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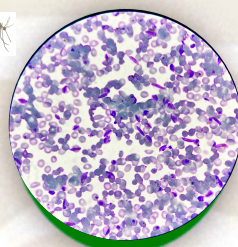
Unraveling the Potential of *Artemisia afra*, *Artemisia annua* and *Erigeron bonariensis* as Culturally Sound Alternative Therapeutic Approaches Against Malaria: Efficacy, Safety and Mode of Action



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Thesis submitted for the degree of PhD in Biomedical and Pharmaceutical Science. Supervisor: Pr. Michel Frédérich, Co-supervisor: Pr. Gisèle Etamé Loe



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Dedication

To the Lahngong's family,
To my mother, Lahngong Magdaline (R.I.P).

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Abstract

The emergence and spread of resistance to antimicrobial agents has increased the probability of treatment failure in the chemotherapy of infectious diseases. Of interest is the potential resistance to antimalarial agents. Resistance has been reported in almost all the major classes of available malarial drugs. The resistance challenge is further compounded by the lack of safer transmission blocking agents. This limitation, which is further aggravated by factors such as cultural impediments to Western medicines, has led to the resurgent and increasing use of traditional remedies, especially *Artemisia annua* and *Artemisia afra*, in the context of malaria, among other ailments.

Although proven to be effective in the management of malaria, the use of these two medicinal plants face tremendous challenges and has been controversially advocated for several reasons including batch-to-batch variations, seasonal and geographical impacts, post-harvest processing methods leading to inconsistency in the metabolite contents. These variations in metabolite composition influence the effectiveness of the medicinal plant. Additionally, there are contradictory reports regarding the chemical composition, especially the presence and role of artemisinin in both plants. This project was conceived to address these problems with its major aim to standardize, validate, and assess the safety of formulations of *Artemisia spp* as potential antimalarial agents to accelerate its elimination in high-transmission settings.

To achieve the aim of this project, firstly, we explore the phytochemical diversity of both plants harvested from different geographical regions and seasons in Cameroon, and the impact of post-harvest handling practices on the metabolite content through HPTLC, HPLC, and GC. In the second phase, we isolated antiplasmodial and gametocytocidal molecules from *A. afra*. Then we proceeded to the development of a formulation of *A. afra* and investigated its efficacy and safety profiles. Additionally, analysis of the phenolic profile of *A. afra* and *A. annua* through HPLC, detected and quantified artemisinin, and performed comparative HPLC-MS metabolomic analysis on *in vitro* *P. falciparum* trophozoites to elucidate the modes of action. This objective was achieved thanks to Dr. Lucia Mamede through her collaboration with Pr. Manuel Llinás (Pennsylvania State University, USA).

In addition, *Erigeron bonariensis*, a medicinal plant with traditional use in Cameroonian folk medicine and other parts of Africa for the management of ailments, including malaria was equally investigated for its antiplasmodial, gametocidal activity and toxicity.

The findings show that *A. annua* and *A. afra* activity profiles are related to their surroundings, with different phytochemical compositions seen for each sample depending on the season and place of origin. High artemisinin contents was retrieved in samples obtained in the rainy for *A. annua* with improved antiplasmodial activity. On the other hand, *A. afra* samples lacked the sesquiterpene lactone and equally demonstrated good antiplasmodial activity. Also, post-harvest handling affected *A. annua* and *A. afra* volatile and polyphenol profiles, and influenced how effective they were against malaria parasites. Sun-dry method showed the best profiles of terpenes and polyphenols for both plants. Furthermore, intriguing dual-stage antiplasmodial and gametocytocidal capabilities were demonstrated by two compounds that were isolated from *A. afra* (IC_{50} values $< 5\mu\text{g/mL}$). Meanwhile, on a hedonic score of 9.0, a formulation of *A. afra* acquired an overall acceptance level of 7.2, and proved to be effective and safe. Ultimately, *A. afra* induced a distinct metabolic response in the parasite, in contrast to *A. annua*, which closely aligned with the parasite response profile generated by purified artemisinin through the redox mechanism of glutathion.

Moreso, *Erigeron bonariensis* showed significant antiplasmodial (IC_{50} $6.02 \pm 1.13 \mu\text{g/mL}$) and gametocytocidal (IC_{50} $1.863 \pm 0.717 \mu\text{g/mL}$) activity and relatively safe of toxicity after an acute and subchronic oral exposure in mice. Generally, the study contributes to the broader exploration of herbal medicine as a sustainable and culturally relevant approach to combating malaria, with potential implications for the development of alternative antimalarial therapies needed to drive malaria elimination efforts. This study shows the divergent mode of action and equally demonstrates that when choosing an *Artemisia* sample as an antimalarial agent we must take into consideration the season, origin and method of drying.

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List of Abbreviations

ACTs	Artemisinin based Combination Therapy
ALT	Alanine Amino Transferase
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
ART	Artemisinin
ARES	Académie de Recherche et d'Enseignement Supérieur
AST	Aspartate Amino Transferase
CA	Chlorogenic Acid
CCM	Complete Culture Medium
CD	Cluster of Differentiation
CQ	Chloroquine
DI-HRMS	Direct Infusion-High Resolution Mass Spectrometry
DMSO	Dimethyl Sulphoxide
DPBAE	Diphenyl-borate diamino-ethanol
DS	Dry Season samples
DV	Digestive Vacuole
ECP	European Copernicus Programme
EDTA	Ethylenediaminetetraacetic Acid
FAS	Fatty Acid Synthesis
GRA	Granulocyte
GC-FID	Gas Chromatography Flame Ionisation Detector
GC-MS	Gas Chromatography-Mass Spectrometry
GSH	Reduced Glutathione
HCT	Hematocrite
HPLC-DAD Detector	High Performance Liquid Chromatography Diode Array Detector
HRP	Histidine Rich Protein
IC ₅₀	50% inhibitory Concentration
iRBC	Infected Red Blood Cell
IRS	Indoor Residual Spray
LC-MS	Liquid Chromatography-Mass Spectrometry
LDL	Low Density Lipoproteins
LLINs	Long lasting Insecticidal Nets
m/z	Mass-to-Charge ratio
MCH	Mean Corpuscular Hemoglobin

MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean corpuscular volume
MoA	Mode of Action
MSI	Multi-Spectral Instrument
NDVI	Normalized Difference Vegetation Index
NIR	Near-Infrared
NMR	Nuclear Magnetic Resonance
OECD	Organization for Economic Cooperation and Development
PCA	Principal Components Analysis
PCV	Packed Cell Volume
PEG	Polyethylene Glycol
PV	Parasitophorus Vacuole
QC	Quality Control
PfEMP1	Plasmodium Erythrocyte Membrane Protein 1
RBC	Red Blood Cells
RF	Retention Factor
ROS	Reactive Oxygen Species
Rpm	Revelation per minute
RT	Retention Time
RS	Rainy Season samples
RPMI	Roswell Park Memorial Institute
SD	Standard Deviation
SDG	Sustainable Development Goals
SEM	Standard Error of Mean
SI	Selectivity Index
TLC	Thin Layer Chromatography
TPCs	Total Polyphenol Contents
UB-IACUC Committee	University of Buea Institutional Animal Care and Use Committee
UPLC	Ultra-High-Pressure Liquid Chromatography
WBC	White Blood Cell
WHO	World Health Organisation

Chapter One

Introduction

1.1 Nature's contribution to the discovery of antimalarials

Natural products are important sources for new drugs and are also good lead compounds suitable for further modification during drug development. The large proportion of natural products in drug discovery has stemmed from the diverse structures and the intricate carbon skeletons of natural products (Beutler, 2009; Lahlou and Lahlou, 2013). More than 50% of all drugs in clinical use today contain certain substances of natural origin; most of these products are from plant sources (Habibi et al., 2022).

Investigations of the indigenous remedies and their possible effects have attracted the attention of many researchers for ages. Drug discovery from natural sources, in the light of ethnopharmacological studies, has an important role in the development of current therapeutic systems. Plants, animals, and minerals are among the natural products that have been the basis for the treatment of many diseases for centuries (Nigussie and Wale, 2022). Recently, much attention has been again paid to pharmacognostical, phytochemical, and pharmacological studies of traditional medicinal plants. Moreover, the biological activity of natural medicines has been investigated in many preclinical and clinical studies, revealing diverse biological effects of a wide range of plant-derived compounds in various classes of chemical groups. Most of the natural sources whose active compounds are currently employed have an ethnomedical use (Tabuti et al., 2023). It is well known that in rural communities, people often use ethnobotanical and ethnomedical alternatives for the management of health-related problems, including malaria. Such alternative remedies have provided leads for the development of drugs useful in therapeutics as practiced in Western medicine. Well-known examples of the seminal contributions of ethnomedicine to the treatment of malaria by the modern medicinal ways include quinine and artemisinin, isolated from the *Cinchona* tree and *Artemisia annua*, respectively (Siqueira-Neto et al., 2023).

For instance, the bark of the *Cinchona* tree from the Rubiaceae family was first used to cure malaria in Peru around 1630 and is widely regarded as the most influential bark medicine in history (Gachelin et al., 2017). *Cinchona* species were employed in traditional medicine against malaria in the 17th century. Described in the London Pharmacopoeia as a traditional remedy against malaria, huge amounts of *Cinchona* bark were sent to regions of North America and Europe where malaria was common, and it equally played

an important role in the European colonisation of tropical regions (Gachelin et al., 2017). However, in 1820, the active component (quinine) was isolated from the bark of the Peruvian native tree (Figure 1), which also contains an array of about 35 different alkaloids (Canales et al., 2020). Although quinine can be synthesised, *Cinchona* trees continue to be the only economically viable source of the medication. Around the 1850s, quinine was the preferred antimalarial medication until the 1940s, following the introduction of synthetic antimalarials. However, it continued to be significant in Third World nations and has resurfaced as an effective treatment for *Plasmodium falciparum* malaria in several regions where the parasite has developed resistance to other antimalarial drugs like chloroquine. To date, quinine is still used to treat malaria in some urgent cases despite the introduction of other effective medicines.

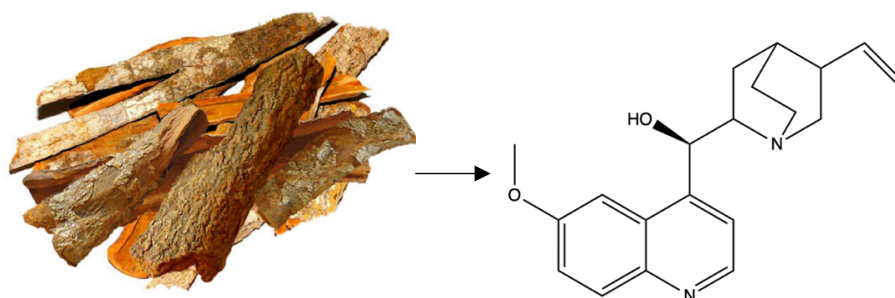


Figure 1: Bark of cinchona tree and chemical structure of quinine

From the use of tree bark to weed in treating malaria, the Chinese utilised the leaves of sweet wormwood (*Artemisia annua*) to achieve the same goal. However, documented records of *A. annua* therapeutic use existed before those of *Cinchona* bark in South America by over a thousand years (Ceravolo et al., 2021; Miller et al., 2022). Derived from the Greek goddess Artemis, *Artemisia* is one of the largest genera in the Asteraceae family, widely distributed and with over 500 species (Mekonen et al., 2020). The well-known *Artemisia* species, *Artemisia annua*, is listed as a treatment for fever and malaria in the People's Republic of China's current pharmacopoeia. For over two millennia, traditional medicine in Asia has utilised *Artemisia annua* in the form of tea or pressed juice to treat fever related to malaria (Mekonen et al., 2020). During ancient times, *A. annua* has been mentioned in Chinese medicinal texts as qinghao. Qinghao was first recorded in the Shennong's

Canon of Materia Medica, the fundamental writing of Chinese herbal medicine (Liu et al., 2006; Yuan et al., 2016). Sweet wormwood's active component, artemisinin (qinghaosu), was discovered to be a potent but unstable antimalarial in the early 1970s. The isolation of the sesquiterpene lactone, artemisinin, from *A. annua* (**Figure 2**), has made the plant to gain international recognition and has been a subject of intensive phytochemical investigation over the past decades.

