirthy mice in the acute toxicity test. Mice were provided by KEMRI animal house and Kept in Makrolon type II cages. All animals had free access to standard pellet diet and tap water *ad libitum*, and was provided with bedding of clean paddy husk.

3.4 Extraction process of powdered leaves of Artemisia afra

3.4.1 Sample preparation

Five samples of leaves of *A. afra* collected from five countries in Africa (Senegal, Kenya, Tanzania, Burundi and South Africa) were grounded and then extracted with four solvents of different polarity ethanol; hexane, dichloromethane, and water.

3.4.2 Ethanolic, Hexane, DCM Extraction Process

Samples of dried and powdered aerial part of *A. afra* weighing each 125 gm were extracted with 600 mL of Ethanol (100%), Hexane (85%) and DCM (99.9%) in a flat bottomed flask and

mixed on an orbital shaker. After gentle maceration for 48 hours, the extracts were filtered through Whatman filter paper n°1. The filtrate was concentrated under reduced pressure using rotary evaporator at 20 rpm and 40°C bath temperature. Finally, concentrated extracts were collected in vials and placed on a water bath at 40°C to evaporate the remaining solvents and stored at room temperature for complete dryness.

3.4.3 Aqueous Extraction

The powdered aerial part of *A. afra* weighing 125 gm was mixed with 800 mL distilled water in a flat bottomed flask on an orbital shaker for 48 hours. After gentle maceration for 48 hours, plant materials were filtered with Whatman paper N°1. The obtained filtrate was frozen at −80°C and lyophilized (freeze-dried) using lyophilizer to collect the crude aqueous extract

3.5 Phytochemical screening

The crude methanolic, hexanolic, DCM and Water extracts of the leaves were tested for the presence of alkaloids, tannins, saponins, terpenoids and glycosides. The qualitative results are expressed as (+) for positive results and (-) for negative results.

3.5.1 Test for alkaloids

Few mg (about 10mg) of each extract was separately stirred with 1% HCl (3mL) on a water bath for 5min and filtered.

Wagner's test was performed: Potassium iodide (2g) and iodine (1,27g) were dissolved in distilled water (5ml), and the solution was diluted to 100 mL with distilled water. Few drops of this solution were added to the filtrate; a brown coloured precipitate indicates the presence of alkaloids (Joshi et *al*., 2013; Abdullah et *al*., 2013).

3.5.2 Test for terpenoids

Salkowski test: the crude extracts (about 10mg for each extract) were separately shaken with chloroform (2mL) followed by the addition of concentrated H_2SO_4 (2mL) along the side of the test tube, the reddish-brown colouration of the interface indicates the presence of terpenoids (Ayoola *et al*., 2008).

3.5.3 Test for tannins

Each extract (about 200mg) was separately stirred with distilled water (3mL) and then filtered. A few drops of 5% ferric chloride was then added. Black or blue-green colouration or

precipitate was taken as a positive result for the presence of tannins (Banso and Adeyemo, 2006).

3.5.4 Test for saponins

Each of the plant extracts (200mg) was separately shaken with distilled water in a test tube. The formation of frothing, which persists on warming in a water bath for 5min showed the presence of saponins (Banso and Adeyemo, 2006).

3.5.5 Test for glycosides

Keller-Killiani test: Each of the plant extract (200mg) was shaken with distilled water (3mL). To this glacial acetic acid (2mL) containing few drops of ferric chloride, was added following by H_2SO_4 (1mL) along the side of the test tube. The formation of a brown ring at the interface gave a positive indication for cardiac glycoside, and a violet ring may appear below the brown ring (Ayoola et al., 2008).

3.6 Determination of total phenolic and total flavonoid contents

3.6.1 Total phenolic content

The total phenolic content contents of the extracts were estimated using the Folin Ciocalteu colourimetric method (Velioglu *et al*., 1998; Cai *et al*., 2004; Chlopicka *et al*., 2012). The calibration curve was plotted by mixing 0.2mL aliquots of 50, 100, 200, 300, 400, 500, 600 ug/mL Gallic acid solutions with 1,25mL of Folin Ciocalteu reagent (diluted ten-fold) and 1mL of sodium carbonate solution 7.5%. The absorbance was measured after 30 min at 765 nm using a UV/Spectrophotometer 6800 GenWay. For each extract 0.2ml (1gm/100mL) aliquot was mixed separately with the same reagent, as performed for the construction of the calibration curve. After 1h, the absorbance was measured to determine the total phenolic contents in all the extracts separately using the formula:

Conc. = C1 x V/M

Where:

Conc. = Total phenolic content in mg of GAE/g of extract. GAE (Gallic acid equivalent)

C1= Concentration of gallic acid established from the calibration curve in mg/ml.

V= Total volume of the extract in ml.

M= weight of the plant extract in g

3.6.2 Total flavonoid content

Total flavonoid content was determined using the aluminium colourimetric method (Chang *et al*., 2002; Rathore *et al*., 2006) with some modifications using the standard rutine. A calibration curve (Figure 8) of rutine was prepared in the range of 0-1000ug/ul. Briefly extract (0,25mL) and standard (0,25mL) were placed in different test tubes and to each 10% of aluminum chloride (50uL) was added and potassium acetate 1M (50uL). 80% of methanol (0,75mL) and distilled water (1,4mL) were added and mixed. Blank was prepared in the same manner where 0.25mL of distilled water was added instead of the sample or standard, and the amount of aluminium chloride was also replaced with distilled water. All tubes were incubated at room temperature for 30min. The absorbance was taken at 415nm. The concentration of flavonoid was expressed as mg rutine per gram of extract. All the test was performed in duplicate.

3.7 Antioxidant activities (DPPH assay)

The assay was done as described by Clark *et al*. in 2013. Briefly, 20 ul of extract diluted appropriately in 2% of DMSO was mixed with 180ul of DPPH in methanol (conc. = 40ug/ml) in wells of a 96- well plate. The plate was kept in the dark for 30min, after which the absorbance of the solution was measured at 490 nm in an ELx800 Absorbance reader. Appropriate blanks were used DMSO and standard (L-ascorbic acid solution in different concentrations 5, 10, 50, 100ug/ml) were run simultaneously with our extracts solutions in triplicates. Several concentration of extracts (10, 30, 100, 300, 1000ug/ml) were accessed.

3.8 *In vitro, in vivo* antimalarial essay and acute oral toxicity

3.8.1 Parasite cultures and *in vitro* antimalarial essay

The different extracts were tested against two *P. falciparum* strains W2 (CQ-resistant) and D6 (CQ-sensitive) lab strains provided by KEMRI (Kenya Medical Research Institute). The parasite was cultivated by a previously described *in vitro* technique (Trager *et al*., 1976). The culture medium consisted of RPMI 1640 supplemented with 10% human serum (Schlichtherle *et al*., 2000). Uninfected human red blood cells group O Rh-positive erythrocytes (<28 days old) served as host cells. Cultures were maintained at 6% hematocrit with changes of culture medium every 48 hours and diluted with uninfected red blood cells when the parasitaemia exceeded 2%. The cultures were incubated at 37 °C in an atmosphere of 3% CO_2, 5% O_2 and 92% N_2 (BOC, Nairobi).

Briefly, aliquots of the culture medium (25µl) were added to all the wells of a 96-well flat-bottomed micro-culture plate. Aliquots of the test solutions (25µl) were added in triplicates to the first wells, start concentration was 200µg/µl following by two-fold dilutions for each sample. 200 µl of parasite culture with 0.5% parasitaemia and 1.5 hematocrits was added to each well. Two hundred microliters of culture media without parasites were added into four wells on the last row of each plate to serve as a background. Parasitized and non-parasitized erythrocytes were incubated at 37°C in a gas mixture, 3% CO2, 5% O2 and 92% N2 for 48 h after which 25 ul of culture medium containing 0.5 µCi of [G-3H] hypoxanthine was added to each well. The culture plates were further incubated for 18 h-24h. At the end of the incubation period, the radio-labelled cultures were harvested onto glass-fiber filters using a 96-well cell harvester.[G-3H]hypoxanthine uptake was determined using a microbeta trilux liquid scintillation and luminescence counter. The drug concentration capable of inhibiting 50% of the *P. falciparum* (IC50) was determined by logarithmic transformation of drug concentration and radioactive counts per minute (CPM) using the formula:

IC50 = antilog (log X1 + [(log Y50 - log Y1) x (log X2 - log X1)] / (log Y2 - log Y1))

Where Y50 is the cpm value midway between parasitized and non-parasitized control cultures and X1, Y1, X2 and Y2 are the concentrations and cpm values for the data points above and below the cpm midpoints (Sixsmith *et al*., 1984).

3.8.2 Oral acute toxicity for *Artemisia afra* plant collected from Burundi

Crude extracts of *A. afra* extracted with dichloromethane; hexane and ethanol were tested for their oral acute toxicity LD_{50} was determined for each extract. Effect of the extracts on the Body weight, organ-weighs and also on the liver where tested. Ethical approval was given by ACUC (Animal Care and Use Committee) in KEMRI (Kenya Medical Research Institute) to carry out that work. The mice were used and handle with respect to animal care and ethics.

3.8.2.1 Experimental animals:

The animals used in this study were provided by KEMRI animal laboratory facility. The animals were kept for 24hours in the acclimatization room before starting the experiment to avoid any stress. Thirty healthy adult male and female of swiss albinos mice weighing between 20- 22g were used for the acute toxicity test. Mice were randomly assigned into 4 groups divided in 10 cages, 3 animal per cages. Male and female were separated to avoid them from mating. The animals were kept in makroloton type II cages and provided with bedding of clean

paddy husk. All animals had free access to water and food. The three extracts of *Artemisia afra* dissolved in 5% DMSO and PBS, were prepared for the acute toxicity test, three concentrations were made for each extract 2500 mg/kg; 2000mg/kg; 1000mg/kg. Selection of doses was made based on the OECD guideline (OECD, 2011). Mice were separated into 10 cages each cage had three mice, 1 cage for control where the mice were inoculated with a mixture of phosphate buffer saline 1X with 5% DMSO and 9 others cages where every mice was inoculated with the extracts with only one single dose for each. The body weight of each mice was recorded before dosing. The first set, from cage 2 to 4 received DCM extract with respectively three designated dosages (1000mg/kg, 2000mg/kg 2500mg/kg of the formulation per body weight), the second set, from cage 5 to 7 received ethanolic extract with respectively three designated dosages (1000mg/kg, 2000mg/kg 2500mg/kg of the formulation per body weight), and the last set, from cage 8 to 10 received hexanolic extract with respectively three designated dosages (1000mg/kg, 2000mg/kg 2500mg/kg of the formulation per body weight) to see a range of toxic effects and mortality rates in 48 hours observation (table 3.1).

Table 3.1: Experimentation on mice

Groups	Group0	Goup1 DCM extracts			Group 2 EtOH extracts			Group3 Hexane extract		
Cages	Cage 1 Control n=3	Cage2 n=3	Cage3 n=3	Cage4 n=3	Cage5 n=3	Cage6 n=3	Cage57 n=3	Cage8 n=3	Cage9 n=3	Cage10 n=3
Dosages (mg/kg)	PBS	1000 mg/kg	2000 mg/kg	2500 mg/kg	1000 mg/kg	2000 mg/kg	250000 mg/kg	1000 mg/kg	2000 mg/kg	2500 mg/kg

n=number of mice; DCM = dichloromethane, EtOH = ethanolic. PBS= phosphate buffer saline

After the single dose inoculation, mice were observed for 14 days toxicity signs and weight of animals were recorded every day. At the end of the experiment, mice were sacrificed using a CO2 chamber and put on dissecting board where the organs were collected, and the blood was drawn by cardiac puncture and kept in a vacuum heparin tube.

The blood samples in the tubes were centrifuged at 2500 rpm for 15 minutes, and the serum was drawn and transferred into other clean vials and kept at -20°C until analysis for clinical chemistry measurements. The organs (Liver, Kidneys; and spleen) were collected and kept in phosphate buffered formalin solution for tissue processing, and the gross pathological observation of these organs was performed to check for any lesions.

3.8.2.2 Tissue harvesting and processing for histopathology

The organs (liver, spleen, and kidney) from the mice were preserved and fixed in 10% neutral buffer formalin solution. They were then dehydrated with different concentration of alcohol from 50% to 100% of Isopropanol. The tissues were dehydrated during 45mn in each solution of Isopropanol from 50% to 80%, then during 1hours in 3 solutions of Isopropanol 80%; 90%; and 100%. After dehydration, the tissues were cleared using xylene to make it clear and transparent. The tissue was taken to several jars containing xylene as follows each taking 30 min Jar I, Jar II, and Jar III of xylene. The tissue was then impregnated with paraffin wax; this was done by using three different Jars of molten paraffin wax or 45mn each.

Next, the tissues were embedding; this was done at the embedding machine by using a metal base then molten wax is poured into it. The plastic tissue tek containing the tissue was opened using a warm forceps and transferred into the wax in the metallic metal base. After a proper orientation of the specimens, all tissue blocks were labelled and allowed to harden at room temperature for 30mn. Tissue blocks were sectioned using microtome into small slices of 5µm which the light of a microscope can pass through. The ribbon of the tissue section was gently collected and laid onto the surface of a water bath heated at 40°C. The straightened tissue in the water bath is fished out by the use of a frosted end glass slide, then labelled and taken to a hot air oven at 60°C to melt the wax and exposed the tissue. The slide was taken to xylene to remove the wax which melted in the hot air oven, then to descending gradient of alcohol and stain with Haematoxylin and Eosin stain method then mounted to DPX. Damage on tissue was assessed using a microscope.

3.8.3 *In vivo* antimalarial test of Burundi extracts

Fifty-six Swiss albinos' mice were used for *in vivo* antimalarial assays. The mice were infected with 2x10 7 of parasitized red blood cells (*P. berghei anka),* and the assay was based on 4 days Peter Test (Peters *et al*., 1975). The parasite was provided by KEMRI and passage several times using donor's mice by intraperitoneal injection (IP) way. Briefly, an aliquot of 0.2 ml of infected blood from donor's mice was used to infect the experimental mice through intraperitoneal injection. The mice were infected with the same amount of parasitized erythrocytes and divided

randomly in a group of 4 individuals. Fourteen groups were made among which negative and positive group.

Two to 4 hours after infection (Day 0) mice were treated daily during 4 days with a single dose of the test sample at a volume of 0.2ml by oral route. The positive group was treated with artemether 10mg/kg of body weight dissolved in tween 80, and the negative group was given of saline buffer constitute with PBS buffer, DMSO (5%) and 10% of tween 80. The remaining experimental groups were treated with *Artemisia afra* extracts. All the extracts were dissolved in DMSO and in Tween 80 and 4 dosages were made for DCM; hexanolic extracts (400 mg/kg, 200 mg/kg, 100 mg/kg, 50 mg/kg) and for ethanolic extracts (200 mg/kg, 100 mg/kg, 50 mg/kg, 25mg/kg).

Parasitaemia was determined daily (24 hours interval) with a thin blood smear sampled from the tail and stain with 10% of Giemsa solution. At the end of the 4 days peter's test, thin blood smear was similarly made (96 h post-infection) for all the animals and stained as described above. Parasitaemia was calculated under a microscope by counting four fields of approximately 200 erythrocytes per field. The difference between the mean value of the control group (taken as 100%) and those of the experimental groups was calculated and expressed as the percentage of Chemo-Suppression, according to the following formula (Gathirwa *et al*., 2007):

%Chemo-Sup. = [(A-B)/A] x 100

A= Mean parasitaemia in the negative control group on day 4.

B= Corresponding parasitaemia in the test group.

3.9 Gas Chromatography Mass Spectrometry (GCMS) Analysis of *Artemisia afra* Plant Extracts collected from Burundi

Artemisia afra collected from Burundi was collected and extracted with three solvents: Dichloromethane, Ethanol, and Hexane. The extracts were dried using rotary evaporator and kept in the oven at 37C until complete evaporation of the solvents. Pure artemisinin crystal was used as a standard to test any presence of the compound in *Artemisia afra* extracts.

3.9.1 Sample Preparation

Samples were dissolved and diluted in suitable organic solvents ie dichloromethane and ethanol extracts were dissolved in methanol solvent, while hexane extract was dissolved in hexane

solvent) and passed through carbon black to remove waxes and chlorophylls. The samples were then filtered through 0.45 µm PTFE filters then transferred to sample vials for GCMS analysis.

3.9.2 Artemisinin standard

1mg of pure artemisinin was dissolved in 2ml of dichloromethane solvent and inject through the GCMS machine to determine the chromatogram peak of the standard and his retention time. This information was used to determine any presence of the compound in the *Artemisia afra* extracts. Same GCMS parameters were used for both for the artemisinin and for the extracts

3.9.3 GCMS Method

A Shimadzu QP 2010-SE GCMS coupled to an autosampler was used for the analysis. Ultrapure He (99.999 %) was used as the carrier gas at a flow rate of 1ml / minute. A BPX5 non-polar capillary column, 30m; 0.25 mm ID; 0.25 µm film thickness, was used for separation. The GC was programmed as follows: 60 ˚C; 10 ˚C /min to 250 ˚C (10 minutes). Total run-time was 30 minutes. Only 1 µL of the sample was injected. The injection was done in split mode, 10:1. Injection was done at 200 ^{0}C. The interface temperature was set at 250 ^{0}C. The EI ion source was set at 200 ^{0}C. Mass analysis was done in full scan mode, 50-550 m/z. A solvent delay time of 2 minutes was used.

3.10 Gene expression study for Fab_Z and Fab_I from *P. falciparum* after exposition of the parasites to the crude extracts.

Cultures of *Plasmodium falciparum* (W2 and D6) with a parasitaemia of 4% were incubated with *Artemisia afra* extracts collected from Burundi, e.g.: Burundi ethanolic, hexane and dichloromethane extract to run an inhibition test.

Artemisia afra solution was made for all the extracts. 100 mg of extracts were dissolved in 200ul of DMS; double distilled water was used to dilute the extracts. 20ml of double distilled water was added to the final concentration of 5ug/ul; then the solution was sterilized by filtering it with a microfilter of 0.45um pore size.

- 6ml of W2 culture were incubated with 666ul of *A. afra* DCM extract
- 6ml of W2 culture were incubated with 666ul of *A. afra* EtOH extract
- 6ml of W2 culture were incubated with 666ul of *A. afra* hexane extract
- 6ml of negative control with 666ul CMS

- 6ml of D6 culture were incubated with 666ul of *A. afra* DCM extract
- 6ml of W2 culture were incubated with 666ul of *A. afra* EtOH extract
- 6ml of W2 culture were incubated with 666ul of *A. afra* hexane extract
- 6ml of W2 culture were incubated with 666ul CMS

The parasite was then kept for 2 days in the incubation room. After the inhibition test is done, the parasite was kept at -80°C. The samples were used later for RNA extraction to run gene expression of FAB_Z and FAB_I in each sample.

3.10.1 RNA extraction:

After the inhibition test was done, the total RNA for all samples (W2 and D6) was extracted by following the method of trizol reagent Invitrogen Company (http://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf).

The parasites were thawed in ice, the whole blood was centrifuged, and the supernatant was discarded. The pellet was lysed using 1ml Trizol, then Incubated at 37°C for 5 min to ensure the complete deproteinization of nucleic acids. 200ul of Chloroform was added, the tube was shaken vigorously by hand followed or using a vortex then centrifuged during 15 min at 12,000g at 4^{o}C. The upper aqueous phase was carefully removed and placed in a new tube. Isopropanol was added (to precipitate the RNA) then mixed and centrifuged at 12,000g at 4^{o}C during 15mn. The supernatant was discarded and the pellet suspended with 1ml of 75% ethanol then vortex briefly and centrifuge for 10mn at 7,500 g 4°C. The supernatant was discard and the pellet air dry for 10mn. The RNA was resuspended with 40 ul of RNAs-free water and incubated in a heat block for 10 minutes at 60°C. The quantity and the quality of the extract respectively will be checked by Nano_Drop. The purity of the RNA sample was defined by the A260/A280 ratio. A ratio between 1.8 and 2.1 is indicative of highly purified RNA. The concentration of the extracted RNA was determined using the following equation:

RNA concentration (µg/µl) = (A260 * 40 * D)/1,000 where D = dilution factor

3.10.2 cDNA synthesis

The RNA samples were normalized by adding RNA free water to get a concentration of 50ug/ul of RNA for all samples; then the RNA was converted into cDNA by reverse transcription (RT). The oligo (DTs) primers were used for the reverse transcription. cDNA synthesis kit from Solis

BioDyne was used for the reaction. The samples were incubated in PCR machine at 50°C during 45 mn to allow the reverse transcription reaction to take place and to be complete, then in 85°C during 5min to inactivate the enzyme and stop the reaction. The cDNA samples were then kept at -20°C awaiting further analysis.

3.10.3 FAB_Z FAB_I primers

Primers for FAB_Z and Fab_I were created using Prime 3 (http://bioinfo.ut.ee/primer3-0.4.0/) and ordered online. The primers were designed to avoid hairpins and self-annealing with a GC content around 40% - 50% and an annealing temperature of 60°C.

A stock solution of 100uM was prepared for each primers using PCR water buffer. 10ul from the stock solution was added to 90 ul of PCR water to prepare a working solution of 10uM.

- FAB_Z primer

OLIGO	start	len	tm	gc%	any_th	3'_th	hairpin	seq
LEFT PRIMER	511	20	58.93	50.00	0.00	0.00	0.00	TTTGCTGGAGTGGATGGAGT
RIGHT PRIMER	687	24	59.91	41.67	0.00	0.00	0.00	CGATAAGGCAAACGTCATTTCTGA

Product size: 177 bp

- FAB_I primer

OLIGO	start	len	tm	gc%	any_th	3'_th	hairpin	seq
LEFT PRIMER	333	22	60.36	50.00	0.00	0.00	0.00	CGGGTGGGGTATTGCTAAAGAA
RIGHT PRIMER	510	20	59.31	50.00	1.61	0.00	0.00	AGAAGCGTCAAAGGGTAGCA

PRODUCT SIZE: 178 bp

3.10.4 Conventional PCR for FAB_I and FAB_Z

The design primers were tested during the conventional PCR, and their parameters set up. 5x FIREpol Master Mix ready to load with syber green dye was used for the conventional PCR. 4ul of a master mix containing DNA polymerase, 5x reaction buffer, 12.5 mM $Mgcl_2$, 1mM dNTPs, blue and yellow dye added into labelled PCR tubes 1ul of primers solutions were added cDNA samples were thawed a 2ul were added in each PCR tubes and topped up with water till 20 ul of the final volume. The sample was placed into the PCR thermocycler, and man run was set with different parameters until we find the right one that works better. The following amplification program was finally used for the two primers (FAB_Z and FAB_I): Initial denaturation at 95°C for 5min and then 40 cycles of denaturation at 95°C for 1 min, annealing

60°C for 1min, extension 72°C for 1min, followed by the final extension at 72°C for 10min, and then held at 4°C. After gel electrophoresis was made to make sure that the primers got correctly amplify with those parameters set up.