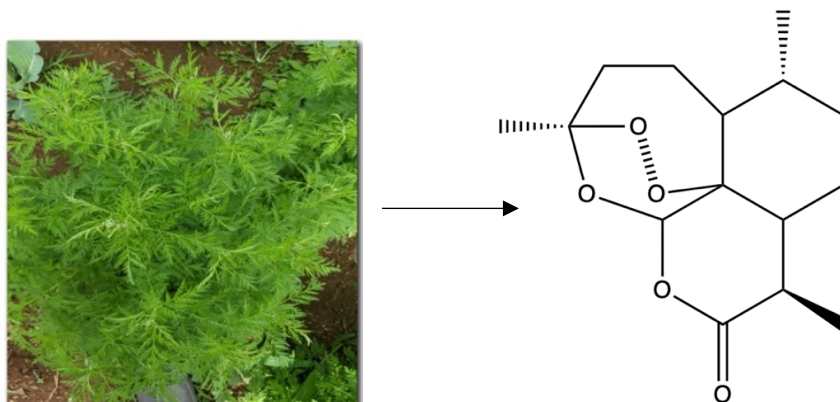


Figure 2: Leaves of *A. annua* and chemical structure of artemisinin

Due to the poor pharmacokinetic parameters of artemisinin (Yaméogo et al., 2020), further research resulted in the development of two stable derivatives: artemether, a methyl-ether derivative, and artesunate, a water-soluble derivative. As prodrugs, artemether and artesunate are transformed by the body into the active metabolite dihydroartemisinin (DHA). Currently, derivatives of artemisinin constitute part of combination therapy known as Artemisinin-based Combination Therapy (ACT), one of the most utilised medicine for the management of malaria (one of the oldest parasitic diseases that still ravage humankind).

1.2 Malaria

1.2.1 Origin, evolution and discovery of malaria

Malaria is one of the oldest parasitic diseases that is still of public health importance even in the present era. Writings and artefacts from antiquity demonstrated the protracted reign of malaria. The parasites that cause

malaria are believed to have emerged in tropical Africa approximately 2.5 million years ago, before spreading to other parts of the world, probably through ancient civilisation and increased mobility of people (Lalremruata et al., 2013). As far back as 800 BC, before the beginning of civilisation, malaria was thought to be one of the major illnesses that ravaged ancient Egypt, with several Egyptian rulers using bed nets that were thought to prevent mosquito bites (Lalremruata et al., 2013). However, the role of mosquitoes in malaria transmission was not known. Later, it was described in several parts of the world, including ancient Greece, where it was referred to as autumn fevers, as described by Hippocrates, among others. According to the belief of the ancient Romans, the word malaria was coined by an Italian physician, Francisco Torti, from *mal aria*, which means bad air originating from pestilential fumes in the swamps (Hempelmann and Krafts, 2013). At the time, they thought that malaria was caused by breathing vapors arising from stagnant water. After originating in Africa, malaria spread to Mesopotamia, the Indian Peninsula and Southeast Asia, India, Mediterranean shores, and reached Northern Europe (Rawal, 2020). At the end of the 15th century, it reached the New World and spread across to North America in the 18th century, where it was introduced by African slaves. Due to its rapid spread, it was declared a pandemic disease with over half of the world population at risk of the disease in the mid-19th century. To date, malaria continues to trouble humankind, especially in Sub-Saharan African countries, with several invasive species identified.

The parasite that causes malaria is a unicellular microorganism (protozoa) belonging to the genus *Plasmodium* with over 156 species of which 5 are known to ultimately cause human malaria, viz, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and the newly identified *Plasmodium knowlesi*. Recent findings suggest that *P. falciparum*, the most virulent species due to its ability to rapidly invade red blood cells, evolved from the African gorilla (Liu et al., 2010; Loy et al., 2016; Sharp et al., 2022). This data supports the hypothesis that malaria parasites came to humans from apes (Sharp et al., 2022) through bites of mosquitoes when the hunting lifestyle shifted to high-density communal living in settlements, thus increasing contacts between these apes, mosquitoes, and humans. Also, findings suggest that human *P. vivax* originated from a *Plasmodium* species that infects chimpanzees and gorillas in Africa (Liu et al., 2010; Loy et al.,

2016; Sharp et al., 2022) while *P. knowlesi* originated in Southeast Asia and evolved from monkeys according to evolutionary analyses of sequence data (Lee et al., 2011). On the other hand, *P. malariae* and *P. ovale* have been suggested to have evolved from closely related chimpanzee, bonobo, and/or gorilla counterparts; however, their origins are still unclear (Ochoa & Pazos, 2010; Sharp et al., 2022). Despite their similarities, these species differ in several ways. In sub-Saharan Africa, for instance, *P. falciparum* is the most deadly and the primary cause of fatalities. *P. vivax*, on the other hand, is more widely distributed geographically and causes less severe infections. Whereas 70% of malaria cases in Southeast Asian regions are caused by *P. knowlesi* (Mueller et al., 2007). Meanwhile, *P. ovale* and *P. malariae* exhibit reduced parasitemia and prevalence, leading to milder and more readily treatable illnesses (Mueller et al., 2007).

The belief that malaria was caused by breathing vapors from stagnant waters was slavishly followed until about 160 years after, that Charles Louis Alphonse Laveran (a French army surgeon) discovered malaria parasites for the first time in malaria patients (Cox, 2010). His findings led to increasing investigation into malaria parasites as many researchers sought to understand and better describe the disease. As such, two terms were used to describe the disease, tertian and quartan fevers (these fevers were due to the rupture of merozoites into the bloodstream) by Camillo Golgi. In the second half of the 19th century, the puzzle of how the disease was transmitted was solved in what is known today as the mosquito-malaria theory (Hempelmann and Krafts, 2013; Lalchandama, 2015). This theory challenged the centuries-old medical belief that poor air, or miasma, was the cause of malaria and instead suggested that insects were the vector of transmission. In the late 1890s, thanks to Sir Ronald Ross, who provided scientific evidence for the transmission of malaria by certain mosquito species by biting humans. This aligned with the earlier postulates of Albert Freeman Africanus that malaria was indeed spread by mosquitoes (Cox, 2010). To this fact, the developmental stages of *Plasmodium* in the mosquito were elucidated, and this revolutionised efforts to control malaria, making it possible to implement vector management techniques that are still essential to preventing malaria today.

1.2.2 Life-cycle and mode of transmission

As described in the earlier works of Sir Ronald Ross, who elucidated the developmental stages of *Plasmodium* in the mosquitoes that play a crucial role in the transmission of malaria, constituting a complex life cycle that involves two hosts (human and mosquitoes). In most cases, malaria is transmitted by dusk and by nocturnally active female *Anopheles* mosquitoes. Interestingly, there are more than 400 different species of *Anopheles* mosquitoes, of which around 30 are malaria vectors of major importance (Pimenta et al., 2015). The intensity of transmission depends on factors related to the parasite, the vector, the human host, and the environment. Transmission is more intense in places where the mosquito lifespan is longer and where it prefers to bite humans rather than other animals. The long lifespan and strong human-biting habit, and the invasive nature of the African vector species are the main reasons why approximately 90% of the world's malaria cases are in Africa. Additionally, transmission also depends on human immunity and climatic conditions that may affect the number and survival of mosquitoes, such as rainfall patterns, temperature, and humidity (World Health Organization, 2018).

The life cycle of the parasite is a complex process occurring in both vertebrate hosts and a mosquito vector, utilizing both sexual and asexual reproduction mechanisms (Sato, 2021). During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host (Figure 1). After entering the bloodstream, the sporozoites migrate to the liver 30–60 minutes later. Once inside hepatocytes, they undergo asexual replication and develop into schizonts, which burst and release merozoites into the bloodstream (Real et al., 2021). Of note, in *P. vivax* and *P. ovale*, a dormant stage (hypnozoites) can persist in the liver (if left untreated) and can cause relapses by invading the bloodstream weeks or even years later. After this initial replication in the liver, also known as the exo-erythrocytic schizogony, which lasts for about 5–16 days depending on the species, the parasites undergo asexual multiplication in the erythrocytes, wherein merozoites invade red blood cells. Also known as the erythrocytic stage, the cycle in blood is known to last for about 48–72 h, depending on the species, and is responsible for the clinical manifestations of the disease. Nonetheless, the parasites undergo several developmental phases throughout their erythrocytic life cycle (Bannister et al., 2000).

Firstly, the ring stage occurs soon after the RBCs are invaded. Here, the merozoites flatten into a ring-shaped disc that contains the majority of the major organelles in a rim of cytoplasm. The nucleus, smooth and rough endoplasmic reticulum (ER), and clusters of vesicles are located in the centre of the structure (**Figure 3**). The parasite feeds on the RBC cytosol that is drawn and broken down inside vacuoles and hemozoin crystals derived from haem following the parasite's digestion of haemoglobin.

Secondly, the trophozoite stage, which is characterised by intracellular alterations as well as changes in cell size and shape, is considered the most metabolically active and growth stage. Internally, the ER and mitochondria get larger, with more proteins and ribosomes, leading to a more complicated Golgi complex. The parasite's primary source of nutrition is found in a single food vacuole, as was evident in the previous stage. During the early trophozoite stage, however, several tiny food vacuoles with hemozoin may be identified, in contrast to the ring stage, suggesting a faster metabolic rate. Later on, these tiny vacuoles combine to form a single, enormous food vacuole, and the trophozoite transforms into a schizont ([Miller et al., 2013](#)). Schizont is an intra-erythrocytic parasite that has undergone or is undergoing recurrent nuclear division during the final stage of development. At least 16 new nuclei are created when the nucleus divides four times. As mentioned in the first stage of development, the parasite experiences cytoplasmic alterations with every nuclear division, signalling the start of a new merozoite stage. A constriction ring separates each merozoite from the schizont after they have all been assembled and with all of their organelles. They are encircled by a flocculent material that contains parasite antigens and congregate inside the parasitophorous vacuole ([Miller et al., 2013](#)). Following these occurrences, hemozoin development proceeds, resulting in a tightly packed vacuole that eventually becomes a compact, spherical mass. The buildup of these parasitic proteins causes the membrane and cytoskeleton of RBCs to distort, resulting in the development of surface knobs and an irregular shape. The RBC membrane and parasitophorous vacuole burst during the last stage of development, allowing the freshly formed merozoites to exit and infect new blood cells. Some merozoites upon entering the RBCs differentiate into male (microgametocytes) and female (macrogametocytes) gametocytes in a process known as gametocyte commitment.

These gametocytes are taken up by the mosquito during a blood meal from the human bloodstream and undergo sexual multiplication in the mosquito in a process known as the sporogony cycle. While in the mosquito's stomach, the microgametes penetrate the macrogametes, generating zygotes. These zygotes, in turn, become motile and elongated (ookinetes) and invade the midgut wall of the mosquito, where they develop into oocysts. The oocysts grow, rupture, and release sporozoites, which pave their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle (Schmidt et al., 2015) (Figure 3).

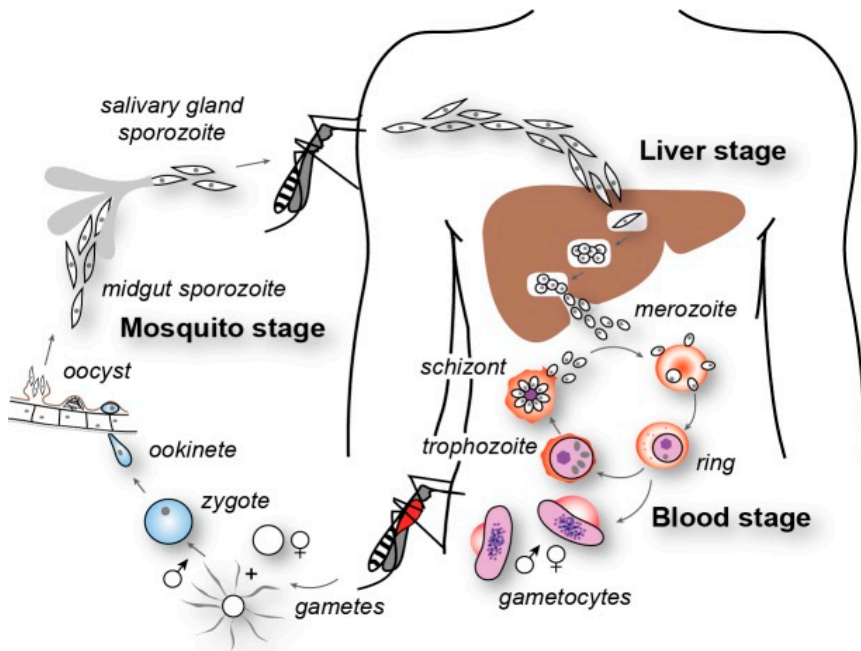


Figure 3: Malaria life cycle (Cowman et al., 2012).