3.10.5 Housekeeping gene for *P. falciparum*

To run gene expression of Fab enzymes: Fab_Z (Beta-hydroxyacyl-Acyl-carrier Protein Dehydratase) and Fab _I (Enoyl Acyl-Carrier-Protein Reductase) we need a housekeeping gene as a reference which is necessary to calculate the expression level of the others genes during real-time PCR. In our study, Actin was chosen as the housekeeping gene for *Plasmodium falciparum*. Primers were made to amplify the actine gene during real-time PCR. Prime 3 was used to make the primers and parameters were set to fit the same parameters than for Fab_z and Fab _I.

- Actin Primers:

OLIGO	start	len	tm	gc%	any_th	3'_th	hairpin	seq
FORWARD PRIMER	813	20	59.89	50.00	0.61	0.00	0.00	AGCAGCAGGAATCCACACAA
REVERSE PRIMER	976	20	60.11	50.00	0.00	0.00	0.00	TGGTTGATGGTGCAAGGGTT

SEQUENCE SIZE: 1131

PRODUCT SIZE: 164

A conventional PCR was run first to test the primers chosen for actin as amplified. The following parameters were used: Initial denaturation at 95°C for 5min and then 40 cycles of denaturation at 95°C for 1 min, annealing 60°C for 1min, extension 72°C for 1min, followed by the final extension at 72°C for 10min, and then held at 4°C. After gel electrophoresis was made to make sure that the primers got correctly amplify

3.10.6 Gel electrophoresis for PCR products

After running the conventional PCR for the genes and the housekeeping gene, a gel was prepared for the PCR product. Agarose gel (2%) in 2x TBE buffer was prepared. Ethidium bromide was included in the gel. Eight µl of each amplification reaction was loaded onto the gel. A molecular weight ladder was included and run at for 40 min.The gel visualized on UV trans-illuminator and photograph gel. The remaining of the 20 µl of the PCR product stored at -20°C.

3.10.7 Real-time PCR

After a success amplification for all the genes (Fab_I and Fab_Z) and the housekeeping gene (Actin) a Real-time PCR was run to study the expression of the genes Fab_I and Fab_Z when exposed to our active extracts *Artemisia afra*. 5x HOT FIREPol EvaGreen qPCR mix plus (No Rox) from Solis BioDyne was used for the quantitative real-time PCR. The qPCR master mix composition was: Hot FIREpol DNA polymerase, 5x Evagreen qPCR buffer, 12.5mM $Mgcl_2$, dNTPs, Evagreen dye, No ROX dye. 96 wells plate for real-time PCR were used to set the reaction (Figure 3.1). In each well 10 µl of total volume solution were prepared each containing 2µl of master mix for qPCR, 0.5 µl for the reverse primers, 0.5µl for the forward primers, 2ul for cDNA and 5µl of water. Each sample was tested in triplicate. LightCycler 96 software was used to set the parameters for Real-time qPCR and to visualize the results

Plate setting:

*	1	2	3	4	5	6	7	8	9	10	11	12
A	U Sample 1 ACTINE	U Sample 1 ACTINE	U Sample 1 ACTINE	U Sample2 ACTINE	U Sample2 ACTINE	U Sample2 ACTINE	U Sample3 ACTINE	U Sample3 ACTINE	U Sample3 ACTINE	U Sample4 ACTINE	U Sample4 ACTINE	U Sample4 ACTINE
B	U Sample5 ACTINE	U Sample5 ACTINE	U Sample5 ACTINE	U Sample6 ACTINE	U Sample6 ACTINE	U Sample6 ACTINE	U Sample7 None	U Sample7 None	U Sample7 None	U Sample8 ACTINE	U Sample8 ACTINE	U Sample8 ACTINE
C	U Sample 1 FABI	U Sample 1 FABI	U Sample 1 FABI	U Sample2 FABI	U Sample2 FABI	U Sample2 FABI	U Sample3 FABI	U Sample3 FABI	U Sample3 FABI	U Sample4 FABI	U Sample4 FABI	U Sample4 FABI
D	U Sample5 None	U Sample5 None	U Sample5 None	U Sample6 FABI	U Sample6 FABI	U Sample6 FABI	U Sample7 FABI	U Sample7 FABI	U Sample7 FABI	U Sample8 FABI	U Sample8 FABI	U Sample8 FABI
E	U Sample 1 FABZ	U Sample 1 FABZ	U Sample 1 FABZ	U Sample2 FABZ	U Sample2 FABZ	U Sample2 FABZ	U Sample3 FABZ	U Sample3 FABZ	U Sample3 FABZ	U Sample4 FABZ	U Sample4 FABZ	U Sample4 FABZ
F	U Sample5 FABZ	U Sample5 FABZ	U Sample5 FABZ	U Sample6 FABZ	U Sample6 FABZ	U Sample6 FABZ	U Sample7 FABZ	U Sample7 FABZ	U Sample7 FABZ	U Sample8 FABZ	U Sample8 FABZ	U Sample8 FABZ
G	- Negative ACTINE	- Negative ACTINE	- Negative ACTINE	- Negative FABI	- Negative FABI	- Negative FABI	- Negative FABZ	- Negative FABZ	- Negative FABZ	-- Empty Empty	-- Empty Empty	-- Empty Empty
H	U Sample 9 ACTINE	U Sample 9 ACTINE	U Sample 9 ACTINE	U Sample 9 FABI	U Sample 9 FABI	U Sample 9 FABI	U Sample 9 FABZ	U Sample 9 FABZ	U Sample 9 FABZ	-- Empty Empty	-- Empty Empty	-- Empty Empty

Figure 3.1: Plate setting for real-time qPCR

Sample 1: cDNA from W2 strain of *P. falciparum* exposed to Hexane extract of *A. afra.*

Sample 2: cDNA from W2 strain of *P. falciparum* exposed to Ethanolic extract of *A. afra.*

Sample 3: cDNA from W2 strain of *P. falciparum* exposed to DCM extract of *A. afra.*

Sample 4: cDNA from W2 strain of *P. falciparum* (negative control, no drug exposition).

Sample 5: cDNA from D6 strain of *P. falciparum* exposed to DCM extract of *A. afra.*

Sample 6: cDNA from D6 strain of *P. falciparum* exposed to Ethanolic extract of *A. afra.*

Sample 7: cDNA from D6 strain of *P. falciparum* exposed to Hexane extract of *A. afra.*

Sample 8: cDNA from D6 strain of *P. falciparum* (negative control, no drug exposition).

Sample 9: Repetition for Sample 4.

Negative control: cDNA plus qPCR master mix and water

Empty wells: Empty (no cDNA, no primers, no master mix, no water)

3.11 Drug screening with Autodock vina in PyRx

After GCMS a library of compounds was prepared based on the GCMS results of *Artemisia afra* extracts from Burundi. The library was screened against two macromolecules (FAB_I FAB_Z) using Autodock Vina in PyRx 0.8 version (Trott *et al*., 2010). PyRx is a Virtual Screening software for Computational Drug Discovery that can be used to screen libraries of compounds against potential drug targets. The software is open access and is available online at http://pyrx.sourceforge.net

3.11.1 Preparation of the library of small molecules

The 3D structures of all the compounds from GCMS were searched from three chemical structure database and downloaded. Three databases were used: ChemSpider (http://www.chemspider.com/), PubChem from NCBI (https://pubchem.ncbi.nlm.nih.gov/search/), and ChEMBL (https://www.ebi.ac.uk/chembl/) from the European Bioinformatics Institute (EBI). The small molecules (Compounds) were downloaded and visualize with Pymol then save in the same file in PDB format.

3.11.2 3D structure of *Plasmodium falciparum* Fab enzymes: Fab Z and Fab I

The 3D structure of the Fab enzymes: Fab_Z and Fab_I were downloaded from the RCSB protein database (PDB), (https://www.rcsb.org/). Fab_Z (PDB entry: 3AZA), (Maity *et al*., 2011); Fab_I (PDB entry: 3LT0), (Maity *et al*., 2010). The macromolecules were crystallized with their ligands. The macromolecules were open in txt format with notepad then all the ligands were removed to free the interaction sites.

3.11.3 Virtual screening with PyRx: Protocol

PyRx (fig7) have the two virtual screening software Autodock 4.0 and Autodock vina. Autodock Vina was used during the drug screening because AutoDock Vina significantly

improves the average accuracy of the binding mode predictions compared to AutoDock 4 and is faster (Trott *et al*. 2010).

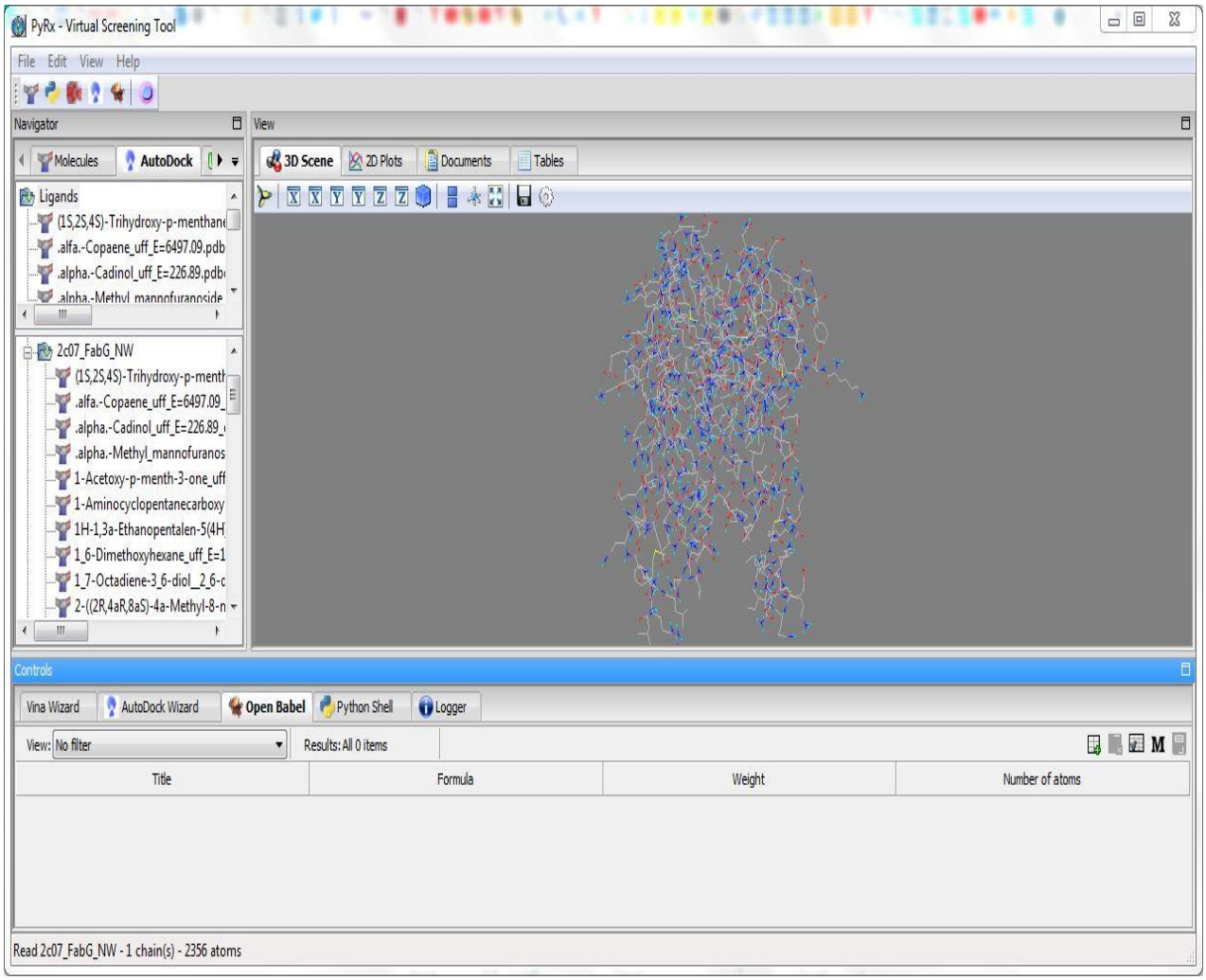


Figure 3.2: PyRx virtual screening interface.

A. Protocol for virtual screening:

a. Preparation of input files for Docking:

- The two macromolecules (Fab_Z and Fab_I) were loaded into PyRx and converted to PDBQT file suitable for docking with Autodock vina (Trott *et al*. 2010).
- All the compounds from GCMS were also downloaded in PDB format, and the library was made.
- The compounds were imported into PyRx by clicking on file then import and to click then on chemical table files-SDF.

- Right-click to one of the loaded compounds and select minimized all. Notice that the title of the molecules has changed. Example: Copaene_uff_E=6497.09. The _uff part corresponds to the force field used for energy minimization, which, by default, is the Universal Force Field (Rappe *et al*., 1992) as implemented in Open Babel software package (0'Boyle *et al*., 2011). The _E=6497.09 part corresponds to the energy of the minimized molecule. The precise value for this energy is not important here. However, this notation is helpful to capture changes made to this molecule before we convert them AutoDock ligand file in the next step.
- Right-click on any of the rows where the ligands are in Open Babel table and select Convert All to AutoDock Ligand (pdbqt).

b. **Virtual screening with Vina wizard**

After converting the molecules in PDBQT files and minimize the energy of the ligands now we can process to the virtual screening with Vina.

Protocol:

- ❖ Select Vina Wizard tab under Controls panel and click on the start button.
- ❖ All the ligands were selected using the shift key for multiple ligand selection.
- ❖ Macromolecule was selected (FabI or FabZ) and click on the forward button on vina wizard.
- ❖ Then we click on the maximized button under vina search space and forward button. This starts Autodock vina and docks each ligand one by one to the selected macromolecule.

After virtual screening is completed, PyRx automatically advances to Analyze Results page, where you can see results of virtual screening computation. AutoDock Vina, by default, outputs 10 best binding modes for each docking run (Sargis et al., 2015). The results are exported in CSV format to be further analyzed. Binding Affinity is expressed in kcal/mol.

3.12 Statistical analysis

All statistical analysis was performed using SPSS. Difference between the level of phytochemical contents were compared statistically by using one-way-ANOVA and Tukey test. $P < 0.05$ are considered as significant. Pearson correlation was used to correlate the antioxidant activity and phytochemical content.

CHAPTER FOUR

4.0 RESULTS

4.1 Phytochemical screening

The phytochemical screening of the crude extracts revealed the presence of some secondary metabolites such as terpenoids, alkaloids, Tannins, and glycosides (Table 4.1). Terpenoids were present in all the extracts; all the plant extracts also showed the presence of glycosides. Tannins are detected in all the water and ethanolic extracts of the plant samples. Alkaloids were detected only in the ethanolic extracts from South Africa and Kenya. The results also showed ethanol, and water solvent extract more phytochemical solvent due to the high polarity.

Tableau 4.1: phytochemicals screening of *Artemisia afra* plant extracts from Burundi, Senegal, South Africa, Kenya and Tanzania.

Phytochemicals Screening					
Tests / Extracts	Terpenoids Salkowski test	Alkaloids Mayer and Wagner test	Tannins test	Saponins	Glycoside Keller-kiliani test
BUR.DCM	+	-	-	-	+
SEN.DCM	+	-	-	-	+
S.A. DCM	+	-	-	-	+
Ken. DCM	+	-	-	-	+
Tanz. DCM	+	-	-	-	+
BUR. ETOH	+	-	+	-	+
SEN.ETOH	+	-	+	-	+
S.A. ETOH	+	+	+	-	+
Ken. ETOH	+	+	+	-	+
Tanz. ETOH	+	-	+	-	+
BUR .Hexane	+	-	-	-	+
SEN. Hexane	+	-	-	-	+
S.A. Hexane	+	-	-	-	+
Ken. Hexane	+	-	-	-	+
Tanz. Hexane	+	-	-	-	+
BUR. Water	+	-	+	+	+

SEN. Water	+	-	+	+	+
S.A. Water	+	-	+	+	+
Ken. Water	+	-	+	+	+
Tanz. Water	+	-	+	+	+

Bur: Burundi; S.A : South Africa; Tanz: Tanzania; SEN: Senegal; Ken = Kenya; DCM: Dichloromethane; ETOH : ethanol

Saponins were found only in the water extracts (Table 4.1). *Artemisia afra* plant was used for decades to treat a lot of affliction by traditional healers and those phytochemical compounds identified in the areal part of the plants may be responsible for the biological activities and their medicinal properties.

4.1 Total phenolic and flavonoids content

4.1.1 Total phenolic

Total phenolic content of the 20 extracts from *A. afra* plant was determined using Folin Ciocalteu method. The results are expressed in mg of GAE/ gramme of extract, the standard curve (Figure 4.1) was determined using different concentrations of gallic acid ranking from 50µg/µl to 500 µg/µl.

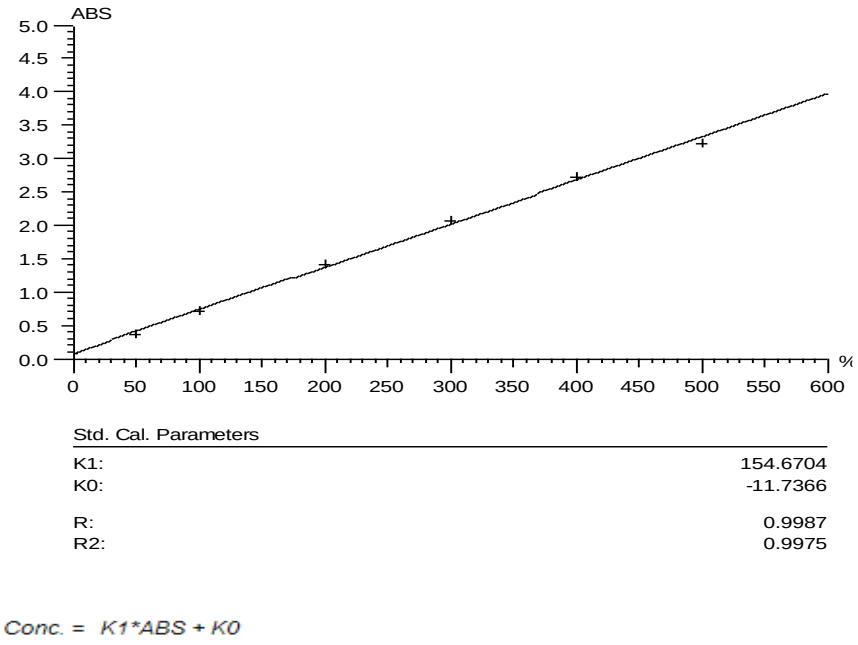


Figure 4.1: Calibration for curve gallic acid standard

One-way-ANOVA were used to compare the results with Tukey test, $P < 0.05$ are considered as significant. The results in table 4.2 revealed that generally, ethanol solvent extracts much better the phenolic compounds than the other solvents, they contain more phenols following by

water extracts then DCM extracts. Hexane extracts were found to have a very low amount of phenols.

After comparison the ethanolic extracts from Burundi, Senegal and South Africa have the highest amount of phenols respectively: 606.9449 mg GAE/ g; 490.5399 mg GAE/g; 513.8178 and they are statically different which mean that Burundi has the highest amount. Tanzania and Kenya have the lowest amount compare to the other with respectively 337.7720 mg GAE/g for Tanzania and 387.8852mg GAE/g for Kenya. Tanzania extract has the lowest amount.

The water extract, has the same scheme Tanzania water extract was found to have the lowest amount 361.7227 mg GAE/g compare the Burundi and South Africa extracts which have the highest yield 606.9449mg GAE/g

Table 4.2: Total phenolic content in *Artemisia afra* extract from Burundi, Senegal, Tanzania, and South Africa.

Extracts / Count.	Burundi	Senegal	Kenya	Tanzania	South Africa
Etoh	606.94±0.00 [0.00]*	490.53±23.27 [0.00]*	387.88±17.81 [0.00]*	337.77±1.22 [0.00]*	513.81±0.00 [0.00]*
DCM	308.77±18.80 [1.00]	316.28±12.35 [1.00]	301.77±1.43 [1.00]	267.30±7.68 [0.406]	247.97±11.64 [0.04]*
Water	606.94±0.00 [0.99]	583.65±23.28 [0.97]	533.13±0.00 [0.007]*	361.72±0.87 [0.00]*	606.94±0.00 [1.00]
Hexane	4.93±1.77 [1.00]	10.97±1.43 [1.00]	36.93±1.04 [0.94]	23.78±2.86 [1.00]	57.70±6.19 [0.24]

* mean that the value of the total phenol is statistically different from the value of the other plant extracts, extracted with the same solvent. Mean+ standard error, P-values are in bracket

For the DCM extracts, the results show that dichloromethane extracts from Burundi, Senegal, and Kenya have a similar amount of phenols the difference is not significative, and the DCM extract from South Africa have the lowest phenols content.

Hexane solvent extracts fewer phenols compare to the other solvents, so the hexanolic extracts had the lowest phenols content, between 57.7026 mg GAE/g (for S. Africa) and 4.9396 mg GAE/g for Burundi hexanolic extracts, and the difference is not significative.

4.1.2 Total Flavonoids content

Total flavonoids were determined using rutin standard; the result was expressed in mg of rutin/g of extract. The standard curve (Figure 4.2) was made using different concentrations of rutin (5, 10, 20 50, 100, 200, 500 µg/µl).

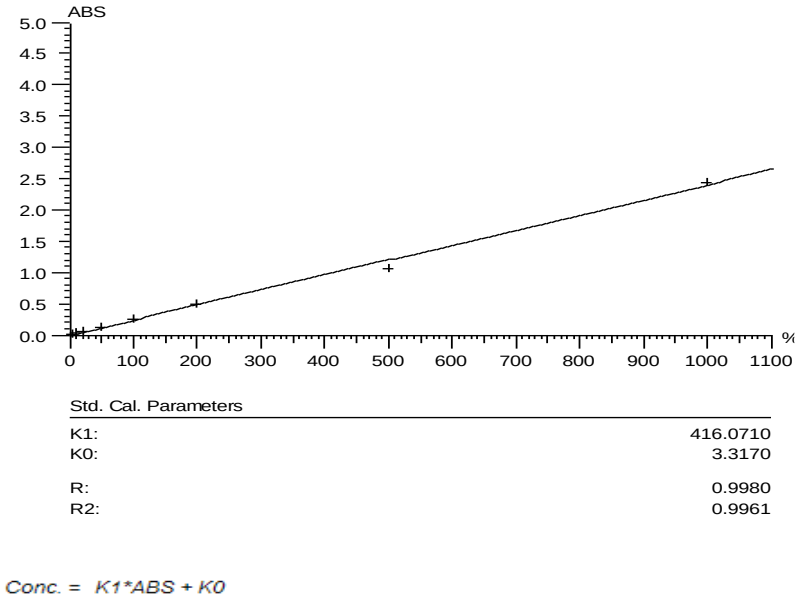


Figure 4.2 Calibration curve for Rutin standard

The results in table 4.3 showed that:

The ethanol solvent extracts had higher flavonoids content compared to the other solvent. Ethanolic extract from Burundi showed the highest flavonoids content (242.4745±2.0594) following by ethanolic extract from Tanzania (165.002±2.1759), Senegal (162.3809±0.1781) South Africa(140.3707±3.4136) and Kenya(139.5384±1.5294) they were significant differences flavonoids content between countries (see table 4.3).

The DCM plant extract had the second highest flavonoids concentration. DCM extract from Burundi had the highest total flavonoids content (143.532±4.74) when compared to the other DCM plant extracts from other countries, and DCM extract from South Africa had the lowest flavonoids concentration (106.7730 ± 01434) compared to the other DCM extracts.

Table 4.3: Total Flavonoids content in *Artemisia afra* extract from Burundi, Senegal, Tanzania, and South Africa.

Extracts / Count.	Burundi	Senegal	Kenya	Tanzania	South Africa
Etoh	242.474±1.45 [0.00]*	162.380±0.12 [0.00]*	139.5385±1.08 [0.00]*	165.0021±1.53 [0.00]*	140.3707±2.41 [0.00]*
DCM	143.532±4.74 [0.00]*	136.5845±.62 [1.00]	131.9661±0.76 [0.99]	108.7493±4.90 [0.07]	106.7730±0.10 [0.04]*
Water	48.8767±1.87 [0.75]	33.3156±0.99 [0.753]	40.0768±0.85 [0.99]	32.4627±20.20 [0.683]	51.7684±3.34 [1.00]
Hexane	28.3112±0.14 [0.95]	39.9728±0.79 [0.99]	54.1608±0.33 [0.85]	50.1457±0.56 [0.99]	56.2619±0.64 [0.69]

* mean that the value of the total phenol is statistically different from the value of the other plant extracts, extracted with the same solvent. Mean + standard deviation; p-values are in brackets

The water extract from Burundi; Senegal, Kenya, Tanzania and South Africa have approximatively the same amount of flavonoids the difference between them is not statistically different. The same observation was done for the hexanolic extracts. However, hexane solvent extracts fewer flavonoids compare to the other solvent.

4.2 DPPH assay results

The DPPH radical scavenging activity of extracts are shown in figure 4.3. The results are expressed in IC50, which mean the concentration that inhibits 50% of the DPPH activity. The results are presented in a diagram which ranks the IC50 from the highest to the lowest.

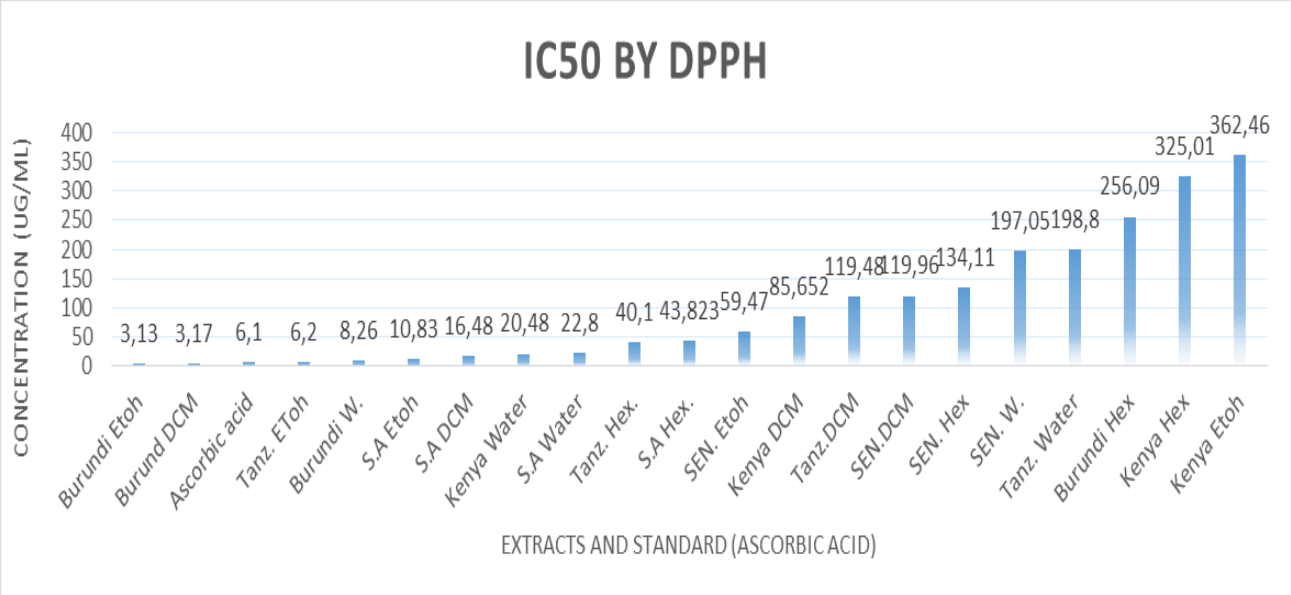


Figure 4.3: DPPH radical scavenging capacity (EC50) of *Artemisia afra* extracts

The IC50 of the standard (Ascorbic acid) was found to be 6.1 µg/mL. The highest IC50 was observed from Burundi ethanolic extract which was: IC50 = 3.13 µg/mL. Burundi DCM extract also had similarly the same IC50 which was: IC50 = 3.17 µg/ml this two may have the same antioxidant activity. Burundi water extract had a high IC50 of 8.26 µg/ mL, this results showed that the water extract from Burundi is more active compared to the other water extracts including Kenya water extract IC50 of 20.48 µgml; South Africa water extract IC50 of 22.8 µg/mL, then Senegal and Tanzania water extract which had a similar IC50: 197.05 and 198.8 µg/mL respectively. Kenya Hexanolic and ethanolic extract had the lowest antioxidant activity compared to the others. Their IC50 were: 325.01 and 362.46 µg/mL respectively.

4.3 Correlation of phytochemical contents with antioxidant activities

To explore the relation between antioxidant activity and phytochemical content for *Artemisia afra* extracts, the correlation coefficient of the total phenolic, total flavonoids and DPPH activity are shown in Table 4.4. Total flavonoids and total phenol are also correlated.

Table 4.4: Correlations between total phenols, total flavonoids and DDPH

		Total Phenols	Total Flavonoids	DPPH
Total Phenols	Pearson Correlation (r)	1	,342*	-,344*
	Sig. (2-tailed)		,015	,015
	N	40	40	40
Total Flavonoids	Pearson Correlation	,342*	1	-,309*
	Sig. (2-tailed)	,015		,026
	N	40	40	40
DPPH	Pearson Correlation (r)	-,344*	-,309*	1
	Sig. (2-tailed)	,015	,026	
	N	40	40	40

*. Correlation is significant at the 0.05 level (1-tailed).

All correlations are globally moderate. Total phenols and total flavonoids are positively correlated with r=0,344. Total phenols and DPPH are negatively correlated with r= -0,344. Total flavonoids and DPPH are also negatively correlated with r= 0,309.

4.4 Results for *in vitro* Antimalarial assay

Activity criteria in the assay were defined as very high when IC50 was below 5, active when between 5 and 10, moderate when between 11 and 50 and low when between 50 and 100 µg/ml. Samples with IC50 >100 µg/mL were considered as inactive. The antimalarial activity of the 5 samples of *Artemisia afra* plants against CQ sensitive/mefloquine-resistant P. falciparum (D6 strain) and CQ resistant/mefloquine-sensitive (W2 strain) are summarized in Table 4.5.

Table 4.5: *In vitro* antimalarial activity; IC50 of the 20 extracts from the 5 samples of *Artemisia afra* plant collected from South Africa, Kenya, Tanzania, Burundi and Senegal (IC50 ± SD (µg/ml))

P. extracts / Countries	ETOH		DCM		HEXANE [b]		WATER	
	IC50s (µg/mL) (M±SD)							
	W2	D6	W2	D6	W2	D6	W2	D6
Burundi	2.66±0.35	7.84±0.64	3.04±0.32	7.92±0.36	0.715±0.10	3.18±0.72	61.9±5.7	75.06±18.8
Senegal	7.26±0.91	17.6±2.55	4.85±1.43	14.57±0.36	11.21±1.7	30.13±1.02	121.8±13.01	72.7±19.3
South Africa	17.24±1.39	22.8±6.56	11.34±1.82	22.15±3.42	29.5±1.04	58.4±2.77	123.3±53.8	149.2±2.4
Kenya	22.18±6.1	44.97±1.5	11.37±2.8	25.28±4.84	31.28±0.59	76.82±6.7	>200	>200
Tanzania	9.64±0.13	24.76±2.57	10.86±2.43	23.5±5.3	29.9±0.47	53.10±2.14	164.2±31.4	140.2±3.01
CQ(control)	74.58±12.1 ng/ml W2 16.95±3.14ng/ml D6							

[b] Forms two immiscible liquid layers, picked on DMSO layer

W2 chloroquine resistant; D6 chloroquine sensitive

The Burundi hexanolic extract were the most active IC50 range: 0.715±0.1087 for W2 and 3.1896±0.728869 for D6 followed by Burundi ethanolic extract IC50 range: 2.666667±0.3597 for W2 and 7.843±0.641 for D6. And Burundi DCM extract IC50 range: 3.049±0.3215 for W2 and 7.9286±0.3638 for D6. Compared to the other plants; *A. afra* from Burundi have the highest antimalarial effect and is highly active with an IC50 lower than 5ug/ml for W2, for D6 is still

very active with an IC50 between 3 and 7µg/ml. Then come the one from Senegal (IC50 for DCM extract range are: 4.8546±1.438 for W2 and 14.5716±0.368 for D6; and IC50 for ETOH extract are: 7.265±0.911 for W2 and 17.699±2.5576 for D6). The IC50 for Senegal hexane extract (11.219±1.786 for W2 and 30.133±1.02675 for D6) is slightly higher compare to Burundi. We also have the extract from South Africa who come third which is also very active but less than the previous ones with an IC50 between 11.341±1.823µg/ml and 17.24±1.395µg/ml for W2 and for D6 where it the activity is moderated; the IC50 is ranking between 22.8±6.564µg/ml and 58.408±2.7736µg/ml. Then comes Tanzania and Kenya.

4.5 *In vivo* acute toxicity results

Thirty albinos swiss mice were used to perform an acute oral toxicity test. Mice were randomly assigned into 10 groups and were housed 3 animal per cages. Single oral dose was given to the mice and toxicity sign was observed mostly during the first critical hours the first 24 hours any toxicity sign was recorded, and the weight of the mice was recorded daily during 14 days.

4.5.1 Effect of acute toxicity of *Artemisia afra* extracts on the weight and behaviour of the mice

After oral administration of the extracts, from the 30 mice used for acute toxicity test, only one died 24 hours after oral administration. That mouse belongs to the group treated with 2500mg/kg of dichloromethane extract (DCM group1). Changes like loss of appetite, hypoactivity, lethargic, dizziness were observed at the dose of 2500mg/kg and disappeared after the washout period after 2 days of observation.

Both the treated and control groups of mice had stable initial body weight during the first week, and during the second week of the observation period, there is a proportional gain in their body weight (figure 4.4, 4.5, and 4.6).

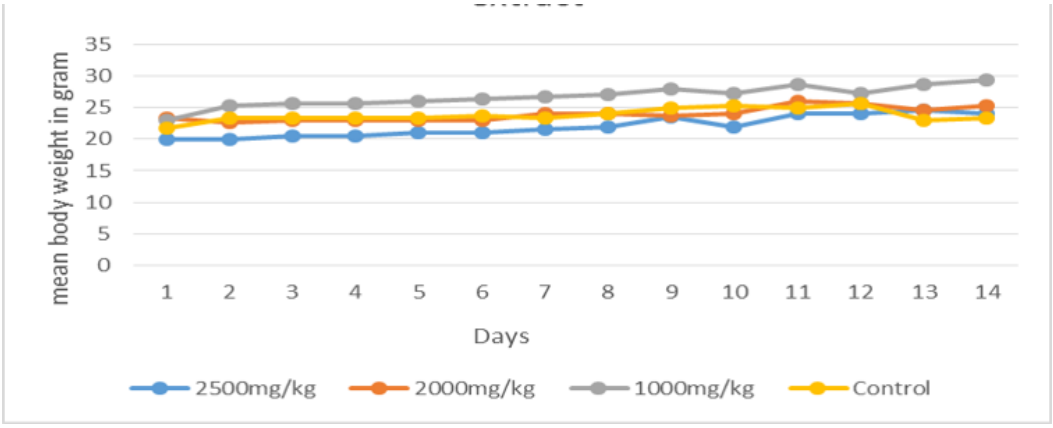


Figure 4.4: Mean body weight change through weeks in mice treated with 2500mg/kg; 2000mg/kg, 1000mg/kg DCM extracts one single dose.

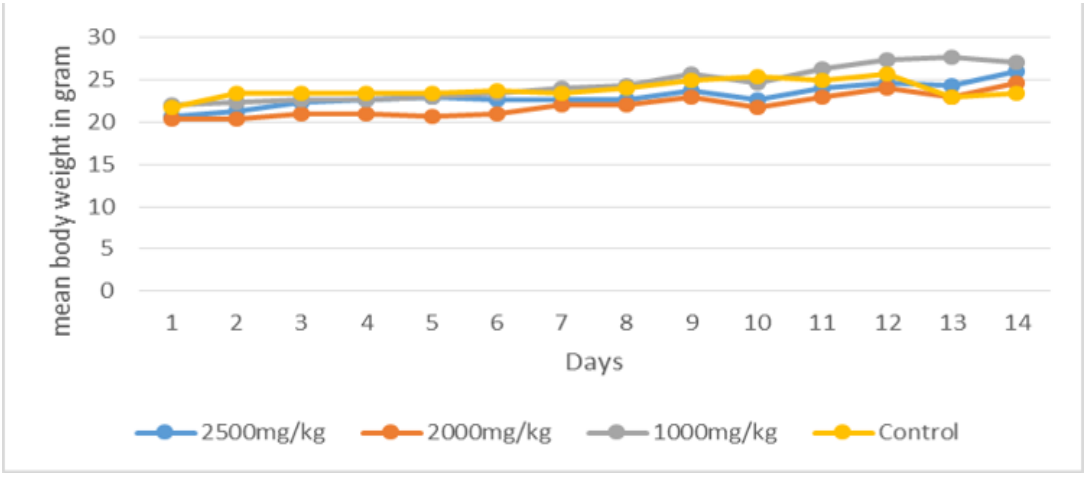


Figure 4.5: Mean body weight change through weeks in mice treated with 2500mg/kg; 2000mg/kg, 1000mg/kg Ethanolic extracts one single dose.

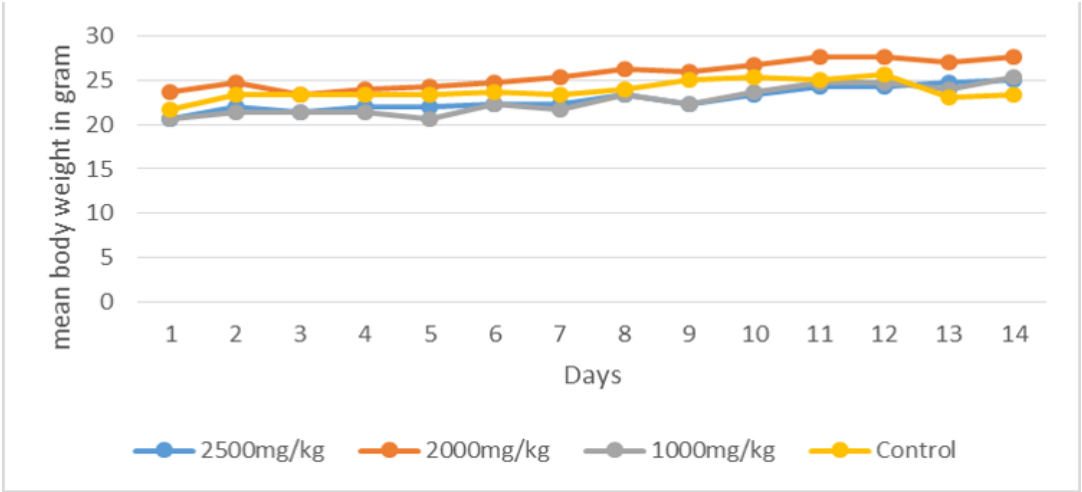


Figure 4.6: Mean body weight change through weeks in mice treated with 2500mg/kg; 2000mg/kg, 1000mg/kg Hexane extracts one single dose.

During 14 days of acute toxicity test weight of the animals were recorded daily. One-way-ANOVA were used to compare the results with Tukey test, $P < 0.05$ are considered as significant. The statistical analysis of the weight between animal treated with extracts and the control showed some slight differences (table 4.6).

Mice treated with DCM extract the difference in body weight was significative ($P< 0.05$) when compared to the control for group1 and group3. Group 1 their weight was slightly small compared to the control, and for the group 3 it was in contrast since showed progressive body weight gain (figure 4.4) in the same time the group3 had received the smallest doses, and the group1 had received the highest doses.

For mice inoculated with ethanolic extract, there was a significant difference in body weight compared to the control. The mice in group 2, had a slight loss of their body weight and a similar observation was reported in those treated with hexanolic extract although there was an increase of their body weight for the mice in group2 treated with 2000mg/kg.

For the last group of mice inoculated with hexanolic extract, when we compared each group to the control, a gain of weight was observed in group 2.

Table 4.6: Mean body weight of mice treated with 3 extracts as compared to the control during 14days. Results presented as the mean of the body weight plus standard Error of the mean (P-values are in bracket)

Groups	Doses mg/kg	DCM (Mean body weight±SDE)	Ethanol (Mean body weight ±SE)	Hexane (Mean body weight ±SE)
Group0	Control	23.8095±.28966	23.8095±,28966	23.8095±,28966
Group1	2500	22.0357±.43998** (,008)	23.095238±(.584)	22.8571±.35414 (.287)
Group2	2000	23.9524±.28996(,993)	21.976190±** (,010)	25.6429±.41561** (.006)
Group3	1000	26.7857±.44145** (,000)	24.500000±(,610)	22.6667±.42510 (.150)

SE standard error of the mean, P-values are in brackets

4.5.2 Effect of acute toxicity of *Artemisia afra* extracts on the organ's weight of the mice

After the 14th day, all the mice were sacrificed. Observation on the gross appearance of internal organs including liver, kidney, spleen, and stomach of treated mice did not show any abnormal changes in texture, shape, size or colour in comparison to that of the control group. No lesion was noted in the internal organs in all groups.

Liver, kidney, and spleen of each experimental group of mice were weight and compared to the control. *A. afra extract* did not produce any significant effect on the weight of the organs. The statistical analysis revealed that there were no significant differences between the organs of the mice inoculated with extracts (DCM, ETOH, and Hexanolic extracts) and the organs of the mice from control (Table 4.7,4.8,4.9).

Table 4.7: Mean weight organs of mice treated with DCM extract as compared to the control (P-values are in bracket).

Doses	Mean weight±SDE for DCM extract		
	Liver	Kidney	Spleen
Control	1.5400±,07572	.3500±01528	.1000±.00000
1000 mg/kg	1,6267±.13383 **(**1.000**)**	.4933±.05487**(**.037**)**	.1033±.01333**(**1.000**)**
2000 mg/kg	1.3067±.08647**(**.829**)**	.3000±.02000**(**.978**)**	.0933±.02404**(**1.000**)**
2500 mg/kg	1.2533±.03756**(**.586**)**	.3433±.00882**(**1.000**)**	.1033±.00882**(**1.000**)**

Table 4.8: Mean weight organs of mice treated with HEX extract as compared to the control (P-values are in bracket).

Doses	Mean weight±SDE for hexane extract		
	Liver	Kidney	Spleen
Control	1.5400±,07572	.3500±,01528	.1000±,00000
1000 mg/kg	1.2867±.06438**(**.746**)**	.2800±.01202**(**.993**)**	.0800±.01000**(**1.000**)**
2000 mg/kg	1.2800±.01732**(**.715**)**	.2800±.01764**(**.442**)**	.0967±.00333**(**1.000**)**
2500 mg/kg	1.1600±.06429**(**.202**)**	.2800±.02082**(**.813**)**	.1000±.00000**(**1.000**)**

Table 4.9: Mean weight organs of mice treated with EtOH extract as compared to the control (P-values are in bracket).

Doses	Mean weight±SDE for Ethanolic extract		
	Liver	Kidney	Spleen
Control	1.5400±.07572	.3500±.01528	.1000±**.**00000
1000 mg/kg	1.4300±**.**12166**(**1.000**)**	.4300±.02517**(**.661**)**	.1867±.06692**(**.419**)**
2000 mg/kg	1.1233±.12252**(**.118**)**	.3500±.03215**(**1.000**)**	.0767±.02186**(**1.000**)**
2500 mg/kg	1.3900±.09018**(**.992**)**	.3300± **.**01528 **(**1.000**)**	.1267±.03180**(**1.000**)**

4.5.3 Effect of acute toxicity of *Artemisia afra* extracts in the biochemical parameters compared to the control group

AST and ALT level were tested in plasma of the mice treated with extracts and compared to the control to detect any sign of toxicity on the liver. One way anova was made followed by a T-test, $P < 0.05$ are considered as significant. When we compared the biochemical parameters between the experimental mice and the control, there were no significant changes for the ALT and AST level in all doses of *Artemisia afra* extracts (Dichloromethane, hexane, and ethanolic) (Table 4.10, 4.11).

Table 4.10: Effect of DCM, EtOH, Hex. Extracts on AST biochemical parameter, mean of experimental groups compared to the control (Expressed in Mean ± SDE, P-value in bracket).

Liver Function Test (AST in u/l)				
Groups	**Doses (mg/kg)**	**DCM**	**Ethanol**	**Hexane**
Control	-	196,3333±25,89938	196,3333±25,89938	196,3333±25,89938
Group1	1000	259,0000±19,07878**(**0,852**)**	189,0000±65,96211**(**1,000**)**	422,3333±63,83399**(**,440**)**
Group2	2000	206,6667±42,58456**(**1,000**)**	305,0000±57,42241**(**,906**)**	508,0000±122,40234 **(**,667**)**
Group3	25000	304,0000±66,39528**(**,951**)**	264,6667±27,50959**(**,901**)**	370,3333±45,03455**(**,397**)**

Table 4.11: Effect of DCM, EtOH, Hex. Extracts on ALT biochemical parameter, mean of experimental groups compared to the control (Expressed in Mean ±SDE, P-value in bracket).

Liver Function Test (ALT in u/l)				
Groups	**Doses (mg/kg)**	**DCM**	**Ethanol**	**Hexane**
Control	-	33,2333±1,6835	33,2333±1,6835	33,2333±1,6835
Group1	1000	41,8000±6,23244**(**0,975**)**	45,2333±13,57895 **(**0,999**)**	63,4000±3,43948 **(**,055**)**
Group2	2000	34,6667±3,10179**(**1,000**)**	51,2000±13,55434 **(**,974**)**	119,0667±36,7249 **(**,0715**)**
Group3	2500	37,0500±2,62694**(**0,994**)**	48,0667±9,71774 **(**0,944**)**	79,2333±18,48480 **(**,673**)**

4.5.4 Acute toxicity effect in mice tissues:

a) Histopathology results on Liver

The liver shows typical hepatolobular architecture, consisting of a central vein with radiating cords of hepatocytes separated by sinusoids; portal areas composed of the portal vein, hepatic artery, and bile duct were situated at the periphery. The hepatocytes were polygonal in shape, with central, lightly stained nucleus and clear nucleolus.