1.2.3 Pathologies associated with malaria

Malaria-infected RBCs lead to structural modification in the morphology of RBCs, developing surface protrusions (knobs) with characteristics of adhering to the capillary and venular endothelium (White et al., 2014). This is mediated by the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is transported to the RBC membrane and serves as a crucial ligand for cytoadhesion (Magowan et al., 2009; Plewes et al., 2018). This cytoadherence property obstructs microcirculation due to sequestered parasitised RBCs in the capillaries and postcapillary venules and in the deep

microvasculature of various tissues and organs (Berendt et al., 1990; Milner, 2018; Ochola et al., 2005; Silamut et al., 1999), resulting in local tissue anoxia with complications varying depending on the tissue. In the brain, it can result in cerebral malaria (Wah et al., 2016), whereas in the kidneys, it may cause renal failure and tubular necrosis, and in the intestines, it can lead to ischemia and ulceration, ultimately causing bleeding. It is important to note that these issues arise at a later, more advanced stage of the illness and are responsible for the fatalities associated with malaria infections (Silamut et al., 1999).

However, the earliest stage possesses a more syndrome-like pattern of symptoms that starts as soon as the erythrocytic stage is completed and the newly generated merozoites infect different RBCs (Miller et al., 2013). The consistent invasion of new RBCs leads to a decreased concentration of RBCs and the development of anaemia, which is proportionate to the intravascular parasitaemia concentration (Miller et al., 2013). Also, the parasites released into the bloodstream by the ruptured RBC activate the host's immune system, ultimately causing the release of different parasitic and endogenous substances, including reactive oxygen intermediates and cytokines. It has been shown that during a malaria attack, immune response to the parasites accounts for an upregulated xanthine oxidase (XO), leading to the production of reactive oxygen species (ROS), which trigger inflammation. Studies also report a correlation between elevated plasma-level activities of XO and cytokine production in malaria patients, especially TNF and interleukin-1 (IL-1) by macrophages (Milner, 2018). The aberrant cytokine production, or a “cytokine storm”, can cause severe systemic inflammatory responses as well as tissue and organ damage in malaria patients (Iwalokun et al., 2006). The hyper-inflammation resulting from aberrant cytokine production also plays a significant role in the pathogenesis and causes symptoms like fever, chills, sweat, and weakness. Although malaria displays many symptoms, it is mainly characterised by heavy fever episodes. This malaria fever is triggered once the parasitic blood concentration surpasses the pyrogenic threshold and enough parasitic toxins are released from a ruptured schizont (Dicko et al., 2005). These toxins, such as hemozoin, activate certain thermoregulating areas of the brain, which in turn leads to elevation of core body temperature. A classical paroxysm for malaria shows a cyclical and intermittent pattern that only lasts for a few hours and alternates between symptom-free periods

and periods of illness. The paroxysm is preceded by a prodromal phase that exhibits flu-like symptoms such as headache, slight fever, muscle pain, and nausea ([Oakley et al., 2011](#)) and can be distinguished into three phases. The cold stage, which lasts roughly 15-60 minutes and is marked by a sharp chill and a throbbing headache. Later on, the hot stage, which is characterised by extreme heat, dry burning skin, and a pounding headache for roughly 2-6 hours, and then the sweating phase, which persists for about 2-4 hours during which the patient perspires and feels weak and exhausted. This ultimately causes a drop in body temperature and marks the beginning of the symptom-free phase until the subsequent paroxysm ([Crutcher and Hoffman, 1996](#)).

1.2.4 Malaria epidemiology: The past and present

If one were to nominate the most infectious parasitic disease in the world, malaria would probably be at the top, hence a disease of public health importance. Although malaria is an ancient parasitic disease, it is still a life-threatening disease in the present era and poses a significant global health threat, especially in Sub-Saharan African countries, despite a steady drop in the disease's prevalence worldwide (**Figure 2**). Over 2.2 billion cases and 12.7 million deaths from malaria globally were reported over the past three decades. Interestingly, the WHO African Region alone accounted for 1.7 billion cases and 12 million fatalities ([World Health Organization, 2018](#)).

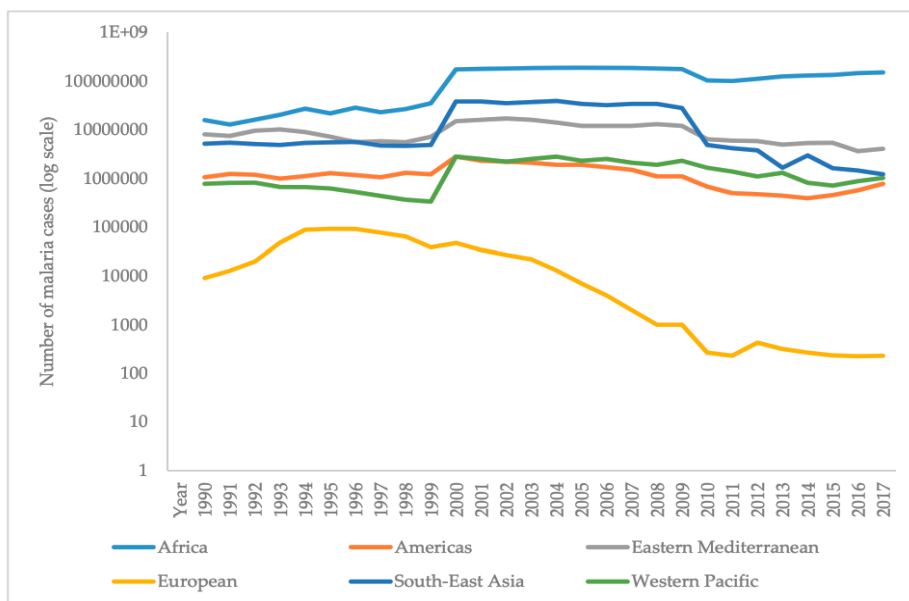


Figure 4: 20 years of global malaria trend (World Health Organization, 2018).

The global malaria burden is lower than it was ten years ago. However, in recent years, the incidence of malaria cases worldwide has increased significantly. The expected number of malaria cases and deaths rose, respectively, by 23 million and 24,000, over the previous five years. According to the latest World Malaria Report, there were about 227 million cases in 2019, and in 2020, the number of cases reported was 241 million, indicating an increase. This increase was observed in later years to over 244 million cases in 2021, with over 249 million cases in 2022. Globally in 2023, almost 263 million estimated malaria cases were reported (Venkatesan, 2025a). However, mortality due to malaria remained more than half a million each year. For instance, in the years 2020, 2021, 2022, and 2023, the estimated number of malaria deaths stood at 625,000, 619,000, 608,000 and 569,000 respectively (World Health Organization, 2022). The WHO African region bears a disproportionately high share of the global malaria burden, with over 95% burden and 96% deaths (Figure 5) shared between 29 countries out of 85 malaria endemic countries (World Health Organization, 2024). The burden of the disease is exceptionally high among the most vulnerable groups, such as children under 5 years old, pregnant women, and migrant laborers traveling to endemic areas (Alehegn et al., 2020; Isiko et al., 2024).

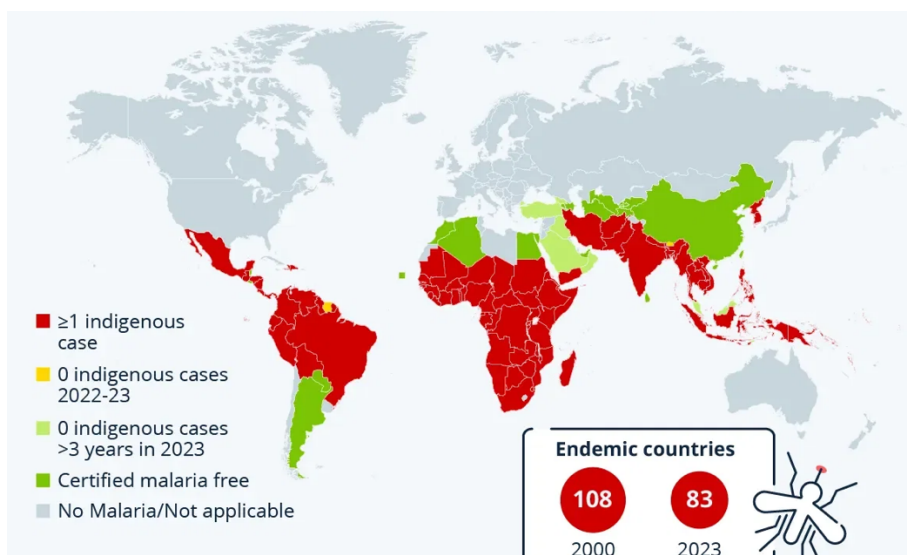


Figure 5: World malaria report: Burden and mortality (World Health Organization, 2024)

Malaria is an important public health threat in Cameroon, with the whole country exposed to the risk of *Plasmodium* transmission (**Figure 6**), with suspected cases in healthcare facilities varying between 3.3 – 3.7 million per year (Antonio-Nkondjio et al., 2019). Cameroon is among the 11 countries with the highest malaria burden, contributing to 70% of global malaria cases and reporting over 5 million cases with over 1.2 million increases in cases reported in the past five years (World Health Organization, 2024). The incidence of malaria is highest in the East, Northwest, North, and Extreme North regions, while malaria mortality remains high in the Center and Adamawa regions, among others (**Figure 6**) (Besem et al., 2024).

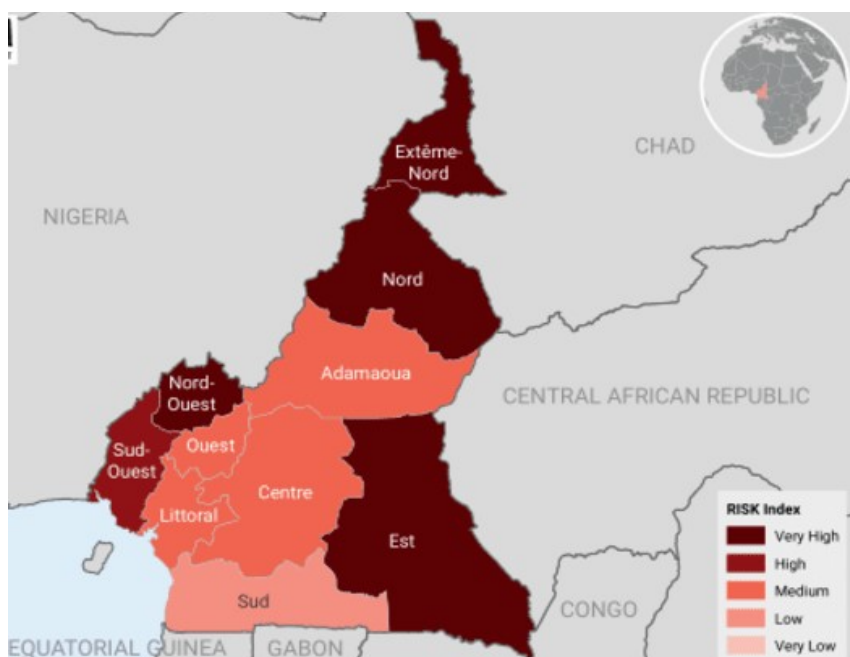


Figure 6: Overall malaria burden in Cameroon.

1.2.5 Efforts in combating malaria

The management of malaria requires concerted approaches either to prevent/or in chemotherapy. Prevention entails the use of vector control measures, including insecticide-treated mosquito nets and indoor residual spraying. Sleeping under an insecticide-treated net (ITN) can reduce contact between mosquitoes and humans by providing both a physical barrier and an insecticidal effect (**Figure 7**).



Figure 7: Mother and child rest under a mosquito net ([World Health Organization, 2022](#))

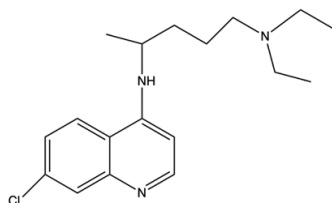
Whether treated with insecticide or not, bed nets provide a physical impediment against insects ([Orok et al., 2021](#)). When treated with insecticide, it provides further protection by killing mosquitoes encountering the net. Pyrethroids, like permethrin and deltamethrin, were the only insecticides used to impregnate bed nets ([Lindsay et al., 2021](#)). In 2019, an estimated 46% of all people at risk of malaria in Africa were protected by an insecticide-treated net, compared to 2% in 2000. Indoor residual spraying (IRS) with insecticides is another powerful way to rapidly reduce malaria transmission (**Figure 8**). It involves spraying the inside of housing structures with an insecticide, typically once or twice per year. IRS is employed to prevent the entry of mosquitoes by covering the walls and floors of a house with insecticide.