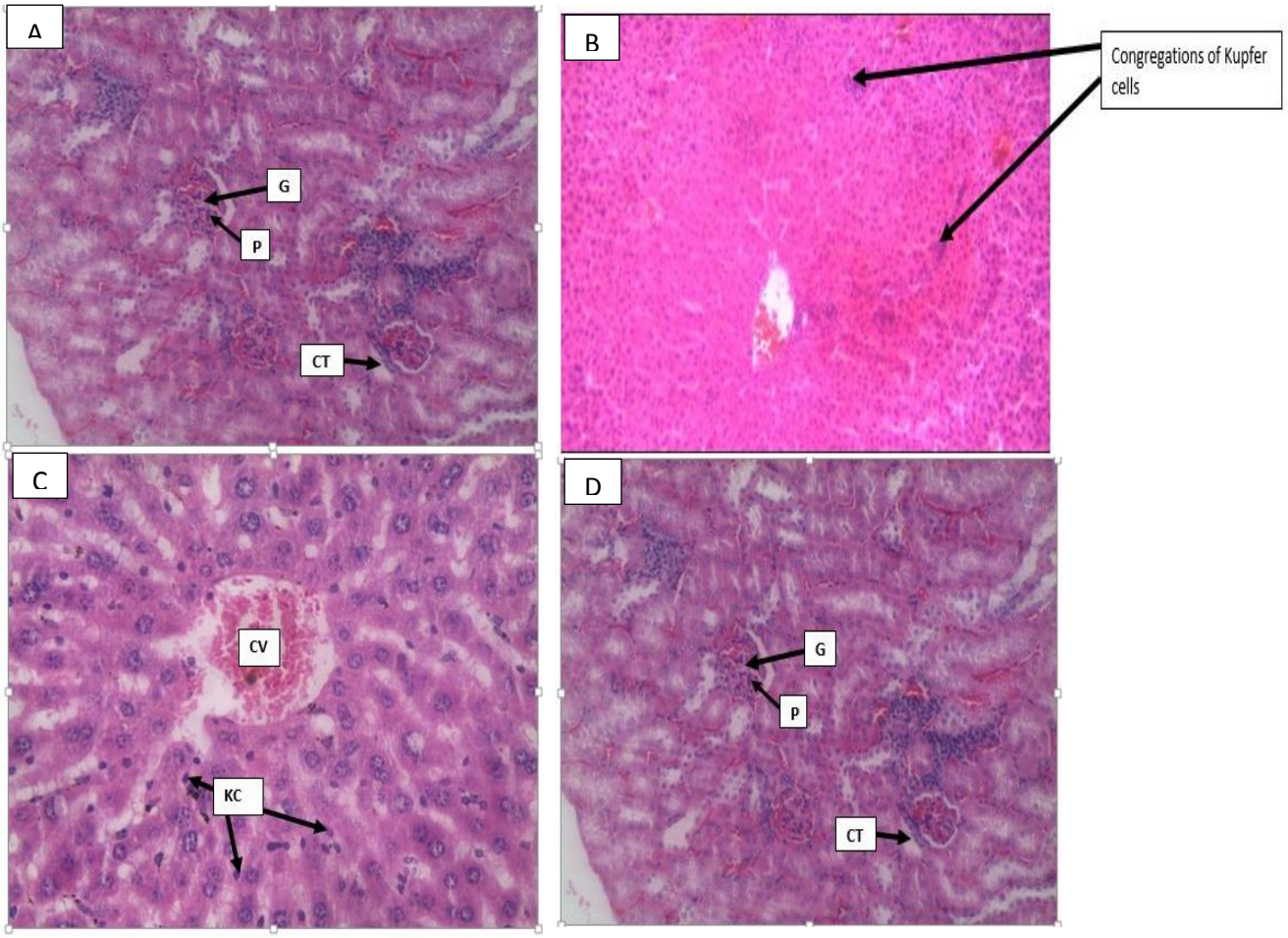


Figure 4.7: Photomicrographs of liver sections of control mice (A) and mice treated with hexane extract (B), ethanolic extract (C) and dichloromethane extract (D) at the dosage of 2500mg/kg

Figure 4.7 (A) Normal ultrastructure**.** A central vein (**CV**) with radiating cords of hepatocytes. Regular sinusoidal (**S**) arrangement around the central vein. A portal triad (**PT**) also clearly visible. Figure 4.7 (C) Mag. X 10 Congested central vein. Focal areas of congregation by Kupfer cells. Note loss of hepatolobular arrangement. Figure 4.7 (C) No histopathological changes were observed. Cords of hepatocytes radiating from the central vein (**CV**)**.** There however appears to be a higher number in kupfer cells **(KC).**

b) Histopathology results on Kidney

Generally the kidney shows normal typical ultrastructure. Features observed include normal proximal and distal tubules (DT), glumerulus (G), blood vessels, and interstitium, Podocytes (P), urinary pole (UP), and Bowman's capsular space (BCS).

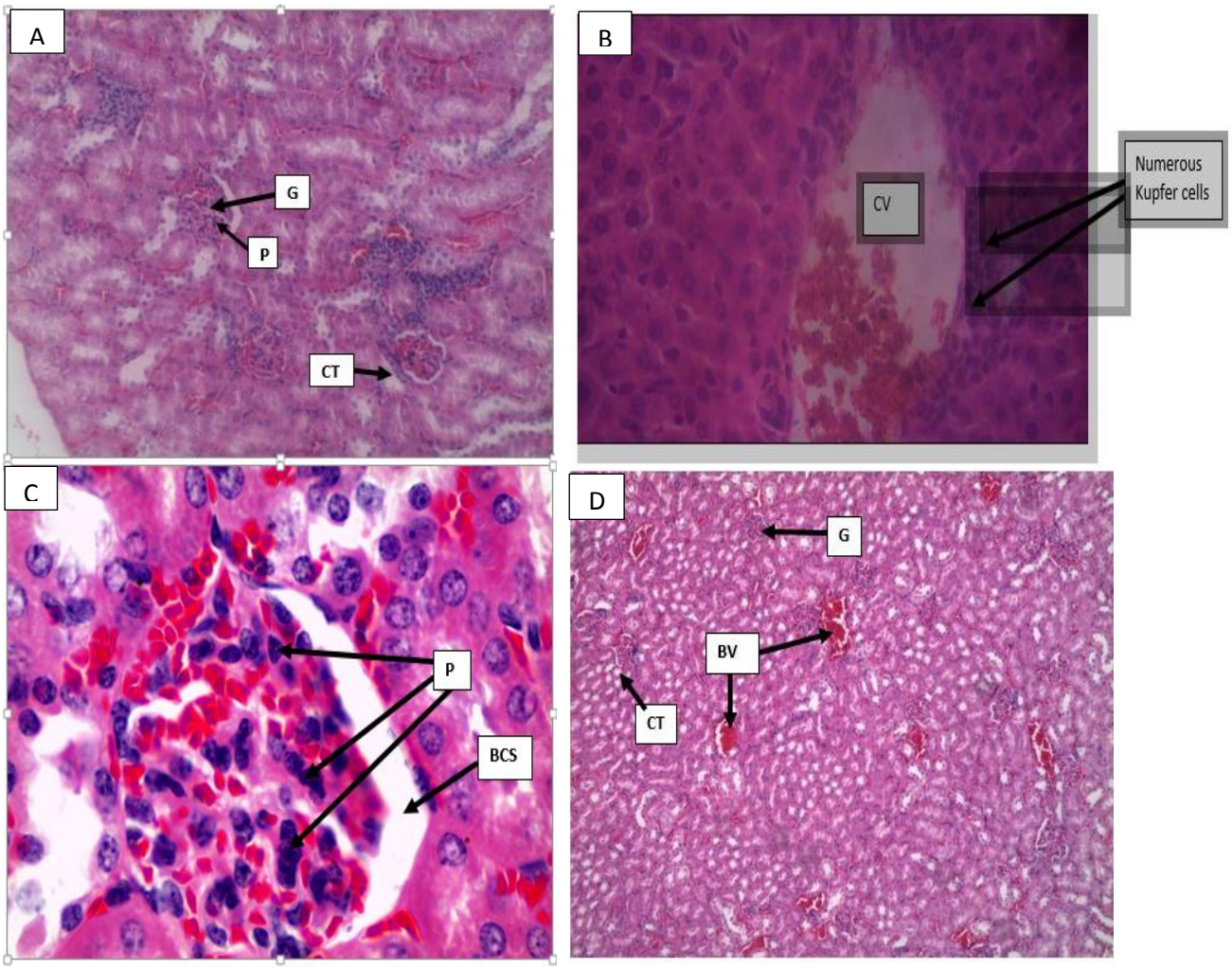


Figure 4.8: Photomicrographs of liver sections of control mice (A) and mice treated with hexane extract (B), ethanolic extract (C) and dichloromethane extract (D) at the dosage of 2500mg/kg

Figure 4.8 (A): (Mag.x20). Normal ultrastructure showing glomeruli (G), podocytes (P) and convoluted tubules (CT). Figure 4.8 (B): Mag. X 40. Central vein (CV) surrounded by numerous Kupfer cells (KC). There is loss of hepatolobular arrangement and absence of clear sinusoids. Figure 4.8 (C) Normal ultrastructure showing glomerulus (G), podocytes (P) and Bowman's corpuscular space (BCS). Figure 4.8 (D): Normal glomeruli (G) and convoluted tubule (CT) structure. Blood vessels (BV) are congested.

4.6 In vivo antimalarial assay for Burundi extracts

Antimalarial effects of extracts of *Artemisia afra* on *Plasmodium berghei* in mice are summarized in table 4.12 and 4.13. The suppression of the Parasitaemia (chemo-suppression) and the ED50 were used as measured of efficacy. Four Dosages were used to do the *in vivo* antimalarial assay. Samples were categorized as highly active when chemo-suppression was above 60% or moderately active between 30 and 60%, but lowly active below 30%. At the dosage of 200mg/kg of body weight for all the different extracts, we got around the same percentage of suppression (ETOH 61.64%; DCM 63.82%; and Hexane 66.89%) all results showed a high suppression of the parasites. When dosage was decreased the parasitaemia changed as follows, at the dosage of 100 mg/kg EtOH extract were the most active and showed moderate suppression compared to the others with a percentage of chemo-suppression which is still high at 57.07% for ETOH extract; following by DCM extract 42.17% and come in last Hexane extract 32.19%. The same observation was observed at the dosage of 50mg/kg of body weight the activity is still moderate for the *A. afra* ethanolic and DCM extracts but low for the hexane extract.

Table 4.12: *In vivo* activity of DCM, ETOH and Hexanolic extracts of *Artemisia afra* on *P. Berghei* infected mice

Dosage / %Chemosup.	**Dosage 1 200mg/ml**	**Dosage 2 100mg/ml**	**Dosage 3 50mg/ml**	**Dosage 4 25mg/ml**
DCM	63.82% ± 6.54%	42.17%±9.66	32.26± 9.25%	5.84% ± 2.20
ETOH	61.64% ±6.04	57.07%±11.97	50.52%±7,93	35.42%±4.3
Dosage	**Dosage 1 400mg/ml**	**Dosage 2 200mg/ml**	**Dosage 3 100mg/ml**	**Dosage 4 50mg/ml**
Hexane	73.23%±13.52	66.89%±5.07	32.19%±23.34	22.86%±18.88
Positive control	88.33%			

The ethanolic extract of *A. afra* has the highest in vivo antimalarial activity with the highest ED50 = 6.43mg/ml following by the DCM extract ED50=11.99mg/ml then came is last the hexanolic extract ED50= 14.79mg/ml.

Table 4.13: ED50 from in vivo antimalarial assay for DCM, ETOH and Hexanolic extracts of *Artemisia afra* collected from Burundi**.**

***A. Afra* extracts**	**Ethanolic**	**DCM**	**Hexane**
ED50 (mg/ml)	**6.43**	**11.99**	**14.79**

4.7 GCMS results of *A. afra* Burundi extracts

After GCMS ran, artemisinin was detected at 20.687 of retention time, peak and mass spectrometry of artemisinin was compared with the mass spectrometry of Burundi ethanolic, hexane and dichloromethane extracts (figure 4.9). The results showed that at the same retention time, no peak from the other extracts was detected.

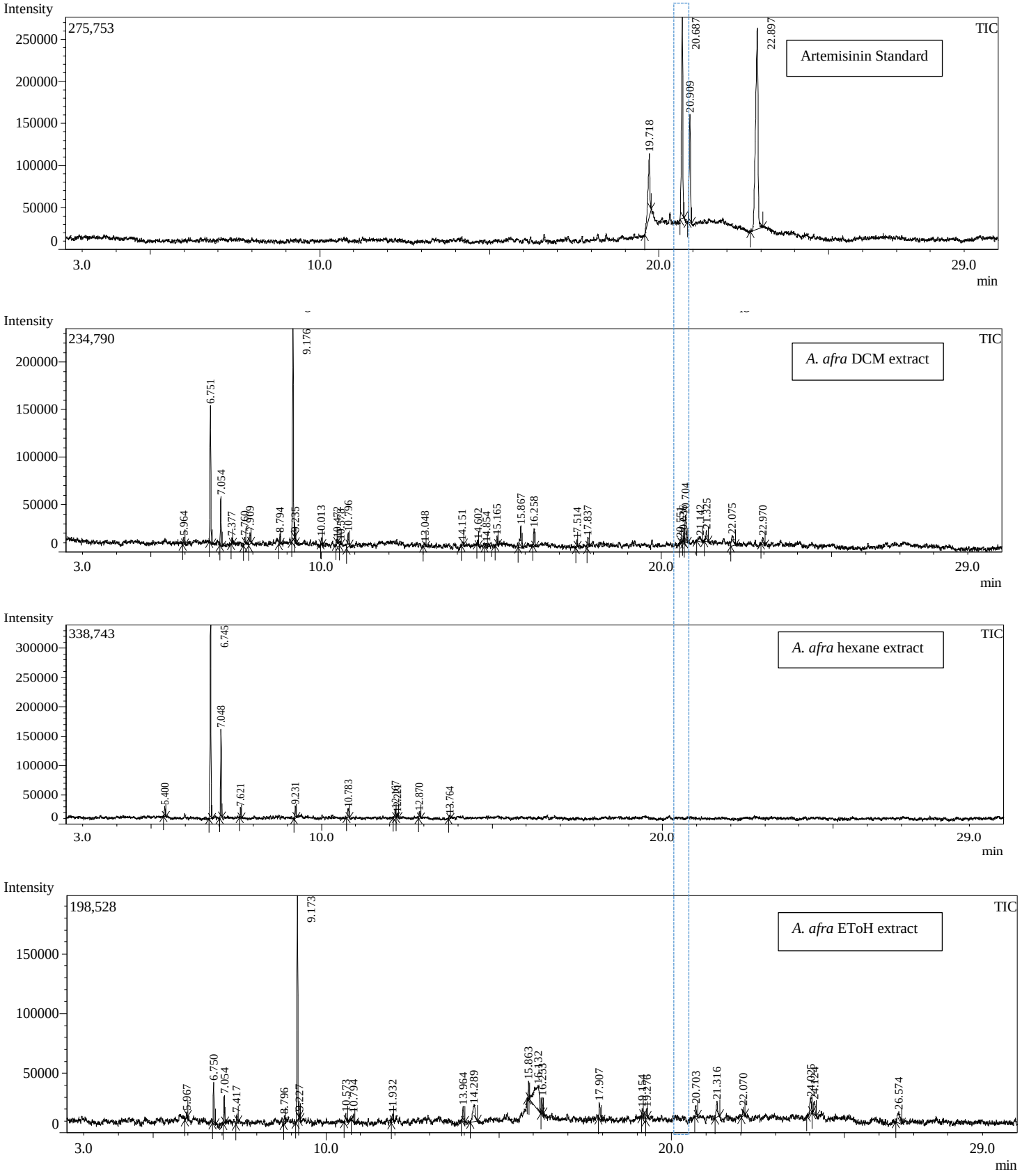


Figure 4.9: Chromatogram of artemisinin standard, dichloromethane extract, hexane and ethanolic extract of *A. afra*

Tableau 4.14: Main compounds detected by **GCMS** from dichloromethane extract, hexane and ethanolic extract of *A. afra* and result for **GCMS** for artemisinin standard

Standard/Extracts	Name of the compounds	Formula	Retention time (s)	Area %
	Cyclodeca[b]furan-2(3H)-one, 3a,4,5,6,7,8,9,11a-octahydro-3,6,10-trimethyl-		19.718	11.91
Artemisinin	Deoxyartemisinin		20.687	25.71
	1,8-Diazabicyclo[5.4.0]undec-7-en-11-one		20.909	13.3
	Bicyclo[4.1.0]heptane, 1-(3-oxo-4-phenylthiobutyl)-2,2,6-trimethyl-		22.897	49.08
A afra DCM	3,3,6-Trimethyl-1,4-heptadien-6-ol		5.964	1.11
extract	Eucalyptol		6.751	19.77
	1,5-Heptadien-4-one, 3,3,6-trimethyl-		7.054	6.87
	Cyclohexanol, 1-methyl-4-(1-methylethenyl)-, cis-		7.377	0.68
	Linalool		7.76	1.06
	5-Isopropyl-2-methylbicyclo[3.1.0]hexan-2-ol #		7.909	1.79
	Camphor		8.794	1.29
	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-		9.176	31.87
	Hexane, 1,6-dimethoxy-		9.235	0.46
	Cyclohexanol, 2-methyl-3-(1-methylethenyl)-, (1.alpha.,2.alpha.,3.alpha.)-		10.013	0.81
	1-Acetoxy-p-menth-3-one		10.472	0.65
	1,7-Octadiene-3,6-diol, 2,6-dimethyl-		10.578	0.56
	Bornyl acetate		10.796	2.63
	(1S,5S,6R)-6-Methyl-2-methylene-6-(4-methylpent-3-en-1-yl)bicyclo[3.1.1]heptane		13.048	1.01
	Methyl (1R,2R,8aS)-2-(methoxycarbonyl)-2-hydroxy-5,5,8a-trimethyl-trans-decalin-1-acetate		14.151	0.52
	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-		14.602	0.52
	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate		14.854	0.71
	Caryophyllene oxide		15.165	1.08
	.alpha.-Cadinol		15.867	5.27
	(1S,4aS,7R,8aS)-1,4a-Dimethyl-7-(prop-1-en-2-yl)decahydronaphthalen-1-ol		16.258	2.86
	Pulegone		17.514	0.96
	2-Pentadecanone, 6,10,14-trimethyl-		17.837	1.64
	6-C14H26		20.551	0.42
	Ethyl 9,12,15-octadecatrienoate		20.636	0.52
	Phytol		20.704	5.28
	Geranyl ethyl ether 2		21.142	1.99
	11alpha-Hydroxyprogesterone		21.325	2.31
	11alpha-Hydroxyprogesterone		22.075	3.77
	1-Aminocyclopentanecarboxylic acid, N-(but-3-yn-1-yloxycarbonyl)-, but-3-yn-1-yl ester		22.97	1.59
A. afra Hexane	Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-, (1S)-		5.4	2.68
extract	Eucalyptol		6.745	55.52
	1,5-Heptadien-4-one, 3,3,6-trimethyl-		7.048	24.19
	Undecane		7.621	2.45
	Dodecane		9.231	3.62

	Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester	10.783	3.72
	.alfa.-Copaene	12.167	2.95
	Tetradecane	12.221	1.24
	Caryophyllene	12.87	2.42
	4a,8-Dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene	13.764	1.21
A. afra Ethanolic extract	3,3,6-Trimethyl-1,4-heptadien-6-ol	5.967	0.45
	Eucalyptol	6.75	5.58
	1,5-Heptadien-4-one, 3,3,6-trimethyl-	7.054	3.23
	2,7-Dimethyl-2,6-octadien-4-ol	7.417	1.01
	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-	8.796	1.02
	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-	9.173	30.12
	3,7-Octadiene-2,6-diol, 2,6-dimethyl-	9.227	0.69
	1,7-Octadiene-3,6-diol, 2,6-dimethyl-	10.573	1.11
	Bornyl acetate	10.794	0.96
	3-Cyclohexene-1-methanol, 2-hydroxy-.alpha.,.alpha.,4-trimethyl-	11.932	1.5
	(1S,2S,4S)-Trihydroxy-p-menthane	13.964	1.99
	4-O-Methylmannose	14.289	6.76
	.alpha.-Cadinol	15.863	1.73
	.alpha.-Methyl mannofuranoside	16.132	18.51
	(1S,4aS,7R,8aS)-1,4a-Dimethyl-7-(prop-1-en-2-yl)decahydronaphthalen-1-ol	16.253	2.06
	Longifolenaldehyde	17.907	3.05
	Pentadecanoic acid	19.154	0.75
	Longifolenaldehyde	19.276	1.56
	Phytol	20.703	1.67
	Corymbolone	21.316	3.57
	5.beta.-Androstan-3.alpha.,11.alpha.,17.beta.-triol	22.07	1.58
	2-((2R,4aR,8aS)-4a-Methyl-8-methylenedecahydronaphthalen-2-yl)prop-2-en-1-ol	24.025	3.35
	1H-1,3a-Ethanopentalen-5(4H)-one, 2,3-dihydro-	24.124	3.18
	Thiourea, 1-(adamantane-1-carbonyl)-3-cyclohexyl-	26.574	4.57

After the GCMS of extracts of *Artemisia afra* from Burundi, the results showed that the ethanolic and dichloromethane extract had a high content of Bicyclo [2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-. The hexane extract had the same compound but at low amount compared to ethanolic and DCM extracts. The major compound in the hexane extract is Eucalyptol (Area 55.52) this compound is also found in DCM extract though at a lower amount (area 19.77) and also in ethanolic extract of *A. afra* (5.59). Hexane extract has fewer compounds compared to the others. Most of the compounds present in the ethanolic extract are also present in methanolic extract.

4.8 Gene expression results

4.8.1 Gel electrophoresis for PCR product

After parasite RNA extraction with trizol from the 8 samples, we got different concentration of RNA for each sample after measuring with a nanodrop. The concentration of RNA was normalized to get equal concentration for the 8 samples (experimentals and controls) before making the cDNA. A conventional PCR was then run to parameter the primers for the Fab enzyme genes and the housekeeping gene (Actine). A gel was run for the PCR product (figure 4.10).

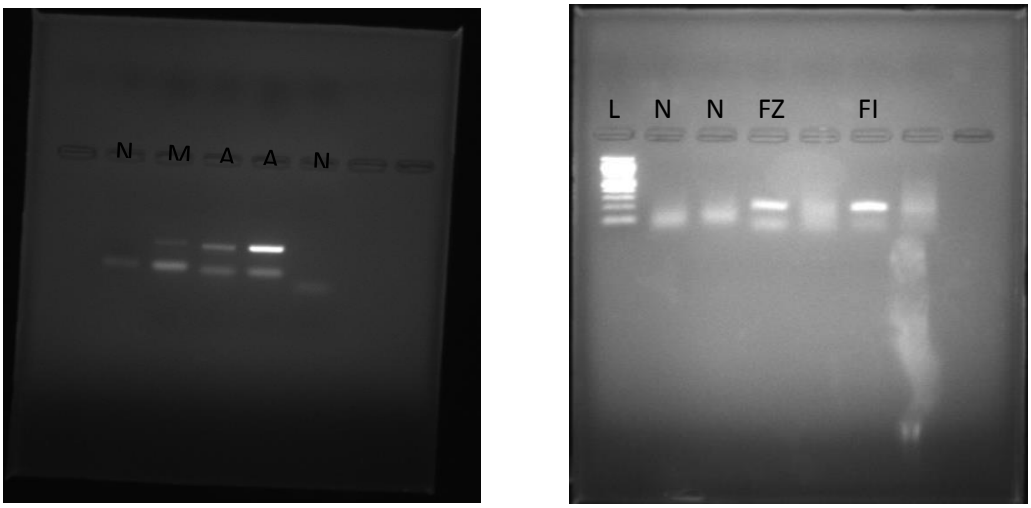


Figure 4.10: Gel electrophoresis of Actine FabI and FabZ

A: Actine

FZ: FabZ

FI: FabI

N: Negative control

L: Ladder

The results showed that the primers had well amplified the Fab enzymes genes and also the housekeeping gene. Many parameters were set to get the best amplification. The amplification was optimum with these parameters: Initial denaturation at 95°C for 5min and then 40 cycles of denaturation at 95°C for 1 min, annealing 60°C for 1min, extension 72°C for 1min, followed by the final extension at 72°C for 10min, and then held at 4°C.

4.8.2 Real-time amplification

The same parameter for the conventional PCR was reported to do the real-time PCR for the gene expression study. The target genes were well amplified as shown in figure 4.11.

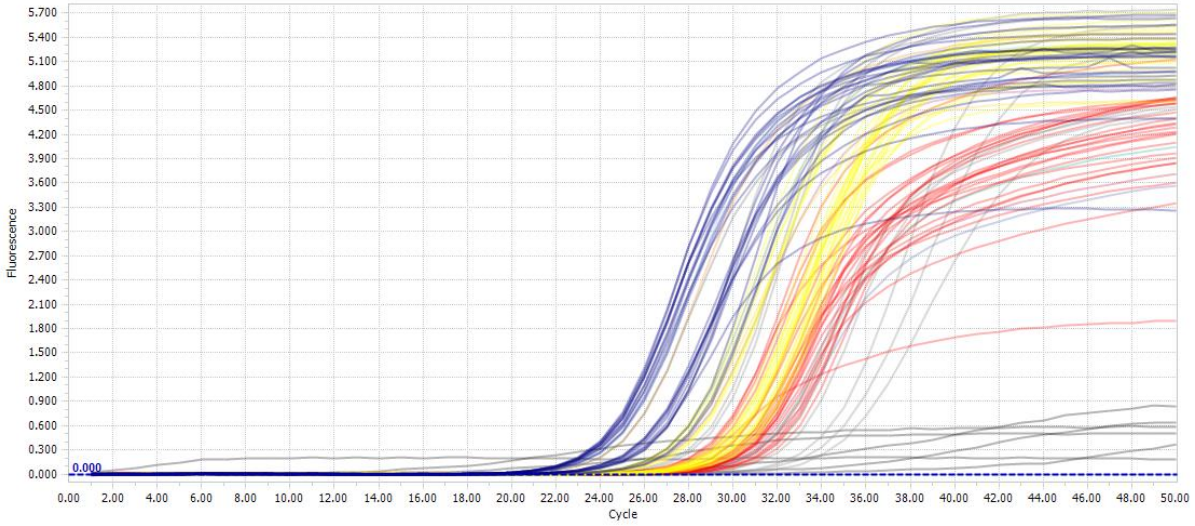


Figure 4.11: Amplification curves for FabI, FabZ and Actine

In blue colour, we have the amplification for the housekeeping gene (Actin), in yellow the amplification for FabI and in red colour the amplification for FabZ, In grey the amplification of the negative control and for the empty wells (that are drawn down of the picture). Actine has started first to be amplify following by FabI then by FabZ. Actine was well amplified and has the best fluorescent curve till 5.7 then followed by Fab I that was also well amplified with a fluorescent curve till 5.7 then for FabZ was also well amplified with a fluorescent curve of 4.8, but the amplification was less compared to the other genes.

4.8.3 Expression for Fab I and FabZ when the parasites are exposed to different extracts of A. afra

The fold change expression of FabI and FabZ was calculated from the Ct (cycle threshold is defined as the number of cycles required for the fluorescent signal to cross the threshold) values generated during the real-time PCR, and the result is showed in table 4.15 and 4.16. The fold change for the untreated sample is 1, for the treated samples when the fold change is greater than 1 it means that the targeted gene is upregulated and when it is lower than 1 it means that the targeted gene is downregulated. The Fab I gene from W2 strain of *P. falciparum* is upregulated with the hexane extract and downregulated by the ethanolic and dichloromethane extracts of the plant. In the second train for D6, we have a different path, FabI is upregulated

by DCM extract and downregulated by hexane and ethanolic extracts. For the two strains, FabI is downregulated by the ethanolic extract of *Artemisia afra* (see table 4.15).

Table 4.15: Fold change expression for FabI after treatment with *A. afra* extracts

Samples	ACTINE Ct Mean	Fab I Average CT	ΔCT (ACTINE)	ΔΔCT (ΔCT treated -ΔCT untreated)	Fold difference F.I 2 ^(-ΔΔCT)	EXTRACTS (A. afra)
P. falciparum W2						
Untreated S4	23.84±0.18	26,58±0.25	2.74±0.3	0±0.3	1	-
Treatment 1	24.84±0.03	27,03±0.10	2.19±0.1	-0.55±0.1	1.464085696	W2 Hexane
Treatment 2	25.36±0.08	29,64±0.47	4.28±0.47	1.54±0.47	0.343885455	W2 ETOH
Treatment 3	23.26±0.07	26,42±0.17	3.16±0.18	0,42±0.18	0.747424624	W2 DCM
P. falciparum D6						
Untreated S8	25,12±0.16	29,35±0.32	4.23±0.35	0±0.35	1	-
Treatment 5	26.59±0.19	29,11±0.50	2.52±0.53	-1.71±0.53	3.271608234	DCM
Treatment 6	23.04±0.07	28,67±0.15	5.63±0.16	1.4±0.16	0.378929142	ETOH
Treatment 7	23.1±0.05	28,39±0.16	5.29±0.16	1.06±0.16	0.47963206	Hexane

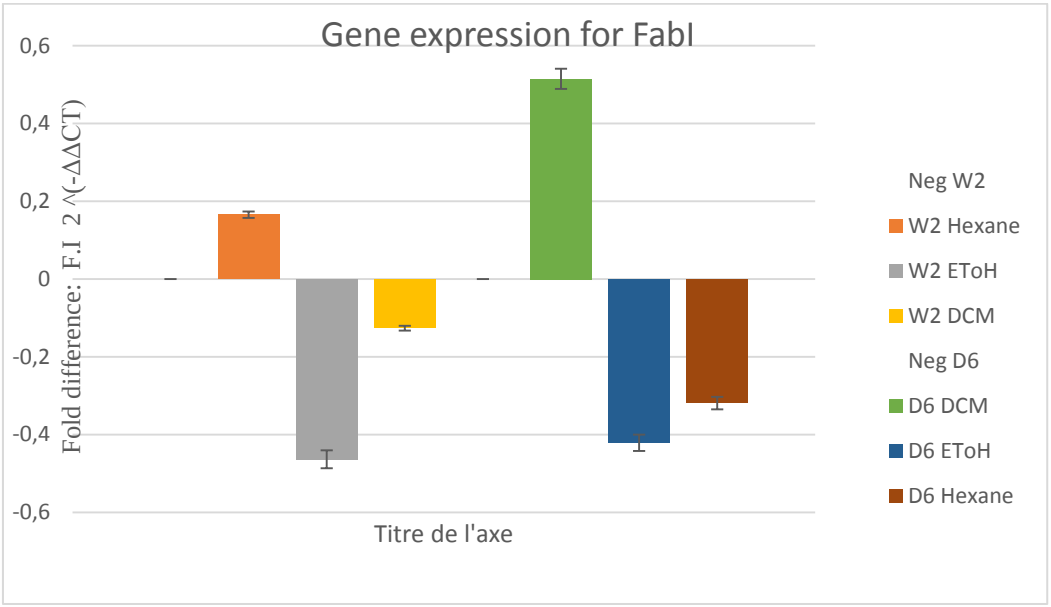


Figure 4.12: Gene expression for Fab I

The logarithm of the fold change for FabI in each treatment was calculated to make a histogram (Figure 4.12). The histogram is centered in 0 the up column showed upregulation, and the down column showed downregulation.

For Fab Z form W2 strain, the gene is upregulation when exposed to the three different extracts. For the D6 strain, the gene is also upregulated when exposed to the DCM extract but downregulated when exposed to the others extracts. Fab Z is upregulated by the DCM extract in the two strains of *P. falciparum.*

Table 4.16: Fold change expression for FabZ after treatment with *A. afra* extracts

Samples	ACTINE Ct Mean	FabZ Average CT	ΔCT (ACTINE_Fab Z)	ΔΔCT F.Z (ΔCT treated -ΔCT untreated)	Fold difference F.Z 2 ^(-ΔΔCT)	EXTRACTS (A. afra)
P. falciparum W2						
Untreated S4	23.84±0.18	30,42±0.2	6.58±0.26	0±0.26	1	-
Treatment 1	24.84±0.03	30,61±0.82	5.77±0.82	-0.81±0.82	1.7	Hexane
Treatment 2	25.36±0.08	30,67±0.51	5.31±0.51	-1.27±0.51	2.4	ETOH
Treatment 3	23.26±0.07	28,29±0.16	5.03±0.17	-1,55±0.17	2,9	DCM
P. falciparum D6						
Untreated S8	25,12±0.16	30,02±0.45	4.9±0.47	0±0.47	1	-
Treatment 5	26.59±0.19	29,23±1.6	2.64±1.6	-2.26±1.6	4.7	DCM
Treatment 6	23.04±0.07	29,5±0.38	6.46±0.38	1.56±0.38	0.3	ETOH
Treatment 7	23.1±0.05	29,65±0.54	6.55±0.54	1.65±0.54	0.3	Hexane

In that figure (figure 4.13) the results for the expression for FabZ are shown in the histogram; this allowed a better view of the scheme of expression for FabZ depending on the extracts where they are exposed. The histogram is centred in 0 the up columns showed upregulation, and the down columns showed downregulation.

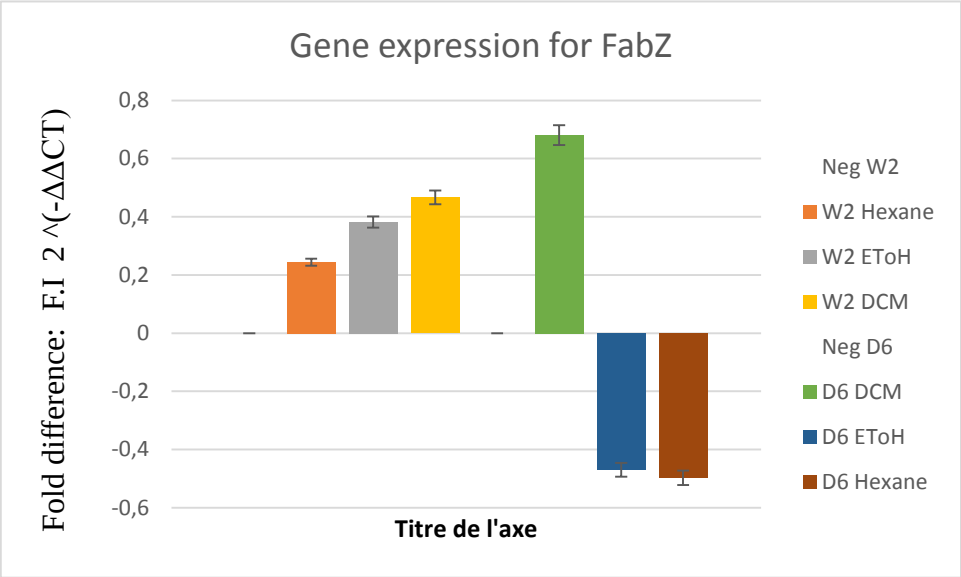


Figure 4.13: Gene expression for Fab Z

4.9 drug screening results:

After GCMS of the ethanolic, hexane and dichloromethane extracts in total, 51 molecules were detected. The affinity of the molecules was tested against FabI and FAB Z with vina. After drug screening, the binding energy for each molecule was determined and expressed in Kcal/mol see table 4.17. The results showed that many compounds have a high affinity with the Fab enzymes.

Table 4.17: *Artemisia afra* compounds from GCMS and Docking energy with FabI and FabZ

Ligands	Binding Energy (Kcal/mol) **(FabI)**	Binding Energy (Kcal/mol) **(FabZ)**
(1S, 2S,4S)-Trihydroxy-p-menthane	-6.4	-6.1
.alfa.-Copaene	-7.9	-7.1
.alpha.-Cadinol	-8.1	-7.4
.alpha.-Methyl_mannofuranoside	-5.7	-5.3
1_6-Dimethoxyhexane	-4.7	-4.9
1_7-Octadiene-3_6-diol__2_6-dimethyl-	-5.7	-6.6
cetoxy-p-menth-3-one	-6.9	-6.9
1-Aminocyclopentanecarboxylic_acid_N-but-3-yn-1-yloxycarbonyl-but-3-yn-1-yl_ester	-7.1	-4.9
1H-1,3a-Ethanopentalen-5(4H)-one_23-dihydro-	-6.7	-6.5
2-((2R,4aR,8aS)-4a-Methyl-8-methylenedecahydronaphthalen-2-yl)prop-2-en-1-ol	-7.9	-6.1

2_2_4-Trimethyl-1_3-pentanediol_diisobutyrate	-6.8	-6.6
2_4H_-Benzofuranone__5_6_7_7a-tetrahydro-4_4_7a-trimethyl-	-7.2	-6.2
2_7-Dimethyl-2_6-octadien-4-ol	-6.3	-6.6
2-Pentadecanone__6_10_14-trimethyl-	-6.2	-5.5
3_7-Octadiene-2_6-diol__2_6-dimethyl-	-6.1	-5.2
3-Cyclohexene-1-methanol__2-hydroxy-.alpha._.alpha._4-trimethyl-	-7.0	-5.8
3-Isopropenyl-2-methylcyclohexanol	-6.7	-6.9
4a_8-Dimethyl-2-_prop-1-en-2-yl_-1,2,3,4,4a,5,6,7-octahydronaphthalene	-8.0	-5.6
4-O-Methylmannose	-6.2	-4.8
5.beta.-Androstan-3.alpha.11.alpha.17.beta.-triol	-9.7	-7.6
6-C14H26_tetradecyne	-5.8	-6.3
Acetic_acid_1_7_7-trimethyl-bicyclo_2.2.1_hept-2-yl_ester	-6.9	-5.9
Aspidospermidin-17-ol, 1-acetyl-19, 21-epoxy-15, 16-dimethoxy	-10.2	-7.6
Bergamotene	-7.5	-8.0
Bicyclo_2.2.1_heptan-2-one__1_7_7-trimethyl-___1S_-	-6.3	-6.7
Bicyclo_221_3d	-6.2	-6.6
Borneol	-6.5	-6.7
Bornyl_acetate	-6.9	-5.9
Camphor	-6.3	-6.7
Caryophyllene	-8.0	-7.3
Caryophyllene_oxide	-8.3	-7.0
Corymbolone	-8.6	-7.3
Dodecane	-5.0	-5.8
Ethyl_9_12_15-octadecatrienoate	-6.6	-5.7
Eucalyptol	-6.4	-5.9
Geranyl_ethyl_ether_2	-5.6	-5.9
Intermedeol_	-7.8	-7.0
Isoartemisia_ketone	-6.0	-6.0
Linalool	-5.8	-6.2
Longifolenaldehyde	-7.9	-7.1
Methyl__1R_2R_8aS_-2-_methoxycarbonyl_-2-hydroxy-5_5_8a-trimethyl-trans-decalin-1-acetate	-8.3	-6.2
Pentadecanoic_acid	-5.6	-4.8
Phytol	-6.4	-7.2
11alpha-Hydroxyprogesterone	-10.7	-10.0
Pulegone	-7.8	-5.7
Tetradecane	-5.2	-5.5
Thiourea, 1-(adamantane-1-carbonyl)-3-cyclohexyl	-9.0	-8.4

Thujanol	-6.3	-6.3
trans--terpineol	-6.8	-6.2
Undecane	-4.7	-5.8

The results showed some hit compounds for FabI and FabZ with high biding energy. 11alpha-Hydroxyprogesterone have high binding energy with FabI (-10.7 kcal/mol) and Fab Z (-10 kcal/mol). The second hit is Aspidospermidin, which also have a high binding energy with FabI (10.2kcal/mol). The third is Thiourea,- which have a binding energy of -9 kcal/mol for FabI and -8.4 kcal/mol for FabZ.

The binding site for Fab I with 11 alpha-hydroxyprogesterone (hit 1) and with Aspidospermidin (hit 2) were shown respectively in figure 4.14 and 4.15. The 3D structure of FabI was download from PDB, and the active sites were determined before docking. After the virtual screening, the ligands were found to bind inside the active site and were in interaction with many residues in the active site (figure 4.14, 4.15).

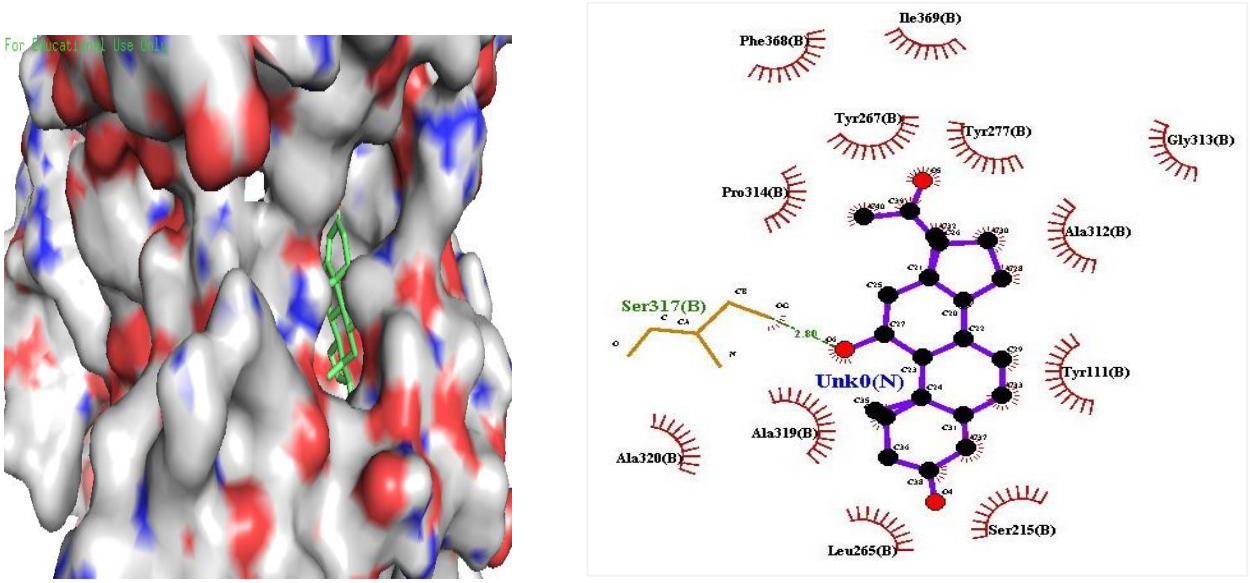


Figure 4.14: Interaction site for FabI (chain B) and hit N°1 (Hydroxyprogesterone)

Residue with hydrophobic interaction: Ile 369, Phe 368, Tyr 267, Tyr 277, Ala 312, Gly 313, Ser 215, Pro 314, Tyr 111, Leu 265, Ala 320, Ala 319 and hydrogen bound with Ser 317.

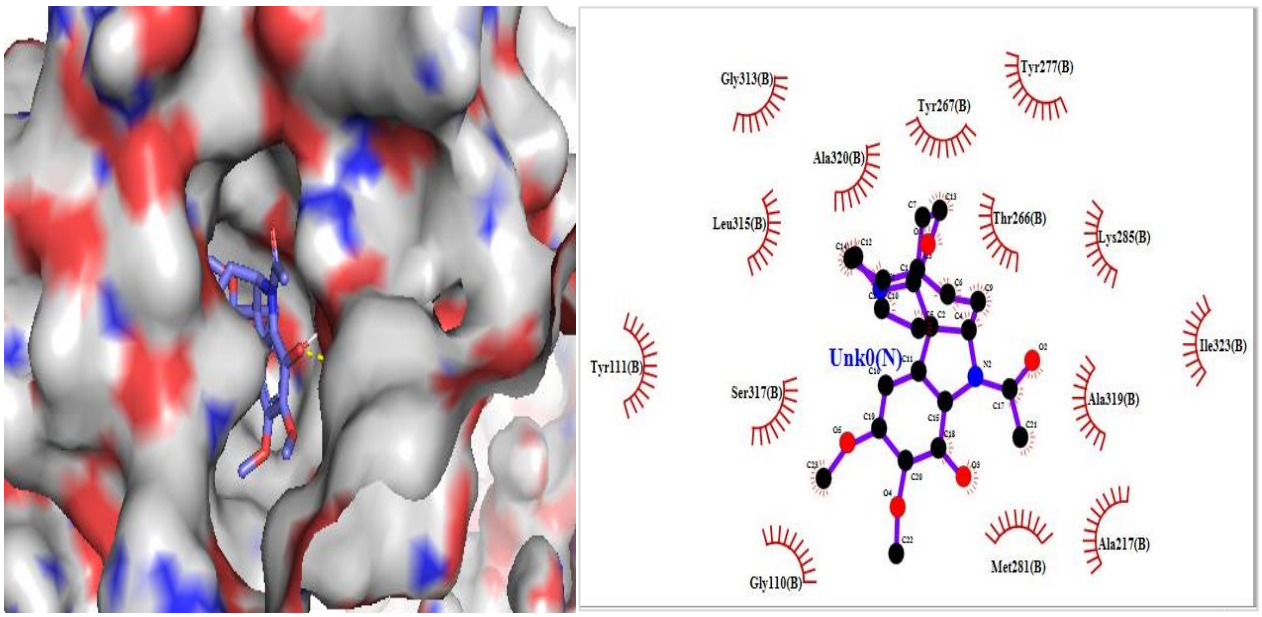
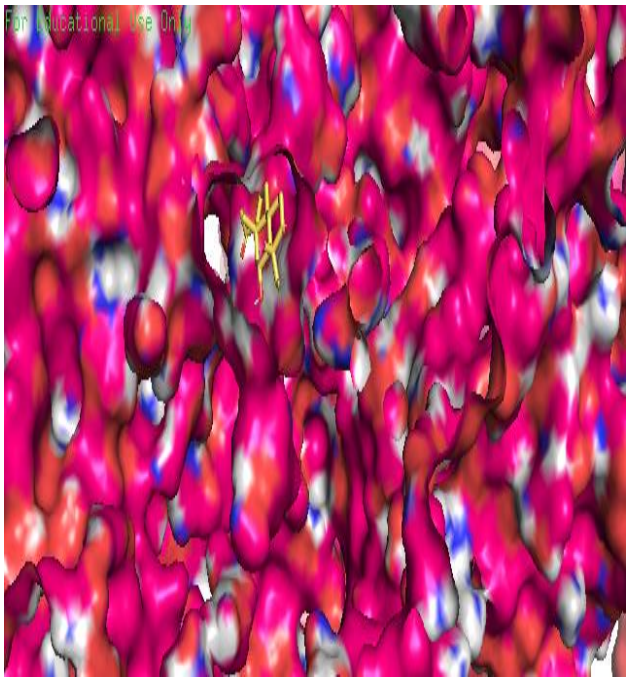


Figure 4.15: Interaction site for FabI (chain B) and hit N°2 (Aspidospermidin).