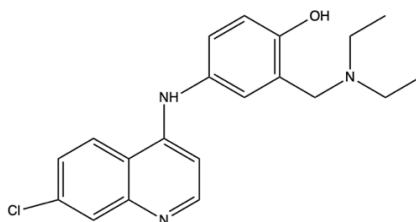
Figure 8: Spraying of inner walls of a house with insecticide ([World Health Organization, 2022](#))

Once the physical barrier is breached, one can get infected with malaria and therefore requires prompt treatment to prevent the disease from progressing to a severe state that can lead to death. Over the years, several antimalaria agents have been developed and can be grouped as quinoline derivatives, antifolates, and artemisinin derivatives based on their chemical structures and/or mechanism of action (**Figures 9, 10 and 11**) ([Belete, 2020](#)). Quinoline derivatives that comprise chloroquine, amodiaquine, quinine, quinidine, mefloquine, primaquine, lumefantrine, and halofantrine are active against the erythrocytic stage of the parasite. Among them, primaquine is active against the hepatic stage of the parasite and gametocytes ([Wampfler et al., 2017](#)). To prevent or delay the spread of antimalarial drug resistance, the World Health Organization recommends the use of combination therapy for all episodes of malaria with at least two effective antimalarial agents having a different mechanism of action ([World Health Organization, 2022](#)). To this effect, Artemisinin-based Combination Therapies (ACTs) were introduced and are now generally accepted as the best treatments for uncomplicated *Falciparum* malaria and constitute a fast-

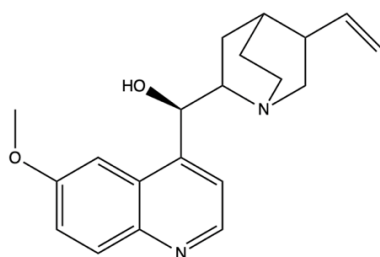
acting derivative (artemether, a methyl-ether derivative, or artesunate, a water-soluble derivative) and a slow-acting blood schizonticide. In addition to ACTs, the World Health Organization also recommends quinine as another treatment regimen for uncomplicated malaria caused by the *P. falciparum* parasite that is resistant to chloroquine. Moreso, quinine is used as a second-line agent for severe malaria when the first-line treatment (artesunate) is unavailable ([World Health Organization, 2025](#)).



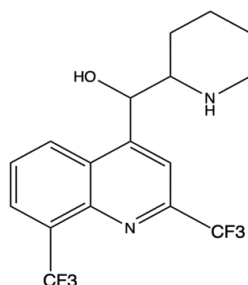
Chloroquine



Amodiaquine

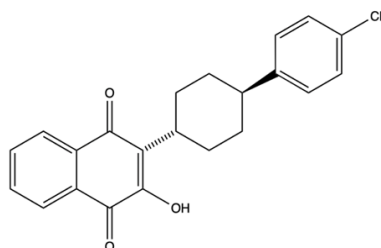


Quinine

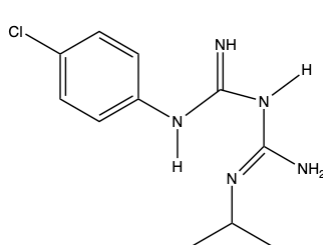


Mefloquine

Figure 9 : Structures of Quinolones



Atovaquone



Proguanil

Figure 10: Structures of antifolates

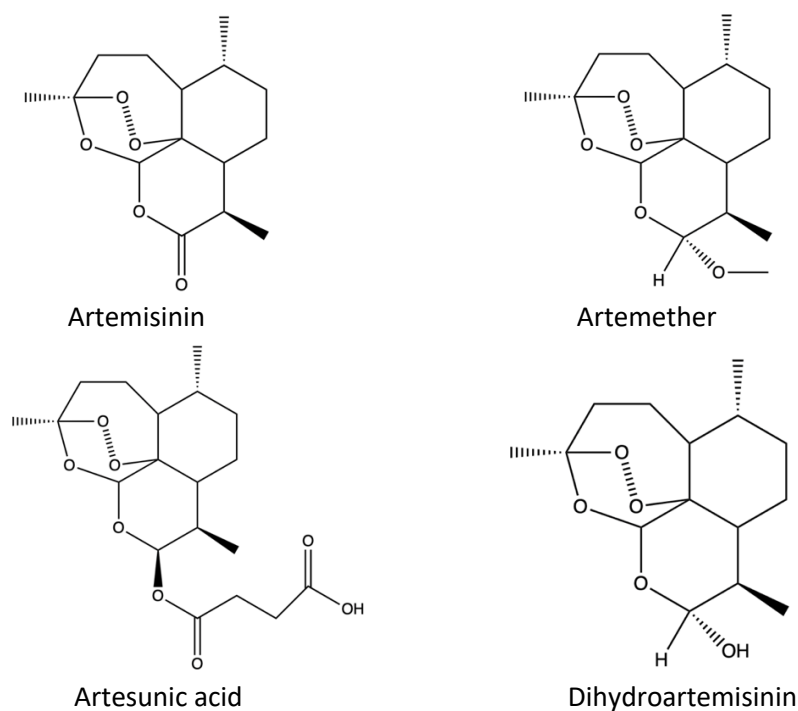


Figure 11: Structure of artemisinin and its derivatives

In addition to drugs used in clinical cure, casual prophylaxis is administered as a drug active against the pre-erythrocytic (liver stage) malaria parasite. These drugs can be discontinued after leaving the malaria-endemic area. Suppressive prophylaxis represents administering drugs that act against asexual blood-stage (erythrocytic) parasites. Depending on the drug chemical class, the duration of administration varies after leaving the area to eliminate asexual parasites emerging from the liver weeks after exposure, unlike causal prophylaxis ([World Health Organization, 2022](#)). For examples, atovaquone and proguanil requires one week after leaving the endemic area. In areas where *P. falciparum* malaria is prevalent, for instance in Sub-Saharan Africa, suppressive prophylaxis is indicated. Whereas, in areas where *P. vivax* co-exists with *P. falciparum* or alone, causal prophylaxis is recommended. Moreover, the approval of the RTS, S vaccine is just in time to maximize the public health benefit of all these efforts and strengthen malaria management strategies ([Laurens, 2020](#)). October 6, 2021, was a historic day when the WHO approved the first malaria vaccine, RTS, S/AS01, for widespread use. The vaccine significantly reduces total malaria cases and the deadly form of

the disease among young children. This is mainly mediated by training of the immune system to recognize the circumsporozoites on the surface of sporozoites which are neutralized via the mobilization of antibodies. The neutralization of sporozoites in the blood stream prevent them from reaching the liver to initiate the pre-erythrocytic stage. World Health Organization, therefore, recommended widespread use of the RTS, S vaccine to immunize children in regions with moderate-to-high transmission of *P. falciparum* malaria, mainly in Sub-Saharan Africa (World Health Organization, 2021). However, future advances need to be made for the RTS, S vaccine, such as improved protection through a schedule of fractionated delayed doses and alternative adjuvants. **Figure 12** summarizes efforts in the fight against malaria from the time the disease was first described till date.

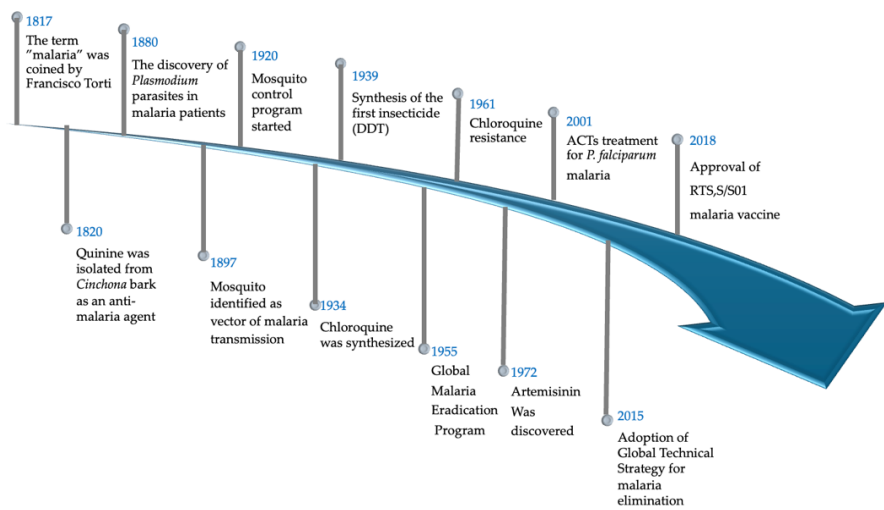


Figure 12. Significant turning points in the history of malaria research and management. From its description in 1817 until the licensing of the RTS, S/AS01 (Mosquirix) malaria vaccine in 2018.

1.2.6 Mechanism of action of antimalarial drugs

The antimalarial mechanism of quinolone derivatives is proposed to be the result of the following two-step activities: the first step involves retarding deposition of heme onto the hemozoin crystal surface by capping the growing hemozoin crystals, and the second step involves complexing with free heme in the lumen of the digestive vacuole. The overall outcome of both steps is killing the parasite by halting heme crystallization leading to the accumulation of heme that is toxic to the parasite after being released from the

hemoglobin (Kapishnikov et al., 2019). Antifolate malarial agents can be grouped as class I and class II, based on their mode of action. Class I antifolate agents act by inhibiting the production of dihydrofolic acid through inhibition of the enzyme dihydropteroate synthase (DHPS) and hence the synthesis of nucleic acids. Class II antifolate agents block the reduction of dihydrofolate to tetrahydrofolate by inhibiting the enzyme dihydrofolate reductase (DHFR) in the parasite. Tetrahydrofolate is important for producing nucleic acids and amino acids. Class II agents have schizonticidal activity, and they act on the asexual form of the parasite. Sulfadoxine is among the class I antifolate agents, while proguanil and pyrimethamine belong to Class II antifolate agents (Kovalev et al., 2022). Artemisinin and its derivatives like artesunate, artemether, arteether, and dihydro-artemisinin, mechanism of action is mainly mediated through generation of free radicals. The malaria parasite is known to be rich in heme since it causes proteolysis of the host cell hemoglobin. Therefore, artemisinin interacts with intra-parasitic heme and gets activated into toxic free radicals through the process. A resulting carbon-centered free radical then kills the parasite by alkylating and denaturing one or more essential malarial proteins. The fact that artemisinin is selectively toxic to the parasite is attributed to its selective interaction with intra-parasitic heme (Shi et al., 2022).

1.2.7 Constraints on malaria control strategies

The wider use of insecticide-treated bed nets, various vector control measures, and preventive intermittent chemotherapeutic courses for vulnerable individuals have all helped reduce the incidence of malaria over the years. However, these efforts and the attempts to eradicate malaria using the powerful 'residual' insecticides and the highly effective antimalarial drugs face tremendous challenges, making the eradication of malaria problematic. One of the major problems in malaria control is the emergence and spread of parasite resistance to the available chemotherapy and multi-drug resistance in most cases, leading to treatment failure (World Health Organization, 2022). For instance, resistance to chloroquine by *P. falciparum* in the majority of endemic regions has been reported. Also, quinine, as well as antifolates such as proguanil and pyrimethamine have been reported not to be effective in some endemic areas due to resistance by the parasite. The emergence of resistance to the recently approved antimalarial medication

Artemisinin-based Combination Therapy (ACTs) exacerbates this even further. Following artesunate monotherapy, artemisinin resistance was observed on the Thailand-Cambodia border in 2008. According to a retrospective study, artemisinin resistance most likely first appeared in 2001, prior to ACTs being widely used in Cambodia ([Dondorp et al., 2010](#); [World Health Organization, 2015](#)). It has been demonstrated that point mutations in the Kelch 13 (K13) propeller gene are linked to the mechanism of resistance to artemisinin medications. Non-synonymous K13 mutations have been however documented in Cameroon and other African nations (Central African Republic, Chad, Kenya, Madagascar, Malawi, Mali, Rwanda, etc.). The most common allele found in Africa is A578S ([World Health Organization, 2015](#)). Subsequent research revealed that *P. falciparum*'s four K13-propeller gene alterations are linked to artemisinin resistance; Y493H, R539T, I543T, and C580Y are among them ([Kamau et al., 2014](#)). In addition to being common in Southeast Asia, PfKelch13 mutations linked to artemisinin resistance have also been found in Guyana, Papua New Guinea, and Rwanda at a notable prevalence (above 5%) ([World Health Organization, 2019](#)). In addition to the resistance challenge, ACT does not ensure complete gametocyte clearance, the form of the parasite responsible for the disease transmission.

Moreso, the expanded insecticide resistance across sub-Saharan African countries has further limited malaria control strategies ([World Health Organization, 2017](#)). In addition to insecticide resistance, ITNs and IRSs are limited to indoor controls, and populations remain exposed to *Plasmodium* transmission during their professional working time and social life. A major weakness of the current malaria control strategies worldwide is that they do not target asymptomatic *Plasmodium* carriers who constitute the vast majority of the infected people in endemic regions and therefore serve as reservoirs that perpetuate the disease transmission over time ([Lindblade et al., 2013](#)).