Residue with hydrophobic interaction: Tyr 277, Tyr 267, Ala 320, Gly 313, Leu 315, Tyr 111, Ser 317, Gly 110, Met 281, Ala217, Ala 319, Ile 323, Lys 285, and Thr 266.

The binding site for FabZ with 11 alpha-hydroxyprogesterone (hit 1) and Thiourea,-(hit2) were shown respectively in figure 4.16 and 4.14. There is mostly hydrophobic interactions with the ligands, and also one hydrogen bind between Thiourea and the residue Tyr 100(G).



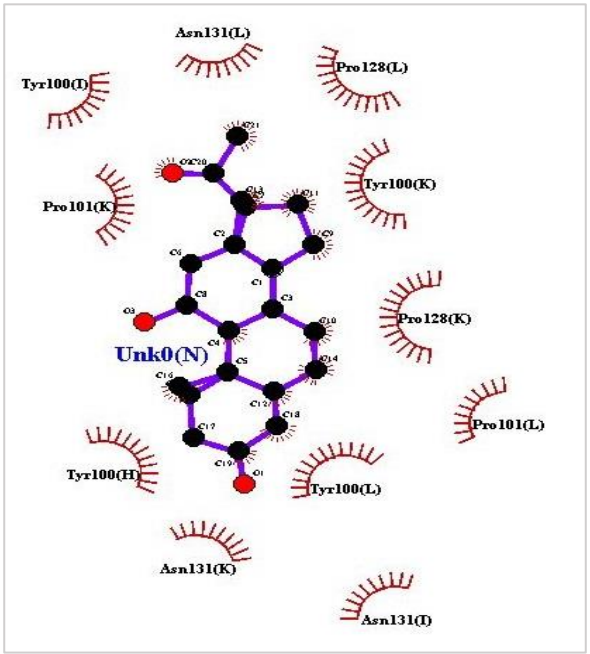


Figure 4.16: Interaction site for FabZ (chain K, L, I, H) and hit N°1 (Hydroxyprogesterone). Residue with hydrophobic interaction: Tyr 100(I), Pro 101(K) 267, Tyr 100(H), Asn 131(K), Asn 131(I), Tyr 100 (L), Pro101(L), Pro 128 (K), Tyr 100(K), Asn 131(L) and Pro 128(L).

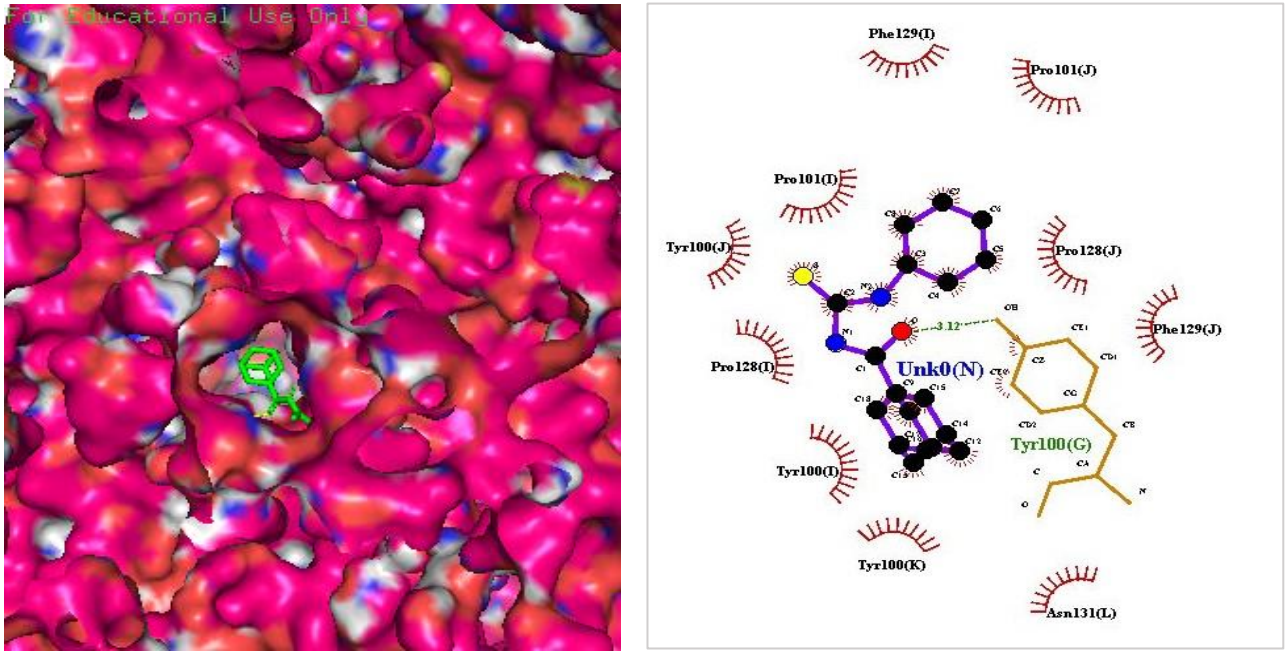


Figure 4.17: Interaction site for FabZ (chain K, L, I, J, G) and hit N°2 (Thiourea).

Residue with hydrophobic interaction: Phe 129(J), Pro 101(J), Pro 101(I), Tyr 100(I), Pro 128(I), Tyr 100 (I), Tyr 100(K), Asn 131 (L), Phe 129(J), Pro 128(J) and one hydrogen bond with Tyr 100(G).

CHAPTER FIVE

DISCUSSION

5.1 Phytochemical screening, Total phenol, and total flavonoid:

5.1.1 Phytochemical screening

The phytochemical screening revealed the presence and absence of different secondary metabolic compounds. Some phytochemical compounds like terpenoids and glycosides were found in all extracts. Previous studies have reported terpenoids such as triterpenes, sesquiterpenes and diterpenes to exhibit antibiotics, insecticidal, anthelmintic and antiseptic activity (Duke *et al*., 1992; Parveen *et al*., 2010) while glycosides are known to treat congestive heart failure and cardiac arrhythmia (Vladimir *et al*., 2001). Alkaloids were only found in the ethanolic extract from South Africa and Kenya; the test was negative for the other extracts. Tannins were detected in all ethanolic and water extracts, which according to earlier studies is reported to have antibacterial (Hisanori *et al*. 2001), antitumor and antiviral activities (Kumar *et al*., 2013). They work by precipitating microbial protein, thus making nutritional protein unavailable for them. Saponins were found in all water extracts. It's believed saponins have a favorable effect on cholesterol, can help boost the immune system, have an antioxidant effect, and may even support bone strength [2]. Phytochemical screening is essential because especially the therapeutic potential uses as medicinal plants.

5.1.2 Total phenols and total flavonoids

This study demonstrated a different level of phenols and flavonoids from the different plant extracts, depending on the solvent used and also depending on the agro-ecological localization of the plant extracts. The difference in phenols and flavonoids may be due to various geographical locations in which sampling was done because this soils and other environmental factors may have a great influence on the phytochemical contents of the plant (Borokini *et al*., 2012). Extensive data are showing that flavonoids and phenols synthesis in the plant is influenced by different factors abiotic and biotic including UV light radiation, drought, ozone, phytopathogens, insect-deterrent (Duangjai *et al*., 2018), and environmental factors (Borokini *et al*., 2012). Globally the results show that *Artemisia afra* plant collected from Burundi had the highest flavonoids and phenols content compared to the other plant collected in different

agro-ecological location. Also, ethanolic extract from Burundi had the highest phenols content and flavonoids content. This study also reported that Ethanolic extract from Burundi had the highest phenols content and flavonoids content. Then the second concentration was the DCM extract from Burundi, which also had high flavonoids content compared to water and hexane extracts. On the other hand, water extract from Burundi was reported to have more phenols compared to the DCM extract. Previous studies have reported phenolic compounds as well as flavonoids to be well-known antioxidant and to contain many other important bioactive agents that have long been known to have human health benefits like, curing and preventing many diseases (Nikolova *et al*., 2005). It can be speculated that the high concentration of phenols and flavonoids of *Artemisia afra* is responsible for their medicinal properties and antioxidant activity. In this study, ethanol and water solvents, extracts had more phenols than DCM and hexane solvent; this could be due to high polarity as reported in earlier studies (Prashant *et al*., 2011).

Furthermore, Burundi had the highest level of flavonoids followed by Tanzania, then Senegal and lowest reported in South Africa and Kenya. These differences in term of phytochemical concentration are due to their different location and may be attributed to different antioxidant activities. The global results indicated that polyphenolics are important components of *A. afra*, and most of the pharmacological effect attributed to this plant could be due to the presence of these valuable constituents. To the best of our knowledge, this is the first study to report total phenol and total flavonoids content results for *Artemisia afra* plant with gallic acid and rutin as standards.

5.2 DDPH and Antimalarial assay:

This study has reported the antimalarial properties of *Artemisia afra* plant and also the antioxidant activity. *Artemisia afra* plant collected from Burundi (hexanolic; ethanolic and DCM extract) expressed the highest antimalarial activity which could be due to high-level content of flavonoids and phenols compared to the others plants collected from Senegal, Kenya, Tanzania and South Africa. The hexane extract from Burundi had the highest antimalarial activity followed by Burundi ethanolic extract, then Burundi DCM extract. On the other hand, Burundi ethanolic extract had the highest antioxidant activity, followed by Burundi DCM extract and lastly Burundi hexane extract. In this study, the antimalarial activities and the antioxidant activity do not always correlate. For example, Burundi hexanolic extract had here the highest antimalarial activity but not the highest antioxidant activity. Research done by

Stephen *et al.* (2012) about the antioxidant activity of methanolic extract of *Artemisia afra* collected in South Africa is in line with our findings. Study done in South Africa by Clarkson *et al*. (2012) about i*n vitro* antiplasmodial test against D10 with extracts of *A. afra* collected in South Africa showed different IC50, for (i) DCM, (ii) MeOH and (iii) Water were 5, 8 and >100 µg/mL indicating non-polar solvent DCM extract to have highest activity against the *P. falciparum.* This results from this study had higher IC50 compared to the study by Clarkson *et al.* (2012). The variation may be due to the difference in extraction technique or the storage of the plant sample.

The water extracts did not show any antimalarial activity, but some study shows that concoction is more active than infusion and also more active than normal water extraction without any heating. Concoction may help in access to essential mineral (Baldwin's team, 2012). This suggests that concoction may extract more active compounds which are more efficacious. This is in line with the *In vitro* antimalarial test done by Gathirwa in 2007, which showed a high activity in *Artemisia afra* water extract (Gathirwa *et al*., 2007). Although the study reported that water extract of *Artemisia afra* extracted without any heating has no antimalarial activity (Gathirwa *et al*., 2007), however, the current research reported high antioxidant activity with Burundi water extract, followed by Kenya water extract. The total phenols and total flavonoids were found to correlate positively, and the correlation between phytochemical compounds and the antioxidant activity was negative, these results indicate that more they the total phenols or flavonoids is high and more the antioxidant activity is high.

5.3 Acute oral toxicity and in vivo antimalarial activity

Based on the previous results *Artemisia afra* collected from Burundi showed the highest antimalarial and antioxidant activities, so the extracts of *A. afra* from Burundi was chosen and tested to check his toxicity but also his In vivo antimalarial activities.

5.3.1Acute oral toxicity

The toxicity was ran using mice toxicity sign and body weight was recorded every day at the end of the test the organs were weight and the blood was collected to test the ALT and AST parameters of the liver.

5.3.2. Body and organs weight

Acute (single dose) toxicity was conducted in mice swiss albinos for a period of 14 days to determine the toxicity of *A. afra* plant extracts collected from Burundi. Most studies have tested toxicity only on the aqueous extract of that plant. The toxicity of *A. afra* extracted with other solvents (DCM; ethanol, hexane…) needs to be investigated because also having high antimalarial activities due to interest in the medicinal value of the plant. In this present study, the ethanolic, hexane, and DCM extract of *A. afra* were tested for their toxicity and also for their antimalarial activity in mice. The result of the acute toxicity did not report mortality with single oral dose up to 2500mg/kg body weight. Only one individual mortality was recorded for the DCM extract at the dosage of 2500mg/kg body weight. The present result, therefore, suggests that the oral LD50 of all the extracts are greater than 2500mg/kg. Others studies after an acute toxicity test have reported an LD_{50} more than 5000mg/kg of body weight but for Aqueous extract of *Artemisia afra* (Idris et al., 2015) that showed that the aqueous extract is much safer. During the 14 days test, the body weight was recorded daily. Body weight changes are indicators of adverse side effects of drugs and chemicals (Hilaly et al., 2004), as the animals that survive cannot lose more than 10% of the initial body weight (Teo *et al*., 2002). An Increase of the body weight for all groups is observed during week 2. Increment in body weight determines the positive health status of the animals (Heywood *et al*., 1983). However, differences between the experimental and control group were noted. A decrease of body weight was observed for the group of mice treated with DCM extract at the dosage of 2500mg/kg and also for ETOH extract at 2000mg/kg compared to the control, but for hexane extract of *A. afra* at the dosage of 2000mg/kg of body weight there is a gain of weight compare to the control. For the other groups, the weight gain is not significantly different from that of the control group. Therefore, the overall weight gain in both treated and control mice might indicate a good health status of the experimental animals. Research done by Eshetu *et al.*, 2016 in the same observation was done during an acute oral administration test of aqueous extract of *Artemisia afra*. For the weight of the organs, there were no significant differences between the experimental groups and the control. Organs weight is an index of swelling, atrophy or hypertrophy (Amresh *et al*., 2008). Comparison of organ weights between control and treated groups have conventionally been used to predict toxic effects of test material (Nisha et al., 2009; Pfeiffer, 1968) and help to know if the organs were exposed to injury or not. However, in the present study, there was no significant change in organ (liver, spleen, and kidney) weights and gross visual examination of the organs of both treated mice and the controls. These showed the

normal architecture, no colour change and no morphological disturbances, indicating that the acute toxicity oral doses of *A. afra* extracts administered had no effect on the organs of the mice and was well tolerated.

5.3.3 Biochemical parameters (ALT and AST)

ALT and AST biochemical test were evaluated using the plasma from the blood of the mice to obtain further toxicity related information, not detected by direct examination of organs and body weight analysis. No statistical differences were noted when we compare the experimental mice with the control, which suggested that the extracts have no effect on the liver function. Generally, liver cell damage is characterised by a rise in serum enzymes like AST, ALT, ALP, etc. (Brautbar *et al*., 2002). In our results AST is more expressed compare to ALT this is due to that AST is expressed in higher concentration in a number of tissues (liver, kidney, heart and pancreas) and it is released slowly compare to ALT (S. Chanda *et al*., 2017 *Artemisia afra* is a promising medicinal plant with a high antimalarial effect. Mostly the acute toxicity for *A. afra* plant is done for the aqueous extract it is also very important to check the toxicity of the plant extracts when others solvents are used for the extraction. The antimalarial effect is more important when the plant is extracted with others solvent for example with methanol or ethanol compare to when it is extracted with water because this solvents (methanol, ethanol) extract more bioactive compounds that may have medicinal importance. The problem using those solvents is they may have toxic effect why toxicity test is important to run for them.

5.3.5 Effects of Acute Administration of the Extract on Histology of the Kidneys and liver

The microscopic architecture of the kidneys and livers in treated mice had similar appearance to that of the controls. Administration of the extracts only caused minimal tissue effect in some animals, mainly characterized by congestion of blood vessels. There were no histopathological changes visible in the kidneys. Minimal changes noticed in the liver include multiplication of Kupfer cells and mild loss of hepatolobular arrangement in some slides. There are mild toxicity signs that have any implication on the health of the mice.

5.4 In vivo antimalarial assay

The *in vivo antimalarial* assay of *A. afra* extracts (EtOH, DCM, and Hexanolic extracts) was found to have high activities. *A. afra* extracted with ethanolic have the highest activities with

an ED50= 6.45mg/ml, following the DCM extract ED50=11.99mg/ml then following by the *A. afra* hexanolic extract (ED50= 14,79mg/ml). The results are in line with an erlier study did by Gathirwa *et al*., who found a high chemo-suppression activity of *A. afra* plant extracted with MeOH during *in vivo* antimalarial activity with *P. berghei anka* (Gathirwa *et al*., 2007). *A. afra* not only has a big antimalarial activity but also was found to inhibit rapid growing of *Mycobacterium aurum* and virulent *Mycobacterium tuberculosis* replication (Siyabulela et al., 2009), to reduce also blood glucose and may have beneficial effects on complications of diabetes (Idris et al., 2015), and also to have potential anticancer properties (L. Spies *et al*., 2012).

5.5 GCMS of *Artemisia afra* extracts

After the *in vitro* and *in vivo* antimalarial test, GCMS was run in all the three extracts: ethanolic; hexane and dichloromethane of *Artemisia afra* leaves from Burundi. 10 compounds were found in hexane extract; the major compound was Eucalyptol (55.5%). Cineole or Eucalyptol has mucolytic, bronchodilating and anti-inflammatory properties and reduces the exacerbation rate in patients suffering from COPD (chronic obstructive pulmonary disease), as well as ameliorates symptoms in patients suffering from asthma and rhinosinusitis (Juergen *et al*., 2013). In the dichloromethane extract, 30 compounds were detected by GCMS, and the major compounds were Borneol (33.5%) and Eucalyptol (21.9%). Borneol is a common ingredient in many traditional Chinese herbal formula and has a wide range of uses, It aids the digestive system by stimulating the production of gastric juices; tones the heart and improves circulation; treats bronchitis, coughs, and colds; can relieve pain caused by rheumatic diseases and sprains; reduces swelling; relieves stress; and can be used as a tonic to promote relaxation and reduce exhaustion (http://acupuncturetoday.com/herbcentral/borneol.php). In the methanolic extract, the major compound was also Borneol (Bicyclo [2.2.1] heptan-2-ol, 1, 7, 7-trimethyl-, (1S-endo) (40.6%). Majority of the compounds found in the ethanolic were also found in the dichloromethane and some also in the hexane extract. The same major compound was found by Josphat *et al.* when analysing the essential oil from *Artemisia afra* by GCMS. The major constituents were 1, 8-cineole (67.4%), terpinen-4-ol (6.5%) and borneol (5.1%) (Josphat *et al*. 1995). The medicinal effect attributed to that plant can be because of the presence of all those active compounds. The first line against malaria is artemisinin and derivatives, which are extracted from *Artemisia annua*. Artemisinin was not detected in all three extracts of *Artemisia afra*, so this means that the antimalarial activity laid on the other active compounds of that plant.

5.6 Gene Expression

Inhibition test was run by incubating during 48hours the parasite cultures with *A. afra* extracts to study the expression level of the *P. falciparum* Fab enzymes (FabI and Fab Z). The fab Enzymes play an important role in fatty acid synthesis pathway (FAS II). The prodigious proliferative capacity of malarial parasites necessitates access to an abundant source of fatty acids (FAs). These carboxylic acid-linked acyl chains are required for the production of lipid species that are essential for parasite membrane and lipid body biogenesis (Palacpac *et al*., 2004). It has been shown that FAS II can be exploited for antimalarial drug discovery (Waller et al., 2003; Perozzo et al., 2002). In our results, FabZ (D6 strain) was found to be downregulated by the ethanolic and hexane extract of *A. afra*. Fab Z is the primary dehydratase that participates in the elongation cycle of saturated as well as unsaturated fatty acid biosynthesis (Kumar et al., 2003). Downregulation of FabZ can lead to parasite death due to his crucial role in the fatty acid biosynthesis. Fab I also play a crucial role during liver-stage of malaria infection (Min Yu et al., 2008). A downregulation of Fab I can lead to a failure of the parasite to complete the liver stage by an inability to form intrahepatic merozoites that normally initiate blood-stage infection. The results showed a downregulation of Fab I by the ethanolic and the DCM extracts of *Artemisia afra* for the W2 strain, and in the D6 strains the gene is downregulated by the hexane and ethanolic extracts; this indicated that *Artemisia afra* could act as a drug with a prophylactic effect that can be able to stop the parasite in the earlier liver stage before the spread into the bloodstream.

5.7 Affinity binding with FabI and FabZ

A library was created based on the compounds found during GCMS and was screened against the two fab enzyme (Fab I and FabZ) to test their potential interactions using Autodock Vina with PyRx.

Autodock Vina is docking software that aims to predict the ligand-protein complex structure by exploring the conformational space of the ligands within the binding site of the protein. A scoring function is then utilised to approximate the free energy of binding between the protein and the ligand in each docking pose (Evanthia *et al*. 2014).

After the virtual screening all compounds were found to display a binding affinity that varies from high to low with the Fab enzymes, this may indicate that the all active compounds of *Artemisia afra* may play a significative rule by interacting with the *P. falciparum* Fab enzymes, which has a significant role in parasite survival during malaria infection. Comparison of the different binding energies for all molecules have shown up some dock hits. It was found that 11 alpha-Hydroxyprogesterone, Aspidospermidin-17-ol, 1-acetyl-19, 21-epoxy-15, 16-dimethoxy, and Thiourea, 1-(adamantane-1-carbonyl)-3-cyclohexyl were the efficient binders, with high binding energy. There is no literature about the pharmacological effects of Aspidospermidin- and Thiourea, 1-(adamantane-1-carbonyl)-3-cyclohexyl. 11alpha-Hydroxyprogesterone has recently been patented for the treatment of skin diseases, particularly psoriasis, it is an important pharmaceutical compound with anti-androgenic and blood-pressure-regulating activity (Nguyen et al., 2012). Those active compounds were also found to interact with most of the residue of the active side of Fab I. However, an in vitro test is necessary to confirm their antimalarial activity.

5.7 Conclusion:

The antimalarial effect of *A. afra* plant has been demonstrated in that study. The results showed a big *in vitro* and also *in vivo* antimalarial activity and no toxicity effect. *A. afra* constitutes a great candidate in the battle against malaria. Due to the inhibition of FabI, this plant can be used as a prophylactic drug to prevent malaria. The drug screening of the active compounds of that plant shown high interaction with the *Plasmodium falciparum* fab enzymes, this useful in the search for new anti-malarial drugs from natural sources.

5.8 Recommendation

- These results provide room for future extensive investigations on the plant.
- Isolation and characterization of the bioactive compounds leading to establishment of novel antimalarial compounds.
- Toxicity test of the actives compounds
- In vitro test of the active compounds
- Use of the herb as profilactic and curative remede against Malaria.
- Overcome drug resistant malaria with *A. afra.*

5.9 Ethical consideration

Permission to carry out this study and ethical clearance was sought from the CBRD Scientific Committee, Scientific Ethics Review Unit (SERU) and Animal Use and Care Committee in KEMRI.

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website.www.kemri.org

KEMRI/RES/7/3/1 March 01, 2019

TO: **NDEYE FATOU KANE,**
PRINCIPAL INVESTIGATOR.

THROUGH: **THE DIRECTOR, CBRD,**
NAIROBI. Forwarded Ukamaji 07/03/2019

Dear Madam,

RE: **KEMRI/SERU/CBRD/189/3756 *(RESUBMSSION II OF INITIAL SUBMISSION)*: EFFECT OF EXTRACTS OF *ARTEMISIA AFRA* PLANTS FROM FIVE DIFFERENT COUNTRIES IN COMBINATION WITH ASIATIC ACID, ARACHIDONIC ACID AND ANTHOCYANIDIN ON *PLASMODIUM* SPECIES.**

Reference is made to your letter dated February 27, 2019. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the following revised study documents on February 27, 2019:

1. Revised proposal (Version 4.0)
2. Response to the comments raised by SERU
3. ACUC ethical approval

This is to inform you that the Committee notes that the following issues raised during the 280th Committee B meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on **October 17, 2018** have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **March 01, 2019** for a period of **one (1) year**. Please note that authorization to conduct this study will automatically expire on **February 29, 2020.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **January 25, 2020**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued. You may embark on the study.

Yours faithfully,

ENOCK KEBENEI,
THE ACTING HEAD
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

References:

Abdoulaye A. Djimde, Amelia W. Maiga, Dinkorma, Ouologuem, Bakary Fofana, Issaka Sagara, Demba Dembele, Sekou Toure, Kassim Sanogo, Souleymane Dama, Bakary Sidibe, and Ogobara K. Doumbo. 2016 'Gametocyte clearance dynamics following oral artesunate treatment of uncomplicated falciparum malaria in Malian children. ' Parasite Journal.; 23: 3. Doi: 10.1051/parasite/2016003

Abdullahi, M.N., Ilyas, N., Ibrahim, H. 2013 Evaluation of phytochemical screening and analgesic activity of aqueous extract of the leaves of Microtrichia perotitii dc (Asteraceae) in mice using hotplate method. Med. Plant. Res.; 3: 37–43.

Acupuncture Today; Borneol (Bing pian); Available from, as of June 24, 2016: http://acupuncturetoday.com/herbcentral/borneol.php

Ágnes Orbán, Ádám Butykai, András Molnár, Zsófia Pröhle, Gergö Fülöp, Tivadar Zelles, Wasan Forsyth, Danika Hill, Ivo Müller, Louis Schofield, Maria Rebelo, Thomas Hänscheid, Stephan Karl, István Kézsmárki ; May 13, 2014 '' Evaluation of a Novel Magneto-Optical Method for the Detection of Malaria Parasites'' http://dx.doi.org/10.1371/journal.pone.0096981

Akkawi M, Jaber S, Lutgen P. 2014 Investigations of Artemisia annua and Artemisia sieberi water extracts inhibitory effect on beta-hematin formation. Medicinal & Aromatic Plants, Volume 3, Issue1.

Amresh G, Singh PN, Rao CV 2008. Toxicological screening of traditional medicine Laghupatha (Cissampelos pareira) in experimental animals. Journal of Ethnopharmacology; 116:454-460.

Arsenault P, Wobbe K, Weathers P 2008 Recent Advances in Artemisinin Production Through Heterologous Expression. Cur Med Chem 15: 2886-2896.

Ayoola, G.A., Coker, H.B., Adesegun, S.A., Adepoju-Bello, A.A., Obaweya, K., Ezennia, E.C., tangbayila, T.O. 2008 Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in southwestern Nigeria. Trop. J. Pharm. Res.; 7: 1019–1024.

Baldwins Team, Infusions v Decoctions - What's the difference?, 2012 downloaded from: https://www.baldwins.co.uk/blog/infusions-v-decoctions-whats-the-difference

Balint G.A, 2001 Artemisinin and its derivatives .An important new class of antimalarial agents. *Pharmacology and Therapeutics*, 90, 261-265.

Banso, A., Adeyemo, S. 2006 Phytochemical screening and antimalarial assessment of Abutilon mauritianum, Bacopa monnifera and Datura stramonium. Biokemistri Res.; 18: 39–44.

Barrett I, Meegan MJ, Hughes RB, Carr M, et al: 2008 Synthesis, biological evaluation, structural-activity relationship, and docking study for a series of benzoxepin-derived estrogen receptor modulators. Bioorg Med Chem., 16: 9554-9573. 10.1016/j.bmc.2008.09.035.

Borokini, T.I., Ayodele, A.E. 2012 Phytochemical screening of Tacca leontopetaloides (L.) Kuntze collected from four geographical locations in Nigeria. Int. J. Modern Bot. Res.; 2: 97–102.

Brautbar N, Williams IIJ. 2002 Industrial solvents and liver toxicity: risk assessment, risk factors, and mechanisms. International Journal of Hygiene and Environmental Health; 205:479-491.

Bunnag, D., Kanda, T., Karbwang, J., Thimasarn, K., Pungpak, S., & Harinasuta, T. (1996). Artemether or artesunate followed by mefloquine as a possible treatment

Burk, O., Arnold, K. A., Nussler, A. K., Schaeffeler, E., Efimova, E., Avery, B. A., ... & Eichelbaum, M. 2005. Antimalarial artemisinin drugs induce cytochrome P450 and MDR1 expression by activation of xenosensors pregnane X receptor and constitutive androstane receptor. *Molecular pharmacology*, *67*(6), 1954-1965.

Burits M, Asres K, Bucar F 2001 The Antioxidant Activity of the Essential Oils of Artemisia afra, Artemisia abyssinica and Juniperus procera. Phytother Res15: 103-108.

Cai, Y.Z., Luo, Q., Sun, M., Corke, H. 2004 Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. Res.; 74: 2157–2184.

Chang, C., Yang, M., Wen, H., Chern, J. 2002Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food Drug Anal. Res. 10: 178–182.

Chlopicka, J., Pasko, P., Gorinstein, S., Jedryas, A., Zagrodzki, P. 2012Total phenolic and total flavonoid content, antioxidant activity and sensory evaluation of pseudocereal breads. LWT –Food Sci. Tech. ; 46: 548–555.

Clarke, Garry & Ting, Kang & Wiart, Christophe & Fry, Jeffrey. 2013 High Correlation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging, Ferric Reducing Activity Potential and Total Phenolics Content Indicates Redundancy in Use of All Three Assays to Screen for Antioxidant Activity of Extracts of Plants from the Malaysian Rainforest. Antioxidants. 2. 1-10. 10.3390/antiox2010001.

Coluzzi M. 1999 The clay feet of the malaria giant and its African roots: hypotheses and inferences about origin, spread and control of Plasmodium falciparum. Parassitologia. ;41:277–283.

CDC: Centers for disease control and prevention, January, 2019 "Malaria's impact worldwide" downloaded from: Global Health, Division of Parasitic Diseases and Malaria

CDC: Centers for disease control and prevention, December 29, 2017" Malaria" Page last reviewed: Content source: Global Health, Division of Parasitic Diseases and Malaria

CJ Puotinen, 2003 Artemisinin, malaria and cancer, NEHA Internat, Winter

Chen MM, Shi L, Sullivan DJ Jr. Haemoproteus and Schistosoma synthesize heme polymers similar to Plasmodium hemozoin and beta-hematin. Mol Biochem Parasitol. Mar;113(1):1-8

Cheng T, Li Q, Zhou Z, Wang Y, Bryant S.H. 2001 Structure-based virtual screening for drug discovery: a problem-centric review. AAPS J. 2012;14(1):133–141. doi: 10.1208/s12248-012-9322-0.

Clarkson, C., Maharaj, V.J., Crouch, N.R., Grace, O.M., Pillay, P., Matsabisa, M.G., Bhagwandin, N., Smith, P.J. & Folb, P.I., 2004. In vitro antiplasmodial activity of medicinal plants native to or naturalised in South Africa. Journal of Ethnopharmacology 92: 177–191.

Clarkson C, Maharaj VJ, Crouch NR, Grace OM, Pillay P, et al. 2012 In vitro antiplasmodial activity of medicinal plants native to or naturalised in South Africa. J Ethnopharmacol ; 92: 177-191.

Dondorp AM, Nosten F, Yi P, et al. 2009 Artemisinin resistance in Plasmodium falciparum malaria. N Engl J Med 2009;361:455-467[Erratum, N Engl J Med;361:1714.]

Druilhe P, Moreno A, Blanc C 2001. A colorimetric in vitro drug sensitivity assay for Plasmodium falciparum based on a highly sensitive double-site lactate deshydrogenase antigen capture enzyme-linked immunosorbent assay. Am. J. Trop. Med. Hyg. 64: 233-241

Duangjai_Tungmunnithum, Areeya_Thongboonyou, Apinan_Pholboon, and Aujana_Yangsabai 2018 "Flavonoids and Other Phenolic Compounds from Medicinal Plants for Pharmaceutical and Medical Aspects: An Overview" Journal Medicine; 5(3): 93. doi: 10.3390/medicines5030093

Dube A. 2006 ''the design, preparation and evaluation of Artemisia afra and placebos in tea bag dosage form suitable for use in clinical trials'', M. Pharm. Thesis: School of Pharmacy, University of the Western Cape, Bellville, South Africa.

Duke, J.A. 1992 Handbook of Phytochemical Constituents of Grass, Herbs and Other Economic Plants. CRC Press, Boca Raton,.

Dyson A In Ashwell A (ed) 1998 Discovering indigenous healing plants of the herb and fragrance gardens at Kirstenbosch National Botanical Garden. Cape Town, National Botanical Institute,) The Printing Press 9-10.

Elizabeth A. Ashley, M.B., B.S., Ph.D., Mehul Dhorda, et al. 2014 'Spread of Artemisinin Resistance in Plasmodium falciparum Malaria' N Engl J Med; 371:411-423 DOI: 10.1056/NEJMoa1314981

Emanuela Ricciotti, PhD and Garret A. FitzGerald, MD.* 2011 Prostaglandins and Inflammation Arterioscler Thromb Vasc Biol.; 31(5): 986–1000. doi: 10.1161/ATVBAHA.110.207449

Elfawal MA, Towler MJ, Reich NG, Weathers PJ, Rich SM. 2015 Dried whole-plant Artemisia annua slows evolution of malaria drug resistance and overcomes resistance to artemisinin. Proc Natl Acad Sci U S A.; 112(3):821-6. Doi: 10.1073/pnas.1413127112.

Elfawal MA, Towler MJ, Reich NG, Golenbock D, Weathers PJ, Rich SM. 2012 Dried whole plant Artemisia annua as an antimalarial therapy. PLoS One; 7: e52746 [PMID: 23289055 DOI: 10.1371/journal.pone.0052746]

El-Sahhar, K.F. & Nassar, R.M. & Farag, H.M. 2010 Morphological and Anatomical Studies of Artemisia vulgaris L. (Asteraceae). Journal of American Science, 6, 806-814.

Eshetu Nikodimos, Mekbeb Afework, Eyasu Makonnen, Asfaw Debella, Wondwossen Ergete, Tesfaye Tolesssa, Evaluation of the Acute and Sub-chronic Toxic Effects of Aqueous Leaf Extracts of Artemisia afra on Liver, Kidney and Some Blood Parameters in Wistar Rats. 2016 Advances in Bioscience and Bioengineering; 1(1): 1-9

Evanthia Lionta, George Spyrou, Demetrios K. Vassilatis and Zoe Cournia, 2014, 'Structure-Based Virtual Screening for Drug Discovery: Principles, Applications and Recent Advances' Current Topics in Medicinal Chemistry, 14, 1923-1938

FAO - Food and Agriculture Organization, (1993) Ecocrop, Data Sheet,Artemisia afra.

Fang H, Tong W, Shi LM, Blair R, et al: 2001 Structure-activity relationships for a large diverse set of natural, synthetic, and environmental estrogens. Chem Res Toxicol., 14: 280-294. 10.1021/tx000208y.

Gathirwa, J.W., Rukunga, G.M., Njagi, E.N.M. et al. (2007) J Nat Med 61: 261. Doi:10.1007/s11418-007-0140-0

G. S. Noland, N. Briones, and D. J. Sullivan Jr., 2003 "The shape and size of hemozoin crystals distinguishes diverse Plasmodium species," Mol. Biochem. Parasitol. 130(2), 91–99

Giao P. T., T Q Binh, P A Kager, H P Long, N Van Thang, N Van Nam and P J de Vries. 2001 Artemisinin for treatment of uncomplicated falciparum malaria: is there a place for monotherapy? Am J Trop Med Hyg, 65 690-695

Guerra CA, Gikandi PW, Tatem AJ, Noor AM, Smith DL, Hay SI, Snow RW. 2008The limits and intensity of Plasmodium falciparum transmission: implications for malaria control and elimination worldwide. PLoS Medicine; 5(2):e38. doi: 10.1371/journal.pmed.0050038.

Greanious Alfred Mavondo, Blessing Nkazimulo Mkhwananzi and Musa Vuyisile Mabandla 2016 ‘’Pre-infection administration of asiatic acid retards parasitaemia induction in Plasmodium berghei murine malaria infected Sprague-Dawley Malaria Journal 15:226 rats’’DOI: 10.1186/s12936-016-1278-6

Greanious Alfred Mavondo, Blessing Nkazimulo Mkhwananzi and Musa Vuyisile Mabandla. 2016 Pre-infection administration of asiatic acid retards parasitaemia induction in Plasmodium berghei murine malaria infected Sprague-Dawley rats. Malaria Journal 15:226 DOI: 10.1186/s12936-016-1278-6 RESEARCH ARTICLE Open Access.

Greenham J Medicinal Plants, (2000) Draft Final Report, The ARD Consortium,USAID Agribusiness Linkages Project, USAID South Africa Grant No. 674-G-00-00-00072-00 1-38.

Hay SI, Guerra CA, Gething PW, Patil AP, Tatem AJ, Noor AM, Kabaria CW, Manh BH, Elyazar IR, Brooker S, Smith DL, Moyeed RA, Snow RW. 2009 A world malaria map: Plasmodium falciparum endemicity in 2007. PLoS Med. ;6:e1000048

Harbach RE. Mosquito taxonomic inventory. http://mosquito-taxonomic-inventory.info.

Hempelmann E. 2007 Hemozoin biocrystallization in Plasmodium falciparum and the antimalarial activity of crystallization inhibitors. Parasitol ; 100:671–676. [PubMed: 17111179]

Heywood, 1983 Long term toxicity. In: Balls M, Riddell RJ, and Worden AN, editors, Animals and alternatives in toxicity testing, London: Academic Press.: p. 79-89.

Heiber, A. and Spielmann, T. (2014). Preparation of Parasite Protein Extracts and Western Blot Analysis. Bio-protocol 4(11): e1136. DOI: 10.21769/BioProtoc.1136; Full Text

Heywood, 1983 Long term toxicity. In: Balls M, Riddell RJ, and Worden AN, editors, Animals and alternatives in toxicity testing, London: Academic Press.: p. 79-89.

Hutchings A, Scott AH, Lewis G, Cunningham A (1996) 'Zulu Medicinal plants: An inventory'. South Africa, University of Natal press, Scottsville: 327: 195-196.

Hien TT, Thuy-Nhien NT, Phu NH, et al. 2012 In vivo susceptibility of Plasmodium falciparum to artesunate in Binh Phuoc Province, Vietnam. Malar J ;11:355-355

Hisanori, A., Kazuyasu, F., Osamu, Y., Takashi, O., Keiji, I. 2001 Antibacterial action of several tannins against Staphylococcus aureus. J. Antimicrob. Chemother.; 48: 487–491.

Hilaly, J. E., Z. H. Israeli, and B. Lyoussi, 2004 Acute and chronic toxicological studies of Ajuga Ivain experimental animals. J.Ethno-pharmacology,. 91: p. 43-50.

HS Banyal, Vanita Devi and Nisha Devi, , 2014, Bioefficacy of Centella asiatica in Plasmodium berghei, Asian J Biological Sciences ISN 1996-3551

Hunt S, Stebbings S, McNamara D.2016 An open-label six-month extension study to investigate the safety and efficacy of an extract of Artemisia annua for managing pain, stiffness and functional limitation associated with osteoarthritis of the hip and knee. N Z Med J. Oct 28; 129(1444):97-102

Huang SY, Zou X: . 2010, Advances and challenges in protein-ligand docking. Int J Mol Sci11: 3016-3034. 10.3390/ijms11083016.

Idris Ahmed Issa and Mohammed Hussein Bule, 2015," Hypoglycemic Effect of Aqueous and Methanolic Extract of Artemisia afra on Alloxan-Induced Diabetic Swiss Albino Mice'',

Hindawi Publishing Corporation Evidence-Based Complementary and Alternative Medicine Volume Article ID 752486, 5 pages http://dx.doi.org/10.1155/2015/752486.

Isah MB, Ibrahim MA, Mohammed A, Aliyu AB, Masola B, Coetzer THA 2016 systematic review of pentacyclic triterpenes and their derivatives as chemotherapeutic agents against tropical parasitic diseases Parasitology.; 143(10):1219-31. doi: 10.1017/S0031182016000718.

Iwu MM, (1993) Handbook of African Medicinal plants. USA, Florida, CRC,Press, 121-122.

Jackson JK, Letchford K. 2016 The Effective Solubilization of Hydrophobic Drugs Using Epigallocatechin Gallate or Tannic Acid-Based Formulations. J Pharm Sci. 2016 Oct; 105(10):3143-52. Doi: 10.1016/j.xphs..06.027.

Josphat Mwangi, Kohath J. Achola, Kipruto A. Sinei, 1995 'Essential Oil Constituents of Artemisia afra Willd' Journal of Essential Oil Research 7(1):97-99 DOI: 10.1080/10412905.1995.9698475.

J Mouton, O Jansen M Frederic, Fr van der Kooy, 2013 Is artemisinin the only antiplasmodial compound in the *Artemisia annua* tea infusion. Planta Med, 79, 468-470.

Juergen Fischer, Uwe Dethlefsen. 'Efficacy of cineole in patients suffering from acute bronchitis: a placebo-controlled double-blind trial' Cough. 2013; 9: 25. Published online 2013 Nov 21. Doi: 10.1186/1745-9974-9-25

Jensen JB, Trager W, Doherty J (1979) Plasmodium falciparum: continuous cultivation in a semi-automated apparatus. Experimental Parasitology 48, 36-41

Jérôme Munyangi, Lucile Cornet-Vernet, Michel Idumbo, Chen Lud, Pierre Lutgen, Christian Perronne, Nadège Ngombe, Jacques Bianga, Bavon Mupenda, Paul Lalukala, Guy Mergeai, Dieudonné Mumba, Melissa Towler, Pamela Weathers. (2019) '*Artemisia annua* and *Artemisia afra* tea infusions *vs.* artesunate-amodiaquine (ASAQ) in treating *Plasmodium falciparum* malaria in a large scale, double blind, randomized clinical trial', Phytomedicine 57 49–56

Katalina Muñoz, Javier Sierra, Geyson Fernandez, Fernando Alzate, Gabriel J. Arango, Cesar Segura, Karent E. Bravo. (2006) Inhibition of a specific antimalarial molecular target and correlation with the activity against Plasmodium falciparum as selection criteria of potential antimalarial from natural sources. Pharmacology online 3: 656-661

Kassa M. Mshana R, Regassa A, Assefa G. 1998; In vitro test of five Ethiopian medicinal plants for antimalarial activity against P. falciparum. Ethiop J Sci. 21:81-89.

Kellenberger E, Rodrigo J, Muller P, Rognan D: 2004 Comparative evaluation of eight docking tools for docking and virtual screening accuracy. Proteins., 57: 225-242. 10.1002/prot.20149.

Kilunga Kubata B, Eguchi N, Urade Y, Yamashita K, Mitamura T, Tai K, et al. 1998 Plasmodium falciparum produces prostaglandins that are pyrogenic, somnogenic, and immunosuppressive substances in humans. J Exp Med.; 188: 1197–1202. Pmid: 9743538 doi: 10.1084/jem.188.6.1197

Kim JS, Kwon CS, Son KH. 2000Inhibition of alpha-glucosidase and amylase by luteolin, a flavonoid. Biosci Biotechnol Biochem. Nov; 64(11):2458-61.

Köppen H. 2009 Virtual screening - what does it give us? Curr. Opin. Drug Discov. Devel. 12(3):397–407

Kraft C, Jenett-Siems K, Siems K, Jakupovic J, Mavi S, Bienzle U, Eich E. 2003 ; In vitro antiplasmodial evaluation of medicinal plants from Zimbabwe 17(2):123-8.

Kursat, M.; Emre, I.; Yılmaz, O.; Cıvelek, S.; Demır, E.; Turkoglu, I. Phytochemical contents of five Artemisia species. Notulae Scientia Biologicae 2015 Vol.7 No.4 pp.495-499 ref.47

Kumar S, Pandey AK. 2013 Chemistry and Biological Activities of Flavonoids: An Overview. The World Scientific J.1-16.

Kumar S. Sharma, Mili Kapoor T. N. C. Ramya, Sanjay Kumar, et al. 2003, Identification, Characterization, and Inhibition of Plasmodium falciparum _-Hydroxyacyl-Acyl Carrier Protein Dehydratase (FabZ), the journal of biological chemistry, DOI 10.1074/jbc.M304283200

Lavecchia A, Di Giovanni C. 2013 Virtual screening strategies in drug discovery: a critical review. Curr. Med. Chem. ;20(23):2839–2860. doi: 10.2174/09298673113209990001

Laid, M. et al. (2008) Sesquiterpene lactones from Algerian Artemisia herba-alba. Phytochemistry Letters, 1, 85-88.