The United Nations Sustainable Development Goal-3 (SDG 3) calls for a 90% decrease in malaria incidence and mortality, as well as the eradication of malaria in at least 35 endemic countries by 2030. This will guarantee well-being and promote healthy lives for all people at all ages ([World Health Organization, 2018](#)). Based on the above-mentioned challenges, the control and elimination of malaria is problematic, and the attainment of SDG3 is

unrealistic. In this view, there is a need to review the current control strategies and the quest for highly efficacious, safer, and cost-effective antimalarial agents that will target both the gametocyte as well as in clinical cure is highly imperative. This will enable curing the disease, breaking transmission cycles, and hence its potential eradication. To address this problem, several approaches are presently being explored to develop new and more efficacious antimalarial agents, which include: screening of synthetic compounds derived from medicinal chemistry, combinatorial chemistry, modification of existing molecules, and exploration of natural products. The project described herein focuses on harnessing the therapeutic potential and valorisation of natural products through a broader exploration of herbal medicine as a sustainable and culturally relevant approach to combating malaria, with potential implications for the development of alternative antimalarial therapies needed to drive malaria elimination efforts.

1.3 Resurgent use of *A. annua* and *A. afra* in the context of malaria

Before the isolation of molecules from natural products and the development into drugs for the treatment of infectious diseases, including malaria, the ethnomedicinal use of medicinal plants has long been practiced for centuries. This approach is based on a body of work that spans several disciplines, such as botany, chemistry, pharmacology, and includes field observations, descriptions of the utilization and bioactivities of folk remedies, botanical identification of the plant material, as well as phytochemical and pharmacological research ([Süntar, 2020](#)). Over the years, there is an increasing interest in the use of medicinal plants for management of health-related problems with about 80% of the global population relying on them for their primary health care, especially in Low- and Middle-Income Countries (LMICs) where access to western medicine is limited among other factors ([World Health Organization, 2002](#); [World Health Organization, 2013](#)). In the context of malaria, the emergence and spread of resistance to the available chemotherapy and multi-drug resistance strains of malaria parasites have increased the probability of treatment failure in malaria control, leading to increased use of medicinal remedies. Additionally, the current treatment is not afforded by all categories of people living in endemic

regions, and it does not ensure complete gametocyte clearance. These limitations are further worsened by the slow pace in the development of new drugs, coupled with cultural impediments to Western medicines, have increased the use of medicinal plants as treatment options, especially in malaria-endemic regions.

Of potential interest is the increasing resurgent use of *Artemisia annua* (native to Southeast Asia) and *Artemisia afra*, closely related species (native to South Africa), both of which are members of the Asteraceae family (Morua et al., 2025). Both plants have a diversity of bioactive compounds, including monoterpenes, diterpenes, sesquiterpenes, flavonoids, phenolic acids, coumarins, and essential oils, amongst others, with a wide range of biological activities, including potent activity against malaria parasites. Several compounds found in both plants have been shown to exhibit direct antiplasmodial activity (luteolin, artemitin, apigenin, rutin, artemisinin, quercetin and its analogues, kaempferol) while others (scopoletin, scopolin, chlorogenic acid, arteanin B) act indirectly by increasing the bioavailability of active components through the modulation of cytochrome P450 enzymes (Blanke et al., 2008; Ferreira & Luthria, 2010a; N. Q. Liu et al., 2009; Shinyuy et al., 2023a; Suberu et al., 2013a; Tasdemir et al., 2006; Weathers, Towler, et al., 2014). Several *in vitro*, *in vivo* and clinical trial data have shown the effectiveness of *A. annua* and *A. afra* including activity against gametocytes, hence, supporting the use of these plants as a culturally sound alternative therapeutic agents for combating malaria (Blanke et al., 2008; Daddy et al., 2017; Elfawal et al., 2012; Moyo et al., 2019a; Mueller et al., 2004; Rath et al., 2004; Walz et al., 2024; Weathers, Towler, et al., 2014). In addition to their promising antimalaria effects, they have been proven to be safe of toxicity and are deemed safe for oral intake, supported by over 2000 years of recorded traditional use and with *in vitro* and *in vivo* studies including clinical trials providing scientific evidence (Feng et al., 2020; Morua et al., 2025; Mukinda and Syce, 2007a). Unlike other medicinal plants, *A. annua* and *A. afra* are easily domesticated and can grow in all hostile environments. These characteristics have made both plants a subject of ethnomedicinal relevance and can be seen in almost every corner of the globe, particularly in malaria-endemic regions, in recent years. In Cameroon, like in other African countries, both *A. afra* and *A. annua* are easily domesticated on large and small scales, in all 10 regions, and sold in local markets for the

management of several ailments, and in particular, malaria (Shinyuy et al., 2025). Thus, directly promoting the United Nations Sustainable Development Goals (SDG 1, 3, 5, 8, 11, and 12), which prioritise poverty alleviation, health and well-being, gender equality, decent employment and economic advancement, responsible consumption and production, and sustainable urban development. These two plant species exemplify how the widespread use of botanical pharmaceuticals can reduce costs, enhance environmental sustainability, and provide economic advantages, particularly for rural communities, by enabling people to manage their healthcare requirements more sustainably (Weathers, 2025).

1.4 Controversy on the use of *A. annua* and *A. afra* in treating malaria

Despite the reported long-term use of *Artemisia* in combating malaria with substantial scientific evidence from *in vitro* to human clinical trials and proven to be effective and safe for treating malaria, *Artemisia spp.*, especially *Artemisia annua*, is controversially advocated for by many researchers, including the World Health Organization. Nonetheless, *Artemisia annua* infusions and decoctions have been used for centuries without resistance development. It should be noted that resistance has only recently emerged in Thailand, where pure artemisinin was used as a monotherapy for several years before the introduction of ACTs (Phyo et al., 2016). Artemisinin Combination Therapies (ACTs) are the most effective method of treating malaria to achieve the recommended dosages of the primary active compound artemisinin (Mueller et al., 2004; World Health Organisation, 2012), thus suggesting that the traditional use of *Artemisia annua* (natural source of artemisinin) should be discouraged. *Artemisia annua* therefore stands at the forefront of the contentious discourse between the single drug–single target paradigm of Western medicine and the holistic methodology of traditional medicinal systems. Most people think that *Plasmodium falciparum* will develop resistance to artemisinin if *Artemisia annua* tea infusion or the entire plant is used for treatment. In fact, some researchers even claim that consumption of whole plant or tea infusions from *A. annua* is a monotherapy. This stems from artemisinin's low levels in *Artemisia annua*, which make quality control and standardisation

challenging, water's poor extraction efficiency, artemisinin's low bioavailability when taken as an infusion, and the high rates of recrudescence in patients receiving the infusion (Mueller et al., 2004). The WHO released a position statement on the use of *Artemisia annua* for the treatment of malaria as a result of this conflict between traditional remedies (*A. annua*) and Western-based medications (artemisinin). This statement's primary suggestion was to avoid using *A. annua* in any form, even as a tea infusion, because of the plant's low natural abundance of artemisinin (0.01–1.34%) (Liu et al., 2006; World Health Organisation, 2012). The World Health Organization argues that non-pharmaceutical sources of artemisinin may deliver sub-therapeutic doses that could exacerbate the resistance challenge faced by ACTs.

In addition to the supposed exacerbation of resistance from consuming *A. annua* in any form, WHO also argues that there are significant differences or variations in the content of *Artemisia* herbal remedies used to treat and prevent malaria, a factor that also affects the use of *A. afra* (despite being devoid of artemisinin) in combating malaria. Additionally, herbal therapies, including *Artemisia*, are frequently insufficient to eradicate all malaria parasites present in a patient's circulation and stop their recurrence. Moreover, it is highly contented that artemisinin resistance may develop and spread more quickly if *Artemisia annua* herbal therapies are widely used.

1.5 What the future holds for *A. annua* and *A. afra*

Using medicinal plants also fits well with the current health care systems in developing countries. There are several drawbacks to use traditional herbal medicines, particularly when treating potentially lethal conditions like malaria. Standardisation of plant cultivation, formulation preparation, dosages, quality control, and proof of clinical safety and efficacy are the primary constraints. For centuries and over three decades, *Artemisia annua* L. and *Artemisia afra* Jacq. Ex Willd., respectively, were used as medicinal herb infusions or powders to treat malaria. Both species have long traditions in their respective ethno-pharmacopeia of Southeast Asia (*A. annua*) and Southern Africa (*A. afra*).

While *A. annua* cultivars have been cultivated and selected for their high artemisinin content, *A. afra* is devoid of this potent sesquiterpene lactone

(Liu et al., 2009). In a recent human double-blind clinical trial and in other previous studies, both plants were reported to stably cure malaria. Importantly, *Artemisia* plants were also reported to cure schistosomiasis (Munyangi et al., 2018), another disease of major public health impact in many Sub-Saharan countries, including Cameroon, suggesting their potential role in integrated disease control programs. However, this findings were retracted due to some methodological issues. Although the World Health Organization has cautioned against the use of non-pharmaceutical sources of artemisinin because of the risk of delivering subtherapeutic doses that could exacerbate this resistance problem (World Health Organization, 2012), there is no scientific evidence to back this hypothesis. It should be noted that using whole plant powder either as an infusion or decoction results in a complex mixture of different phytochemicals that can have a totum effect against malaria parasites. The totum effect involves interactions that lead to a higher bioavailability, decreased metabolism, reduced excretion, and resistance reversal. The totum effect was illustrated by giving *P. berghei*-infected mice an ethanol-based whole plant extract of *A. annua*. This resulted in an ED₅₀ reached with 35.1 mg /kg artemisinin from the whole plant extract, while when applying only artemisinin, the same effect was only reached with an ED₅₀ of 122 mg/kg. This indicates that less artemisinin is needed when using the whole plant extract and may be explained by the fact that other metabolites could enhance pharmacodynamic and/or pharmacokinetic properties of artemisinin (Rasoanaivo et al., 2011). In this context, the use of *A. annua* in the management of malaria should rather focus on standardizing dosage regimens, preservation methods, quality control, and development of pharmaceutical monographs to provide sufficient data geared towards ensuring consistent efficacy.

On the other hand, recent investigations have however, provided evidence that *Artemisia afra* infusions, with no artemisinin content, effectively and efficiently cure malaria, including activity againsts *Plasmodium* gametocytes without adverse effects (Moyo et al., 2019; Shinyuy et al., 2023; Snider and Weathers, 2020), suggesting its use without the above-mentioned WHO threat to ACTs in emptying malaria reservoirs. In this regard, and with reported data on the safety profile, *in vitro* and *in vivo* studies, supports the use of *A. afra* as a culturally sound therapeutic/prophylaxis alternative for combating malaria. However, much scientific evidence is needed to provide

data on the human clinical trial, standardisation, impact of both geographical location and season of harvest, and post-harvest handling methods on metabolite contents is needed to improve and ensure consistent efficacy. This aspect forms the basis of this project, whose aim is to generate data on the above-mentioned points, hence valorising these two medicinal plants to drive malaria elimination efforts and the attainment of United Nations SGD-3 and the World Health Organisation Global Technical Strategy for malaria elimination.

1.6 Plants used for the treatment of malaria in Cameroon

In addition to *A. afra* and *A. annua* (Asteraceae family) as traditionally used medicinal plant in Cameroon for treatment and or in prophylaxis of malaria, there are numerous other medicinal plants belonging to different plant families employed as therapeutic alternatives for management of malaria. The Cameroonian terrestrial ecosystem has a diverse flora with a large number of plants identified with potentials as traditional remedy against malaria. In addition, these antimalarial medicinal plants served as reservoirs of bioactive molecules suitable for further drug development. It is worth noting that the use of these medicinal plants is mostly practiced in rural communities where access to modern medicines is limited and coupled to other limitations like cultural impediment to Western medicines (Titanji et al., 2008). In these communities, whole plant or plant parts are mostly prepared as decoctions and infusions with most remedies comprising of two or more plant species (Titanji et al., 2008). Ethnobotanical and ethnopharmacological studies have identified a couple of antimalarial medicinal plants including but not limited to, Annonaceae (e.g. *Enantia chlorantha*, stem bark), Anacardiaceae (e.g. *Mangifera indica*, leaves), Rubiaceae (e.g. *Coffea arabica*, stem bark, leaves or roots are used in combination with other plants), Asteraceae (e.g. *Conyza sumariensis*, leaves) (Titanji et al., 2008). Amongst these plant families, the Asteraceae, has gained significant attention over the past decades especially, the genus *Artemisia*. During the Covid-19 pandemic, there was significant media attention on the use of plants from the Asteraceae family as traditional remedies against the virus (Van der Kooy, 2024). This trend led to their widespread adoption in several regions, including areas where malaria is endemic. Consequently, the cultivation, processing, and commercialization

of these medicinal plants, both on large and small scales, became increasingly common across Cameroon, particularly for malaria treatment once Covid-19 was no longer a major concern. However, in the Adamawa region of Cameroon, the commercialization of *Artemisia* species was relatively limited compared to other regions. This was mainly because the local population has long relied on *Erigeron bonariensis* (a member of the Asteraceae family) for malaria treatment. Although, *E. bonariensis* has a long history of use for management of malaria, there is limited data to justify both its efficacy against malaria parasites and safety profiles. Although traditional herbal remedies like *E. bonariensis* may offer sources of new drugs, thorough toxicological evaluations are vital. Natural products are not inherently safe and can cause a range of adverse effects, including those impacting the liver and kidneys (Stournaras et al., 2021). A comprehensive approach combining traditional knowledge with modern pharmacological analysis is essential for realizing the benefits of traditional remedies while minimizing risks (Thomford et al., 2018).

1.7 Chemistry and pharmacology of *Erigeron bonariensis*

Erigeron bonariensis (L). formerly known as *Conyza bonariensis* (L), is a plant native to South America but has spread to various regions around the world. While it is often considered a weed due to its ability to grow rapidly and compete with cultivated crops, *E. bonariensis* is of ethopharmacological relevance due to its antimalarial properties. Botanically, *E. bonariensis* is an herbaceous annual plant that typically grows up to 1-2 meters in height. It has a slender stem covered with fine hairs, which gives it a hairy or fuzzy appearance. The leaves are alternate, elongated, and serrated, with a gray-green color. The plant produces numerous small flowers that are arranged in clusters, and the mature seeds are equipped with feathery structures that aid in wind dispersal. From an ethomedicinal point of view, *E. bonariensis* have been traditionally used in folk medicine for their potential therapeutic properties. For instance, the leaves and aerial parts of the plant have been employed in traditional medicine for their reputed diuretic, expectorant, and anti-inflammatory effects (Carvalho et al., 2013). Infusions or decoctions made from the plant have been used to treat respiratory conditions, such as coughs, bronchitis, and asthma. However, it is important to note that scientific studies on the medicinal properties of *E. bonariensis* are limited, and further research is needed to validate these traditional claims. The

antioxidant and antimicrobial properties of its phytochemicals may have applications in the development of natural cosmetics, functional foods, or herbal supplements. The presence of bioactive compounds, including flavonoids, sesquiterpene lactones, phenolic acids, terpenoids and alkaloids, (Zhang et al., 2018), explains its biological activity against several diseases/conditions including antioxidant, antimicrobial, antidiabetic, anticancer and hepatoprotective effects (Silva et al., 2019; Fernandes et al., 2016; Barros et al., 2017). *E. bonariensis* has equally demonstrated its potential as traditional remedy for the management of malaria. However, further research is needed to investigate the safety, efficacy, and commercial viability of utilizing *E. bonariensis* as a natural product.

1.8 Phytochemistry and pharmacological properties of *A. annua* and *A. afra*

Artemisia annua is one of the most extensively studied medicinal plants over the past decades and has a diverse phytochemical composition. In addition to *A. annua*, much attention has been given to *A. afra*, a closely related species that lacks or with trace amounts of artemisinin. Both plants are rich in monoterpenes, diterpenes, triterpenes, sesquiterpenes, flavonoids, phenolic acids, coumarins, essential oils, long-chain fatty acids, and minerals (Rafiri et al., 2024; Sharifi-Rad et al., 2022; Shinyuy et al., 2023; Sotenjwa et al., 2020). Due to their phytochemical diversity, both plants have been shown to demonstrate a wide range of biological activities and have been proven to be of importance in human health, animal health, and to the environment (Figure 13). *Artemisia annua* and *Artemisia afra* medicinal plants with historical applications and with encouraging scientific validation, correspond with the One Health paradigm by exhibiting advantages for both human and animal health, while their cultivation may yield beneficial environmental effects (Liu et al., 2009; Morua et al., 2025; Swamikannu et al., 2025; Weathers, 2025).

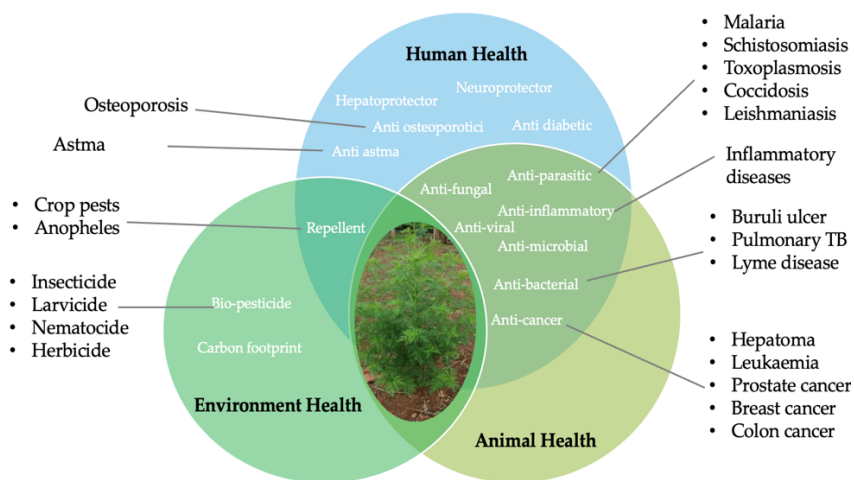


Figure 13: Biological activity of *Artemisia afra* and *Artemisia annua*

1.8.1 Foreword

Indigenous to Southeast Asia, *Artemisia annua* gained international recognition following the isolation of artemisinin, one of the most potent antimalarial compounds, from its leaves. This breakthrough sparked global interest in the plant, leading to extensive phytochemical investigations aimed at identifying other bioactive compounds with therapeutic potential. As a result, numerous constituents exhibiting diverse biological activities have been isolated and characterized from *A. annua*, including antiplasmodial, anti-inflammatory, and immunomodulatory properties.

In parallel, *Artemisia afra*, a closely related species native to Africa, has also attracted significant scientific attention over the past two decades. Although it contains little to no artemisinin content, *A. afra* has demonstrated considerable ethnomedicinal relevance, particularly in malaria-endemic regions. Both species exhibit rich and complex phytochemical profiles, underscoring their potential roles in integrated disease management strategies. With regards to the work conducted on the chemical composition and antiplasmodial properties of both plants, it was essential to have an overview of the existing literature on the secondary metabolite composition and antiplasmodial properties. The following section presents a comprehensive overview of the chemical profile and biological activity of *A. annua* and *A. afra*.

1.8.2 Chemistry and antiparasitological properties – Article (published)



metabolites



Review

Secondary Metabolites Isolated from *Artemisia afra* and *Artemisia annua* and Their Anti-Malarial, Anti-Inflammatory and Immunomodulating Properties – Pharmacokinetics and Pharmacodynamics: A Review

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Abstract: There are over 500 species of the genus *Artemisia* in the Asteraceae family distributed over the globe, with varying potentials to treat different ailments. Following the isolation of artemisinin (a potent anti-malarial compound with a sesquiterpene backbone) from *Artemisia annua*, the phytochemical composition of this species has been of interest over recent decades. Additionally, the number of phytochemical investigations of other species, including those of *Artemisia afra* in a search for new molecules with pharmacological potentials, has increased in recent years. This has led to the isolation of several compounds from both species, including a majority of monoterpenes, sesquiterpenes, and polyphenols with varying pharmacological activities. This review aims to discuss the most important compounds present in both plant species with anti-malarial properties, anti-inflammatory potentials, and immunomodulating properties, with an emphasis on their pharmacokinetics and pharmacodynamics properties. Additionally, the toxicity of both plants and their anti-malaria properties, including those of other species in the genus *Artemisia*, is discussed. As such, data were collected via a thorough literature search in web databases, such as ResearchGate, ScienceDirect, Google scholar, PubMed, Phytochemical and Ethnobotanical databases, up to 2022. A distinction was made between compounds involved in a direct anti-plasmodial activity and those expressing anti-inflammatory and immunomodulating activities or anti-fever properties. For pharmacokinetics activities, a distinction was made between compounds influencing bioavailability (CYP effect or P-Glycoprotein effect) and those affecting the stability of pharmacodynamic active components.

Keywords: malaria; artemisinin; metabolites; *Artemisia afra*; *Artemisia annua*

Figure 14: Screenshot of the cover page of our published article.

1. Introduction

If one were to nominate the most infectious parasitic disease in the world, malaria would be at the top of the list, as it is a disease of public health importance with serious socio-economic and human consequences. Malaria is a devastating disease, affecting over 241 million people worldwide annually, with 627,000 deaths ([World Health Organization, 2022](#)). There were about 14 million more cases of malaria and 69,000 more deaths in 2020 than there were in 2019, most of which were in Sub-Saharan African countries. Approximately 95% of the world's malaria burden is concentrated in 11 countries, with 10 of them being in Sub-Saharan Africa with 96% of the death rate; over 80% of these are among children under the age of five and pregnant women ([World Health Organization, 2022](#)). This is due to increasing antimalarial drug resistance to the chemotherapy employed at present. The situation is further aggravated by not only Histidine-Rich Protein-2 gene (HRP2 gene) deletion, making diagnosis problematic, but also by mosquito resistance to insecticides and invasive vector species (*Anopheles stephensi*) in the Horn of Africa ([World Health Organization, 2022](#)). The fight against this life-threatening disease is one of the goals of the World Health Organization's (WHO) Global Technical Strategy to reduce its transmission and help create a world that is free of malaria by 2030. The terrestrial ecosystem has been a source of many lead compounds that have undergone chemical derivatization and drug development to produce antimicrobial molecules, including those used to fight malaria ([Abad et al., 2012](#)). Numerous studies have been conducted on natural products such as crude extracts or pure compounds isolated from medicinal plants. Ethnobotanical and ethnomedical alternatives for the management of health problems, including malaria, have been practiced for many years and are used by 80% of the global population in primary health care ([Bora et al., 2010](#); [Milani et al., 2011](#)). These alternative remedies have provided leads for the development of drugs that are useful in therapeutics such as those in used in Western medicine. A well-known example of the seminal contribution of ethnomedicine to the treatment of malaria in the modern medicinal way includes artemisinin, which is isolated from *A. annua* ([Jeremić et al., 1973](#)). Following the isolation of this well-known antimalarial molecule, intensive research on the phytochemistry of this species has resulted in the isolation of almost 600 secondary metabolites in recent decades ([Brown et al., 2010](#)).

The emergence of resistance to all current malaria drugs including artemisinin has increased the use of *A. annua* and *A. afra* by the population, especially in areas of high endemicity. *A. annua* has been used in China since ancient times as a herbal product taken in the form of infusions or decoction for the alleviation of intermittent fevers associated with malaria, amongst other diseases (Klayman et al., 1985). *A. annua* is documented in the Pharmacopeia of the People's Republic of China and is now domesticated in other parts of Asia, Europe, Australia, and America, where it is used to fight other ailments (Klayman et al., 1985; Ćavar et al., 2011; Mueller et al., 2000; Gouveia et al., 2013). However, the WHO has cautioned against the use of non-pharmaceutical sources of artemisinin because of the risk of delivering sub-therapeutic doses of artemisinin that could potentiate antimalarial drug resistance (Price et al., 1996). Hence, the emergence of *A. afra* which contains negligible or no artemisinin content but kills malaria parasites (Weathers et al., 2014) including the gametocytes (Karl et al., 2011; Moyo et al., 2019). *A. afra* has a long history of use as a herbal product in South Africa for the treatment of ailments, including malaria (van Wyk, 2011) and it is cultivated across the African continent for the management of malaria and other diseases (Mueller et al., 2000; Weathers et al., 2014). About 400 secondary metabolites have been isolated from *A. afra* with a wide range of biological activities against various diseases, including malaria (Mueller et al., 2000). Being a member of the Asteraceae family, the genus *Artemisia* is one of the largest and most dispersed genera, with over 500 species, which are predominantly located in different geographical regions due to their ability to thrive, survive and persist in almost all habitat types (Price et al., 1996). The ethnobotanical and ethnomedicinal importance, potential phytotherapeutic application and traditional use of this plant genus have been reviewed by Parada et al. (2019) and Wright et al. (2017). In this review, the main objective is to discuss the main secondary metabolites isolated from *A. afra* and *A. annua* and their anti-malarial and anti-inflammatory potentials, with an emphasis on their pharmacokinetics and pharmacodynamic importance.