Lambros C, Vanderberg JP; 1979 Synchronization of Plasmodium falciparum erythrocytic stages in culture. J Parasitol. Jun; 65(3):418-20.

Liu, N.Q., Van der Kooy, F., Verpoorte, R., 2009. Artemisia afra: a potential flagship for African medicinal plants? South African Journal of Botany 75, 185–195.

Libbey LM, Sturtz G (1989) 'Unusual essential oils grown in Oregon. I. Artemisia afra Jacq. 'JEOR 1: 29-31.

Lutgen P, Munyangi J, Cornet Vernet Lucile, 2016 Artemisia plants, deadly weapons against tropical diseases Int J Clin. Trials, 1: 109 http://dx.doi.org/10.15344/ijcrt/2016/109

Lucy N Kangethe, Sabah Omar , Joseph K Nganga , Kimani Francis , Johnson Kinyua and Ahmed Hassanali 2016 Comparative Effects of Repeated In Vitro Exposures of Plasmodium Falciparum to Sub-Lethal Doses of Pure Artemisinin and Artemisia Annua Phytochemical Blend''; The Scientific Pages of Infectious Diseases

Lu et al. 2017 Emergence of indigenous artemisinin-resistant Plasmodium falciparum in Africa. N Engl J Med; 376:991-993.

Leepika Tuli and Habtom W. Ressom 2009 'LC–MS Based Detection of Differential Protein Expression' J Proteomics Bioinform. Author manuscript; available in PMC 2010 Oct 2. Published in final edited form as J Proteomics Bioinform; 2: 416–438. doi: 10.4172/jpb.1000102

L. Spies, T.C. Koekemoer, A.A. Sowemimo, E.D. Goosen, M. Van de Venter, (2012) ' Caspase-dependent apoptosis is induced by Artemisia afra Jacq. Ex Willd in a mitochondria-dependent manner after G2/M arrest'' South African Journal of Botany.

Maria Rebelo, Claudia Sousa,Howard M. Shapiro, Maria M. Mota, Martin P. Grobusch, and Thomas Hänscheid, 2013 'A Novel Flow Cytometric Hemozoin Detection Assay for Real-Time Sensitivity Testing of Plasmodium falciparum'' PLoS One.; 8(4): e61606. Published online 2013 Apr 24. doi: 10.1371/journal.pone.0061606

Malaria Site '' Malaria drugs'', downloaded from https://www.malariasite.com/malaria-drugs/

M Carter, Hemozoin: a paradigm for biomineralization in disease. Thesis Aug 2009, Vanderbilt University

Min Yu,T. R. Santha Kumar, Louis J. Nkrumah et al., 2008 'The Fatty Acid Biosynthesis Enzyme FabI Plays a Key Role in the Development of Liver-Stage Malarial Parasites' Journal: Cell Host & Microbe

Mostafa A. Elfawal, Melissa J. Towler, Nicholas G. Reich, Douglas Golenbock, Pamela J. Weathers, Stephen M. Rich. 2012 Dried Whole Plant Artemisia annua as an Antimalarial Therapy, PLOS. http://dx.doi.org/10.1371/journal.pone.0052746)

Moges Kassa, Robert Mshana, Azeb Regassa and Getachew Assefa 1998. In vitro test of 5 Ethiopian Medicinal plants for antimalarial activity against Plasmodium falciparum. SINET: Ethiop. J.Sci. 21(1): 81-89.

M. Laid, M-E. F. Hegazy and A. A. Ahmed 2008. Sesquiterpene lactones from Algerian Artemisia herbaalba. Phytochemistry lett., 1, 85-88.

Megan S. J. Arnold, Jessica A. Engel, Ming Jang Chua, Gillian M. Fisher, Tina S. Skinner-Adams and Katherine T. Andrews, 2016 .Adaptation of the [3H]Hypoxanthine Uptake Assay for In Vitro-Cultured Plasmodium knowlesi Malaria Parasites; Antimicrob. Agents Chemother. vol. 60 no. 7 4361-4363

Mathieu Rougemont, Madeleine Van Saanen, Roland Sahli, Hans Peter Hinrikson, Jacques Bille and Katia Jaton. 2004 Detection of Four *Plasmodium* Species in Blood from Humans by 18S rRNA Gene Subunit-Based and Species-Specific Real-Time PCR Assays. J. Clin. Microbiol., 42(12):5636.

Maity K. et al. 2010, X-ray crystallographic analysis of the complexes of enoyl acyl carrier protein reductase of Plasmodium falciparum with triclosan variants to elucidate the importance of different functional groups in enzyme inhibition. Iubmb Life, 62, 467-476. PubMed id: 20503440

Mucciarelli M, Maffei M Ch. (2002) 'Introduction to the genus Artemisia' In Wright CW (ed) Medicinal and Aromatic Plants - Industrial Profiles, Taylor & Francis, London, ISBN:04152721212 1-50.

Mukinda JT (2005) 'Acute and chronic toxicity of the flavonoid- containing plant, Artemisia afra in rodents', Thesis, University of the Western Cape.

Mwangi JW, Achola JK, Sinei KA, Lwande W, Laurent R '(1995) 'Essential oil constituents of Artemisia afra Willd JEOR 7: 97-99. ISSN:1041-2905

Nibert E, Wink M., (2010) 'Volatile components of four Ethiopia Artemisa species extracts and their in vitro antitrypanosomal and cytotoxic activities'.Phytomedicine 17: 369-374.

K.Maity et al. 2011, Structural basis for the functional and inhibitory mechanisms of β-hydroxyacyl-acyl carrier protein dehydratase (FabZ) of Plasmodium falciparum. J Struct Biol, 176, 238-249. PubMed id: 21843645

Machault V, Vignolles C, Borchi F, Vounatsou P, Pages F, Briolant S, Lacaux JP, Rogier C. 2011The use of remotely sensed environmental data in the study of malaria. Geospatial Health. ;5(2):151–168.

McLaughlin, J.L., 1991. Crown-gall Tumors in Potato Discs and Brine Shrimp Lethality: Two Simple Bioassays for Higher Plant Screening and Fractionation. In: Methods in Plant Biochemistry, Hostettmann, K. (Ed.). Academic Press, London.

Morris GM, Lim-Wilby M: 2008 Molecular docking. Methods Mol Biol., 443: 365-382. 10.1007/978-1-59745-177-2_19.

Munyangi and Idumbo, 2015 ‘’ artemisia afra: successfull clinical trials in maniema, rdc’’ https://www.iwerliewen.org/news/informations/item/artemisia-afra-successfull-clinical-trials-in-maniema-rdc,

Nadia Ponts, Duk-Won D. Chung, and Karine G. Le Roch; 2012; Strand-specific RNA-seq applied to malaria samples, Published in final edited form as: Methods Mol Biol. 883: 59–73. doi:10.1007/978-1-61779-839-9_4.

Nguyen, Kim Thoa; Virus, Cornelia; Günnewich, Nils; Hannemann, Frank; Bernhardt, Rita. . 2012 "Changing the Regioselectivity of a P450 from C15 to C11 Hydroxylation of Progesterone". ChemBioChem,13 (8):1161-1166. doi:10.1002/cbic.201100811. ISSN 1439-4227

Nikolova, M.T., and Ivancheva, 2005 S.V. *Acta Biol.Szeged;*.

Nisha A, Muthukumar SP, Venkateswaran G. 2009 Safety evaluation of arachidonic acid rich Mortierella Alpina biomass in albino rats-a subchronic study. Regulatory Toxicology and Pharmacology; 53: 186-194.

NkuitchouChougouoK. Rosine D., Kouamouo Jonas, Titilayo O. Johnson, Djeungoue P. Marie-Ange, Chuisseu Pascal, Jaryum, Kouemeni Lysette, Lutgen Pierre , Tane Pierre, Moudipa F. Paul. 2016 Comparative study of Hepatoprotective and Antioxidant Activities of Artesunate and Artemisia annua Flavonoids on rats hepatocytes. Pharmacognosy conference Sao Paolo,.

Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM. 2008 Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med ;359:2619-2620

O'Boyle,N.M., et al. 2011, Open Babel: An open chemical toolbox. J Cheminform. doi:10.1186/1758-2946-3-33.

OECD Guideline for testing of chemicals; ‘’Acute oral toxicity-Acute toxic class method’’, 2001, PDF available at https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd_gl423.pdf

Pagola S, Stephens P, Bohle D, Kosar A, Madsen S 2000 The structure of malaria pigment β-hematin. Nature 404: 307- 310.

Pamela J Weathers, Melissa Towler, Ahmed Hassanali, Pierre Lutgen, Patrick Ogwang Engeu ,2014 Dried-leaf Artemisia annua : A practical malaria therapeutic for developing countries? Zorld journal of pharmacology.

Parveen, M., Ghalib, R.M., Khanam, Z., Mehdi, S.H., Ali, M. Res. 2010 A novel antimicrobial agent from the leaves of Peltophorum vogelianum (Benth.). Nat. Prod.; 24: 1268–1273

Patil GV, Dass SK, Chandra R (2011) Artemisia afra and Modern Diseases. J Pharmacogenom Pharmacoproteomics 2:105. doi:10.4172/2153-0645.1000105.

Patil Et Al., J Pharmacogenom ..." N.p., n.d. Web. 17 Jun. 2019 https://www.longdom.org/open-access/artemisia-afra-and-modern-diseases-2153-0645.

Peters W, Portus JH, Robinson BL (1975). ‘’The chemotherapy of rodent malaria, XXII. The value of drug-resistant strains of Plasmodium berghei in screening for blood schizontocidal activity.’’ Ann Trop Med Parasitol 69:155–171

Palacpac NM, Hiramine Y, Mi-ichi F, Torii M, Kita K, Hiramatsu R, Horii T, Mitamura T. 2004; Developmental-stage-specific triacylglycerol biosynthesis, degradation and trafficking as lipid bodies in Plasmodium falciparum-infected erythrocytes. J Cell Sci. 117:1469–1480.

Petrenko R, Meller J: Molecular dynamics. 2009, In Encyclopedia of Life Sciences, John Wiley & Sons

Perozzo, R.; Kuo, M.; Sidhu, A. b. S.; Valiyaveettil, J. T.; Bittman, R.; *et al.* 2002 ‘Structural elucidation of the specificity of the antibacterial agent triclosan for malarial enoyl acyl carrier protein reductase.’ J.Biol. Chem., 277, 13106-13114.

Petrenko R, Meller 2009 J: Molecular dynamics., In Encyclopedia of Life Sciences, John Wiley & Sons

Pfeiffer CJ. 1968 A mathematical evaluation of the thymic weight parameter. Toxicology and Applied Pharmacology; 13:220-227.

P. Moyo, M.E. Botha, S. Nondaba, J. Niemand, V.J. Maharaj, J.N. Eloff, A.I. Louw, L. Birkholtz . 2016 In vitro inhibition of Plasmodium falciparum early and late stage gametocyte viability by extracts from eight traditionally used South African plant species, Journal of Ethnopharmacology . 185 235-242

Pierre Lutgen, 2016 "Tannins in Artemisia: hidden treasure for prophylaxis" https://malariaworld.org/blog/tannins-artemisia-hidden-treasure-prophylaxis

Pierre Lutgen, 2016 '' Artemisia afra will save Africa '' https://malariaworld.org/blog/artemisia-afra-will-save-africa

Pierre Lutgen, 2015 'Artemisia afra and luteolin' https://malariaworld.org/

P. Moyo, M.E. Botha, S. Nondaba, J. Niemand, V.J. Maharaj, J.N. Eloff, A.I. Louw, L. Birkholtz . 2016 In vitro inhibition of Plasmodium falciparum early and late stage gametocyte viability by extracts from eight traditionally used South African plant species, Journal of Ethnopharmacology . 185 235-242

Phyo AP, Nkhoma S, Stepniewska K, et al. 2012 Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. Lancet ;379:1960-1966

Prashant Tiwari, Bimlesh Kumar, Mandeep Kaur, Gurpreet , Harleen Kaur, 2011 " Phytochemical screening and Extraction: A Review", Internationale Pharmaceutica Scienca Journal, Date of Submission: Date of Acceptance: 22-02-2011

Rappe,A.K.,et al. (1992) UFF, a full periodic table force field for molecular mechanics and molecular dynamics simulations J. Am. Chem. Soc.114,10024–10035.

Rathore D (2006) Strategies for Malaria Control. VBI Scientific Annual Report 49-53.

Rath K, Taxis K, Walz G, Gleiter GH, Li SM, Heide L (2004). Pharma-cokinetic study of artemisinin after oral intake of a traditional preparation of Artemisia annua L. (annual wormwood). Am. J Trop. Med. Hyg. 70(2): 128-132.

Reddy A.S, Pati S.P, Kumar P.P, Pradeep H.N, Sastry G.N. 2007 Virtual screening in drug discovery — a computational perspective. Curr. Protein Pept. Sci. ;8(4):329–351. doi: 10.2174/138920307781369427.

Rosangela Frita, Maria Rebelo, Ana Pamplona, Ana M Vigario, Maria M Mota, Martin P Grobusch and Thomas Hänscheid, 011'' Simple flow cytometric detection of haemozoin containing leukocytes and erythrocytes for research on diagnosis, immunology and drug sensitivity testing '', Malaria Journal 2011 10:74 DOI: 10.1186/1475-2875-10-74© Frita et al; licensee BioMed Central Ltd. 2 Received: 1 February 2011Accepted: 31 March 2011Published: 31 March 2011

Ripphausen P, Nisius B, Bajorath J: S2011tate-of-the-art in ligand-based virtual screening. Drug Discov Today. 2011, 16: 372-376. 10.1016/j.drudis..02.011.

Sargis Dallakyan Arthur J Olson, 2015 'Small-Molecule Library Screening by Docking with PyRx'. **Series:** Methods In Molecular Biology > **Book:** Chemical Biology. Methods in molecular biology (Clifton, N.J.) 1263:243-250 DOI: 10.1007/978-1-4939-2269-7_19 Source PubMed Project: PyRx Virtual Screening Tool

Schlichtherle M, Wahlgren M, Perlmann H, Scherf A. 2000 Methods in malaria research, 3rd edtn. MR4/ATCC, Manassas,. pp1–74

Sevil Toroglu. 2007 In vitro antimicrobial activity and antagonistic effect of essential oils from plant species. Journal of Environmental Biology, 28(3) 551-559 (2007)

Sixsmith DG, Watkins WM, Chulay JD, Spencer HC (1984) In vitro anti-malarial activity of tetrahydrofoliate dehydrogenase inhibitors. Am J Trop Med Hyg 33:772–776

Siyabulela Nutella, Pete Smitha, Lungile Matikab, James Mukunda, Hiram Arends, Nasiema Allieb, D. Mark Estes, Wilfred Mabuselae, Peter Folba, Lafras Steyn, Quinton Johnson, William R. Folk, James Synced,e, and Muazzam. 2009 December; '' efficacy of *Artemisia afra* phytotherapy in experimental tuberculosis'', Elsevier Tuberculosis (Edinb). 89(Suppl 1): S33–S40. doi:10.1016/S1472-9792(09)70009-5.

Snounou G, Viriyakosol S, Zhu XP, et al. 1993; High sensitivity detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol 61:315-320.

Stach, A. et al. 2007 Prevalence of Artemisia Species Pollinosis in Western Poland: Impact of Climate Change on Aerobiological Trends, 1995-2004. J Investing Alllergol Clin Immunol, 17, 39-47.

Status report on artemisinin resistance. Geneva: January 2014 World Health Organization, (http://www.who.int/malaria/publications/atoz/status_rep_artemisinin_resistance_jan2014.pdf).

Stephen O Amoo, Adeyemi O Aremu, Mack Moyo, and Johannes Van Staden, 2012 " Antioxidant and acetylcholinesterase-inhibitory properties of long-term stored medicinal plants" J. BioMedCentral,. doi: 10.1186/1472-6882-12-87

Strategy for malaria elimination in the GMS (2015–2030). 2015 Geneva: World Health Organization; (http://www.wpro.who.int/mvp/documents/strat_mal_elim_gms/en/).

Suberu JO, Gorka AP, Jacobs L, Roepe PD, Sullivan N, 2013 Anti-Plasmodial Polyvalent Interactions in Artemisia annua L. Aqueous Extract – Possible Synergistic and Resistance Mechanisms. PLOS doi/10.1371/annotation/57ae25b0-d2c8-444b-ab62-f047c5f3e01e)

Sullivan D 2000 Hemozoin, ABiocrystal Synthesized during the Degradation of haemoglobin. Miscellaneous Biopolymers and Biodegradation of Polymers9: 129-137

Sunmonu, T.O. and Afolayan, A.J. (2012). Evaluation of polyphenolic content and antioxidant activity of Artemisia afra Jacq. Ex Willd. aqueous extract. Pakistan Journal of Nutrition 11(7): 520–525.

Svensson US, Mäki-Jouppila M, Hoffmann KJ, Ashton M. 2003. Characterization of the human liver in vitro metabolic pattern of artemisinin and auto-induction in the rat by use of nonlinear mixed effects modelling. Biopharm Drug Dispos.;24(2):71-85.

S. Chanda, J. Parekh, Y. Vaghasiya, R. Dave, Y. Baravalia and R. Nair, 2017 medicinal plants - from traditional use to toxicity assessment: a review, international journal of pharmaceutical sciences and research.

Tasdemir D, Lack G, Brun R, Rüedi P, Scapozza L, Perozzo R. 2006. Inhibition of Plasmodium falciparum fatty acid biosynthesis: evaluation of FabG, FabZ, and FabI as drug targets for flavonoids. J Med Chem. 1;49(11):3345-53.

Takken W, Scott TW. 2003, Ecological Aspects for Application of Genetically Modified Mosquitoes. Dordrecht: Kluwer Academic Publishers;. pp. 75–90.

Teo S, Stirling D, Thomas S, Hoberman A, Kiorpes A, Khetani V. 2002 A 90-day oral gavage toxicity study of D-methylphenidate and D, L-methylphenidate in Sprague Dawley rats. Toxicology; 179:183-196.

Timothy J.C. Anderson, Shalini Nair, Marina McDew-White, Ian H. Cheeseman, Standwell Nkhoma, Fatma Bilgic, Rose McGready, Elizabeth Ashley, Aung Pyae Phyo, Nicholas J. White, François Nosten. 2016 Why are there so many independent origins of artemisinin resistance in malaria parasites? doi: http://dx.doi.org/10.1101/056291.

Trager W, Jensen JB. 1976; Human malaria parasites in continuous culture. Science Res. 193: 673–675

Trott O., A. J. Olson, AutoDock Vina: 2010, improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading, Journal of Computational Chemistry 455-461.

Van der Walt, L. Artemisia afra. (2004) URL: http://www.plantzafrica.com/plantab/artemisafra.htm [Seen: 23.01.2012]

Van der Walt L) Artemisia afra, (2004) page downloaded from the South African Biodiverisity Institute's Plant Information, accessed from plantzafrica.com

Van Wyk, B. E. (2008), A broad review of commercially important southern African medicinal plants. Journal of Ethnopharmacology 119, 342-355.

Van Wyk BE, van Oudtshoorn B, Gericke N, (1997), 'Medicinal Plants of South Africa. 1st Ed. Briza, South Africa, Briza Publications, Pretoria, South Africa ISBN:978-1-875093-37-3.

Van Wyk BE, Van OB, Gericke N (2000) Medicinal plants of South Africa. 2nd Ed. Briza Publications, Pretoria, ,South Africa, ISBN: 1875093095.

Velioglu, Y.S., Mazza, G., Gao, L., Oomah, B.D. 1998 Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J. Agric. Food Chem.; 46: 4113–4117.

Vladimir, K., Ludmila, M. 2001 Glycosides in medicine: the role of glycosidic residue in biological activity. Curr. Med. Chem.; 8: 1303– 1328.

Watt JM, Breyer-Brandwijk MG (1962) 'The medicinal and poisonous plants of Southern and Eastern Africa', 2nd Ed. London, Livingstone, 199-202.

Waller, R. F.; Ralph, S. A.; Reed, M. B.; Su, V.; Douglas, J. D.; *et al.* 2003, 'A type II pathway for fatty acid biosynthesis presents drug targets in Plasmodium falciparum.' Antimicrob. Agents Chemother. 47,297-301.

Weissbuch I, Leiserowitz L (2008) Interplay between malaria, crystalline hemozoin formation, and antimalarial drug action and design. Chem Rev 108:4899-4914.

White NJ, Olliaro PL (1996) Strategies for the prevention of antimalarial drug resistance: rationale for combination chemotherapy for malaria. Parasitology Today 12: 399-401.

White NJ. 1996 The treatment of malaria. NEJM. 12.9.;335:11:800-806

Willoughby JA Sr, Sundar SN, Cheung M, Tin AS, Modiano J, et al. (2009) Artemisinin blocks prostate cancer growth and cell cycle progression by disrupting Sp1 interactions with the cyclin-dependent kinase-4 (CDK4) promoter and inhibiting CDK4 gene expression. J Biol Chem 284: 2203-2213.

Worku T, Rubiolo P (1996) Major constituents of Artemisia afra oil. JEOR 8:55-357.

W. Peters and B. L. Robinson, 1999. "Malaria," in Handbook of Animal Models of Infection, O. Zak and M. A. Sande, Eds., pp. 757–773, Academic Press, London, UK, View at Google Scholar

www.iwerliewen.org/news/informations/item/high-solubility-and-stability-of-artemisinin-in-aqueous-infusions '' HIGH SOLUBILITY AND STABILITY OF ARTEMISININ IN AQUEOUS INFUSIONS'

Wright, C. W. (2002) Artemisia. TJ International Limited, London.

WHO (2006) Indoor residual spraying Use of indoor residual spraying for scaling up global malaria control and elimination WHO/HTM/MAL/2006.1112

WHO (2010). Global report on antimalarial drug efficacy and drug resistance 2000-2010. Retrieved from http://whqlibdoc.who.int/publications/2010/9789241500470_eng.pdf?ua=1.

World Malaria Report. 2011.http://www.who.int/malaria/world_malaria_report_2011/9789241564403_eng.pdf.

WHO, world malaria report, WMR (2012).

WHO releases 2016 World Malaria Report. http://www.wwarn.org/news/news-articles/who-releases-2016-world-malaria-report,

WHO (2017); Global Malaria Programme Report; ''Artemisinin and artemisinin-based

combination therapy resistance ‘’ http://www.who.int/malaria/areas/drug_resistance/en/

WHO (2017), Malaria, https://www.who.int/news-room/fact-sheets/detail/malaria

WHO (2019); https://www.who.int/news-room/fact-sheets/detail/malaria

WHO, 2019 “WHO and partners launch new country-led response to put stalled malaria control efforts back on track”, 19 November 2018 News release Maputo/Geneva. https://www.who.int/news-room/detail/19-11-2018-who-and-partners-launch-new-country-led-response-to-put-stalled-malaria-control-efforts-back-on-track

WHO, 2019 ‘Q&A on the malaria vaccine implementation programme (MVIP)‘. https://www.who.int/malaria/media/malaria-vaccine-implementation-qa/en/