2. Methodology

2.1 Literature Search

A thorough review of the literature in web databases relating to secondary metabolites isolated from *A. annua* and *A. afra* and their anti-malaria, anti-inflammatory and immunomodulating properties, with a focus on their pharmacokinetics and pharmacodynamics properties, was performed. A literature search of the research in web databases was conducted, and data up to 2022 were collected from published journal articles in international scientific databases, including Ethnomedicinal, ResearchGate, ScienceDirect, Google scholar, PubMed, Phytochemical and Ethnobotanical, etc., reporting on the traditional use, phytochemistry, anti-malaria, anti-inflammatory, immunomodulating, pharmacokinetics and pharmacodynamics of *A. annua* and *A. afra* and their secondary metabolites. The following key terms were employed for the literature search: Malaria/burden/*A.annua*/*A. afra*, botanical description, geographical distribution/Secondary metabolites/*Asteraceae* family/Anti-malarial, anti-inflammatory/Immunomodulation/Indigenous use/Herbal Remedies/Ethnobotany/Ethnopharmacology/Ethnomedicine/Ethnomedicinal/Ethnopharmaceutical/phytochemistry/Cytochrome/Cytochrome P450/Pharmacology/pharmacokinetics, pharmacodynamics, bioavailability/Resistance/Artemisinin Combination Therapy. About 200 published research articles were studied, and chemical structures of bioactive compounds were drawn using a scientifically accepted program ChemDraw.

3. Results

3.1. Botanical Description and Distribution

Artemisia annua L. is also referred to as sweet wormwood, sweet annie, and annual wormwood (English) or qinghao (Chinese). According to the Pharmacopeia of the People's Republic of China ([Chinese Pharmacopoeia, 2010](#)), a plant containing artemisinin in the *Asteraceae* family is termed qinghao, meaning "the dry" above-ground parts. *A. annua* is a large, annually grown, highly aromatic herbaceous herb that can grow up to 2 m tall, with a single stem covered with fine grey-green hairs. It has aromatic leaves which are deeply dissected. Despite it being widely distributed throughout the world due to its ability to treat a wide range of diseases, traditionally, *A. annua* originated in China ([Ma et al., 2006](#)). Just like most of

the members of the Asteraceae family, *A. annua* is highly adaptable, growing in almost all hostile environments, such as forest margins, roadsides, hillsides, dry river valleys, forest meadows, rocky slopes, semiarid climates, and grasslands. However, it was reported that the best growing condition for this plant is in a humid and subtropical monsoon climate with average temperatures of 17.6–28.4 °C (Ferreira et al., 1995). *A. annua* grows in most parts of the temperate and subtropical parts of the central, southern, and eastern parts of Europe and Asia. Extending to the south-eastern part of Asia, this plant is also seen growing across the Mediterranean and northern parts of Africa. It has also been spread in the United States, North America, and Canada (Ma et al., 2006), **Figure 15**.

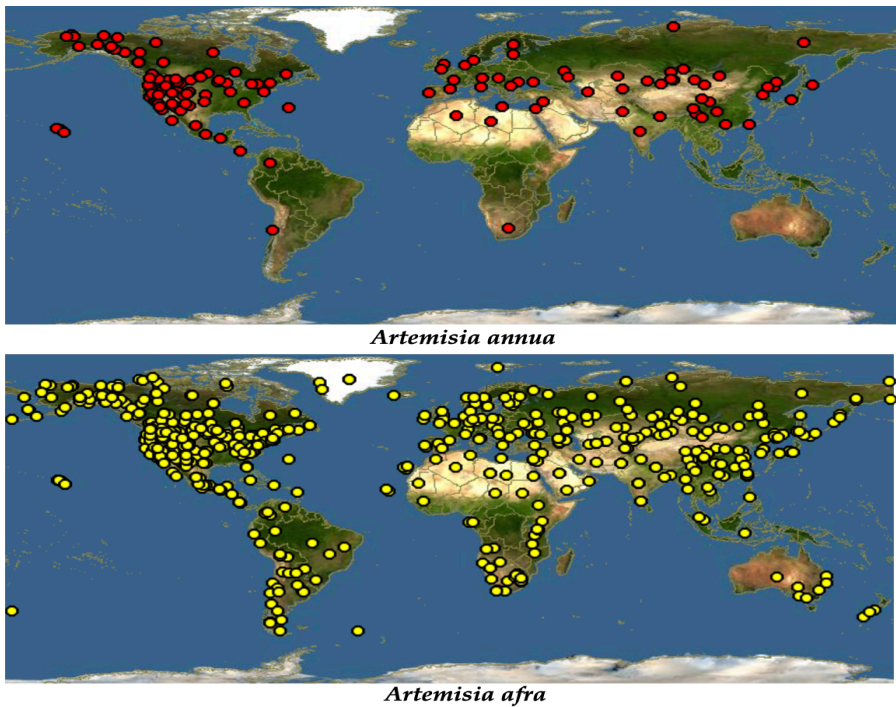


Figure 15. Global distribution of *A. annua* and *A. afra*.

Additionally, referred to as African wormwood, *A. afra* (Jac. ex Willd) is described as having different common names in different societies (Roberts, 1990). Being an indigenous herb to Africa, *A. afra* grows across the African continent in areas extending from Cederberg Mountains in the Cape to tropical East Africa and continuing through to the north, including Ethiopia

(Roberts, 1990). Described as a condiment (Chinese Pharmacopoeia, 2010), it has been spotted in almost every corner in the world due to its high medicinal potential (**Figure 1**). *A. afra* grows up to a height of at least 2 m. It is a woody perennial herb with finely divided oval-shaped leaves, and it has an aromatic smell, and alternately arranged, silver-grey leaves in the adaxial region and light green leaves in the axial region (van Wyk, 2011).

3.2. Phytochemistry of *A. afra* and *A. annua*

For decades, the phytochemical investigation of *A. annua* has been at the forefront of research due to the isolation of artemisinin, a potent anti-malarial compound belonging to the sesquiterpene lactone class (Jeremić et al., 1973). Further research has led to the isolation of almost six hundred secondary metabolites, the majority of which are terpenoids, polyphenols and coumarins, as summarized in the Supplementary Materials (**Supplementary Tables S1–S11**). There have been several publications on the phytochemistry of *A. annua* over the previous decade. **Table 1** summarizes the secondary metabolites isolated from both plant species over the previous decade. As such, several new secondary metabolites have been identified and characterized, with most of them belonging to the monoterpenes and sesquiterpenes classes and a few polyphenols.

Table 1. Secondary metabolites isolated from *A. annua* and *A. afra* in the previous decade.

Plant	Compound Name	Classification	Reference
<i>A. annua</i>	Abeo-amorphane sesquiterpene	Sesquiterpene	(Czechowski et al., 2018)
	(Z)-7-Acetoxy-methyl-11-methyl-3-ethylenedodeca-1,6,10-triene	Sesquiterpenes	(Sotenjwa et al., 2020)
	Apigenin-6-C-hexoside-8-C-pentoside.	Flavonoid	(Gouveia & Castilho, 2013)
	Arteannoides A to E	Sesquiterpene	(Qin et al., 2021)
	Arteannoides F to R	Sesquiterpene	(Qin et al., 2018)
	Arteannoides U to Z	Sesquiterpenes	(Qin et al., 2021)
	Artemisinic acid, 6 α -peroxy ester	Sesquiterpene	(Czechowski et al., 2018)
	Artemisiannuside A	Coumarin glycoside	(Wang et al., 2018)
	Artemanin A	Sesquiterpene	(Takenaka et al., 2020)
	Artemanin B	Sesquiterpene	
	Arteannuin P	Monoterpene	
	Arteannuin Q	Monoterpene	
	Arteannuin S	Sesquiterpene	
	Arteannuin T	Sesquiterpene	
	Arteannuin U	Sesquiterpene	(Czechowski et al., 2018; Han et al., 2008)
	Arteannuin V	Sesquiterpene	
	Arteannuin W	Sesquiterpene	
	Arteannuin Y	Sesquiterpene	
	Arteannuin Z	Monoterpene	

<i>A. annua</i>	Cadinanolide	Sesquiterpene	(Wang et al., 2018)
	Caffeoylcoumaroyltartaric acid	Phenolic acid	(Gouveia & Castilho, 2013)
	n-Cos-(Z)-9-enoic acid.	unsaturated fatty acid	(Goel et al., 2019)
	n-Cos-(Z)-10-enoic acid	unsaturated fatty acid	
	3-p-o-Coumaroyl-5-O-caffeoylquinic acid	Flavonoid	(Gouveia & Castilho, 2013)
	Chrysoeriol rutinoside	Flavonoid	(Carbonara et al., 2012)
	Cis-melilotoside	Coumarin	(Mouton & van de kooy, 2013)
	Dehydroarteannuin L	Sesquiterpene	(Czechowski et al., 2018)
	Deoxyartemistene	Sesquiterpene	
	Dihydroxy-dimethoxyl-O-hexoside	Sugar	(Gouveia & Castilho, 2013)
	3,4-Dihydroxybenzyl 2',3',4'-trihydroxybenzoate	dihydroxybenzyl ester	(Goel et al., 2019)
	3,4-Dihydroxybenzyl 2',3',4'- trihydroxybenzoate	dihydroxybenzyl ester	
	4,4'-β-D-dixylopyranoside		
	4α, 5α-Epoxy-6α- hydroxyartemisinic acid methyl ester	Sesquiterpene	(Czechowski et al., 2018)
	3-(2-(2,5-dihydrofuran-3-yl) ethyl)-2,2-Dimethyl-methylenecyclohexan-1-one	Sesquiterpene	
	(R)-15,16-Didehydrocoriolic acid	-	(Wang et al., 2018)
	Eriodictyol-7-O-hexoside.	Flavonoid	(Gouveia & Castilho, 2013)
	Epi-11-hydroxy-arteannuin I	Sesquiterpene	(Czechowski et al., 2018)
	6,7-Epoxy-6,7-dihydro-β-farnesene	Monoterpene	
	n-Heptadecanyl-β-D-glucopyranoside	aliphatic	(Goel et al., 2019)

	alcoholic glucoside	
n-Heptadecanyl linoleate	unsaturated fatty acid	
Homoeriodictyol	-	(Wang et al., 2018)
5 β -Hydroperoxy-eudesma-4(15),11-diene	Sesquiterpene	
7 α -Hydroxy-artemisinic acid	Sesquiterpene	
11-Hydroxy-arteanuin I	Sesquiterpene	
6 α -Hydroxy-arteanuin J	Sesquiterpene	
(E)-7-Hydroxy-2,7-dimethylocta-2,5-dien-4-one	Monoterpene	(Czechowski et al., 2018)
(E)-7-Hydroperoxy-2,7-dimethylocta-2,5-dien-4-one	Monoterpene	
6- Hydroxy- γ -humulene	Monoterpene	
Isoarteanuin A	Sesquiterpene	
Isodocosanol	Aliphatic alcohol	(Goel et al., 2019)
Isorhamnetin-O-hexoside	Flavonoid	(Gouveia & Castilho, 2013)
Isononadecano	Aliphatic alcohol	(Goel et al., 2019)
Luteolin 7-O-pentoside	Flavonoid	(Gouveia & Castilho, 2013)
1-Octacosanol	Aliphatic alcohol	
n-Octadecanyl n-octadec-9,12, - dienoate.	unsaturated fatty acid	(Goel et al., 2019)
n-Nonacosanyl n-octadec-9, 12-dienoate.	unsaturated fatty acid	
($\pm$)-Qinghaocoumarin A	Coumarin	
Qinghaocoumarin B	Coumarin	(Li et al., 2019)
Qinghaolignan A.	Lignan	

<i>A. annua</i>	Qinghaolignan B	Lignan	
	Qinghaosu I and III	Sesquiterpene	
	Quinic acid	Organic acid	(Gouveia & Castilho, 2013)
	Rosmarinic acid	Phenolic acid	(Czechowski et al., 2018)
	<i>Trans</i> -melilotoside	Coumarin	(Mouton & van de Kooy , 2013)
	5, 7, 4'- Trimethoxy-8,3"-dihydroxyflavone	Flavonoid	(Wang et al., 2018)
<i>A. afra</i>	Trimethoxy-coumarin	Coumarin	(Youyou et al., 2015)
	Artemin	Sesquiterpene	(Gakuba, 2009)
	Artesin	Sesquiterpene	
	Arabinose	Sugar	(Braünlich et al., 2018)
	Fucose	Sugar	
	1 α ,4 α -Dihydroxybishopsolicepolide	Guaianolide	(Moyo et al., 2019)
	2,4-Dihydroxy-6 methoxyacetophenone	-	(Gakuba, 2009)
	Galacturonic acid	Organic acid	
	Galactose	Sugar	(Braünlich et al., 2018)
	Glucose	Sugar	
	Glucuronic acid	Organic acid	
	<i>p</i> -Hydroxyacetophenone	-	(Gakuba, 2009)
	Mannose	Sugar	(Braünlich et al., 2018)
	Maritimin	Sesquiterpene	(Gakuba, 2009)
	4-O-Methylglucuronic acid,	Organic acid	(Braünlich et al., 2018)
	Reynosin	Sesquiterpene	(Gakuba, 2009)

Rhamnose	Sugar	(Braünlich et al., 2018)
Rutin	Flavonoid	
Santolinifolide	Sesquiterpene	(Gakuba, 2009)
Santolinifolide A	Sesquiterpene	
Scopolin	Coumarin	(Sotenjwa et al., 2020)
Yomogiartemin	Guaianolide	(Han et al., 2022)

For instance, [Qin et al. \(2018\)](#), isolated five new sesquiterpene compounds from *A. annua*: Arteannoid A, which is a sesquiterpenoid dimer composed of two cadinene sesquiterpenoid units; Arteannoids B and C, which are rearranged heterodimers of the cadinene sesquiterpene and one phenylpropanoid unit and two new rearranged cadinene sesquiterpenoids (Arteannoides D and E). In addition, thirteen new sesquiterpenoid compounds (Arteannoides F–R) were isolated from the aerial part of *A. annua* ([Qin et al., 2020](#)). Arteannoid H is a new eudesmane-type sesquiterpenoid ([Qin et al., 2018](#)). A comparative phytochemical investigation of two chemotypes of *A. annua* (high- and low-artemisinin-producing chemotypes, HAP and LAP, respectively) via UPLC, GC-MS and NMR also lead to the isolation and characterization of twenty-six novel compounds belonging to monoterpenes and sesquiterpenes classes. Nineteen of these secondary metabolites were highly oxygenated amorphane sesquiterpenes, which are the most diverse and abundant subclass ([Czechowski et al., 2018](#)). [De Magalhães et al. \(2016\)](#) also confirmed the presence of phenolic acid (Rosmarinic acid) in the leaves of *A. annua*. [Mouton and Van der Kooy, \(2013\)](#) isolated two compounds (melilotosides) with antityphoid potentials. [Goel et al. \(2007\)](#) also characterized nine new aliphatic compounds from the aerial part of *A. annua*. As it is a member of the same genus, *A. afra* shares almost the same pattern of secondary metabolites, which is dominated by terpenoids, polyphenols and coumarins, as summarized in the Supplementary Materials (**Supplementary Tables S1–S6**). While *A. annua* cultivars have been cultivated and selected for their high artemisinin content, *A. afra* is devoid of this potent sesquiterpene lactone ([Snider et al., 2020](#)). However, other studies have shown that there are cultivars of *A. afra* with negligible contents of artemisinin ([Karl et al., 2011](#)). Research on the phytochemistry of *A. afra* has led to the isolation of almost five hundred secondary metabolites comprising monoterpenes, diterpenes, triterpenes, sesquiterpenes, guaianolides, glaucolides, coumarins and polyphenols (**Supplementary Tables S1–S11**). Surprisingly, a very limited amount of work on phytochemistry has been conducted on *A. afra* over the previous decade despite its multi-biological activity against infections ([van Wyk, 2011](#)). However, [Gabuka et al. \(2009\)](#) isolated five new sesquiterpenes from this species and two other compounds (p-hydroxy acetophenone and 2,4-dihydroxy-6 methoxy acetophenone). [Sotenjwa et al. \(2020\)](#) also isolated two

new compounds, a flavonoid (rutin) and a coumarin (scopolin), from this species.

3.3. Anti-Malarial Properties, Anti-Inflammatory and Immunomodulating Effects of *A. annua* and *A. afra*

According to the Chinese culture, *A. annua* has been known to alleviate fevers since the second century (Klayman et al., 1985; Mueller et al., 2000). It is a highly efficacious medicinal plant against malaria due to the presence of artemisinin and is traditionally used in infusions or decoctions for the treatment of malaria in China (Braünlich et al., 2018). Ethnopharmacological studies for the use of *A. annua* in Traditional Chinese Medicine (TCM) prompted the isolation and characterization of artemisinin (Jeremić et al., 1973) which is a sesquiterpene with remarkable efficacy against the erythrocytic stages of the malaria parasite. Several in vitro and in vivo studies on the crude plant material and/or infusions have been reported, showing good antiplasmodial activities (de Ridder et al., 2008; Gruessner et al., 2021). For example, Diawara et al. (2015) confirmed the in vitro anti-malarial activity of aqueous and hydroalcoholic crude extracts of *A. annua*, with an IC₅₀ of 4.95 nM for chloroquine resistance against *P. falciparum*. Recent investigations on the effect of tea infusions of *A. annua* on pre-erythrocytic and erythrocytic stages showed growth inhibition of various *Plasmodium* species in a dose-dependent manner (Mueller et al., 2000). In addition, Snider et al. (2020) confirmed the anti-parasitic activity of a tea infusion with *A. annua* against the early and late stages of *P. falciparum* gametocytes, as well as against the asexual forms. **Table 2** summarizes the reports on anti-malarial properties

Table 2. *Artemisia* spp. with reported anti-malarial properties either *in vitro*, *in vivo* or in clinical trials.

Plant species	Plant part /extract	Model	Stage of parasite	IC ₅₀ or %Inhibition	Reference
<i>Artemisia absinthium</i>	Leaves/CF	In vitro	Erythrocytic	0.42 µg/mL	(Dua et al., 2011)
	Leaves/MeOH	In vitro	Erythrocytic	20.00 µg/mL	(Abdou et al., 2022)
	Leaves/EtOH	In vivo	Erythrocytic	60 % inhibition at 100 mg/Kg/day	(Abdou et al., 2022)
<i>Artemisia abyssinica</i>	Leaves/PE	In vitro	Erythrocytic	2.10 µg/mL	(Tasdemir et al., 2015)
<i>Artemisia afra</i>	Leaves/ethanolic	In vitro	Erythrocytic	2.66 µg/mL	(Kane et al., 2019)
	Leaves/hexanolic	In vitro	Erythrocytic	0.71 µg/mL	(de Donno et al., 2012)
	Leaves/CF	In vitro	Erythrocytic	8.55 µg/mL	(Liu et al., 2010)
	Leaves/DCM	In vitro	Erythrocytic	6.58 µg Artemisinin/L	(Gruessner & Weathers, 2021)
	Leaves/tea infusion	In vitro	Erythrocytic	100 % inhibition of parasite growth at 4 g/L	(Snider & Weathers, 2021)
	Leaves/tea infusion	In vitro	Pre- ythrocytic	100 % inhibition of parasite growth at 4 g/L	(Snider & Weathers, 2021)

	Leaves and twigs/tea infusion	In vitro	Erythrocytic	Significant decrease in parasitemia at 5 g/L	(Snider & Weathers, 2021)
	Leaves and twigs/tea infusion	In vitro	Gametocytes	Significant decrease in gametocytaemia at 5 g/L	(Snider & Weathers, 2021)
	Leaves/hydro-ethanol	In vitro	erythrocytic	0.46 µg/mL	(Abdou et al., 2022)
	Leaves/DCM	In vivo	Erythrocytic	94.28 % inhibition at a dose of 200 mg/kg	(Abdou et al., 2022)
<i>Artemisia armeniaca</i>	Aerial part/PE	In vitro	Erythrocytic	0.90 mg/mL	(Mojarrab et al., 2016)
	Aerial part/DCM	In vitro	Erythrocytic	1.04 mg/mL	(Mojarrab et al., 2016)
<i>Artemisia annua</i>	Leaves/Twigs	Leaves/crude extracts	Erythrocytic	0.51, 0.52, and 1.11 µg/mL for W, B, and R respectively	(Sankhuan et al., 2022)
	Leaves/tea infusion	Leaves/tea infusion	Pre- ythrocytic	85 % inhibition of parasite growth at 10 g/L	(Ashraf et al., 2022)
	Leaves/tea infusion	In vitro	Erythrocytic	100 % inhibition of parasite	(Ashraf et al., 2022)

			growth at 10 g/L	
Leaves and twigs/tea infusion	In vitro	Erythrocytic	Significant decrease in parasitemia at 5 g/L	(Snider & Weathers, 2021)
Leaves and twigs/tea infusion	In vitro	Gametocytes	Significant decrease in gametocytaemia at 5 g/L	(Snider & Weathers, 2021)
Leaves/tea infusion	In vitro	Erythrocytic	7.21 µg artemisinin/L	(Gruessner & Weathers, 2021)
Leaves/DCM	In vitro	Erythrocytic	3.79 µg artemisinin/L	(Gruessner & Weathers, 2021)
Leaves/Methanol	In vitro	Erythrocytic	3.00 artemisinin/L	(Gruessner & Weathers, 2021)
Leaves/aqueous	In vitro	Erythrocytic	4.95 nM	(Mojarab et al., 2014)
Leaves/ethanolic	In vivo	Erythrocytic	80 % inhibition at 20 mg/kg artemisinin per day for 5 days.	(Mojarab et al., 2016)
Leaves/ethanolic	In vitro	Erythrocytic	3.27 nM	(Mojarab et al., 2016)
Leaves/hexane	In vivo	Erythrocytic	26 % suppression of parasitemia after 4	(Mojarab et al., 2016)

				days of treatment at 75 mg/kg/day of extract	
<i>Artemisia aucheri</i>	Leaves/tea infusion	In vitro	Erythrocytic	0.095 µg/mL	(Papa et al., 2013)
	Leaves/tea infusion	In vitro	Erythrocytic	1.11 µg/mL and 0.88 µg/mL for CQ- sensitive and CQ- resistant strains, respectively	(De Donno et al., 2012)
	Leaves/DCM	In vivo	Erythrocytic	83.28 % inhibition at a dose of 200 mg/kg body weight	(Abdou et al., 2922)
	Leaves/tea	Clinical trial	Erythrocytic	92 % inhibition of parasitemia at 5 g/L	(Weathers, Elfawal, et al., 2014)
	Leaves/Acetone	In vitro	Gametocidal	< 10 µg/mL	(P. Moyo et al., 2016)
	Leaves/DCM	In vitro	Erythrocytic	3.04 µg/mL	(De Donno et al., 2012)
	Leaves/DCM	In vitro	Erythrocytic	1.00 mg/mL	(Afshar et al., 2011a)
	Aerial part/DCM	In vitro	Erythrocytic	1.95 mg/mL	(Mojarrab et al., 2014)

<i>Artemisia biennis</i>	Aerial part/DCM	In vitro	Erythrocytic	5.2 µg/mL	(Ortet et al., 2010)
	Leaves/DCM	In vitro	Erythrocytic	0.78 mg/mL	(Afshar et al., 2011b)
<i>Artemisia gorgonum</i>	Aerial part/EtOH	In vitro	Erythrocytic	2.64 mg/mL	(Mojarrab et al., 2016)
	Leaves/Hexane	In vitro	Erythrocytic	4.40 µg/mL	(Tasdemir et al., 2015)
<i>Artemisia indica</i>	Aerial part/Combination of EtOH, MeOH, PE (1:1:1)	In vivo	Erythrocytic	-	(Rustaiyan et al., 2009)
	Leaves/CF	In vitro	Erythrocytic	7.09 µg/mL	(Panda et al., 2018)
	Leaves/PE	In vitro	Erythrocytic	10.24 µg/mL	(Panda et al., 2018)
	Leaves/Hexane	In vitro	Erythrocytic	9.88 µg/mL	(Panda et al., 2018)
	Leaves/MeOH	In vitro	Erythrocytic	5.76 µg/mL	(Panda et al., 2018)
	Leaves/EtOH	In vitro	Erythrocytic	11.37 µg/mL	(Panda et al., 2018)
	Leaves/EtOAC	In vitro	Erythrocytic	1.35 mg/mL	(Mojarrab et al., 2015)

<i>judaica</i>	Leaves/DCM	In vitro	Erythrocytic	9.02 mg/mL	(Mojarrab et al., 2015)
<i>Artemisia roxburghiana</i>	Leaves/DCM	In vitro	Erythrocytic	1.93 mg/mL	(Mojarrab et al., 2015)
<i>A. scoparia</i>	Leaves/DCM	In vitro	Erythrocytic	0.78 mg/mL	(Afshar et al., 2011)
<i>Artemisia zbera</i>	Leaves/PE	In vitro	Erythrocytic	2.88 mg/mL	(Mojarrab et al., 2015)
<i>Artemisia icigera</i>	Leaves/DCM	In vitro	Erythrocytic	1.00 mg/mL	(Afshar et al., 2011)
<i>Artemisia turanica</i>	Leaves/DCM	In vitro	Erythrocytic	0.92 mg/mL	(Mojarrab et al., 2015)
<i>Artemisia turcomanica</i>	Aerial part/Methanol	In vivo	Erythrocytic	82.40 % inhibition at 500 mg/kg	(Adugna et al., 2014)
	Leaves/DCM	In vitro	Erythrocytic	4.90 µg/mL	(Tasdemir et al., 2015)
	Leaves/H ₂ O	In vitro	Erythrocytic	20 µg/mL	
	Leaves/Acetone	In vitro	Erythrocytic	1.9 µg/mL	
	Aerial part/Ethanol	In vivo	Erythrocytic	87.30 % inhibition at 1000 mg/kg	(Bamunuarachchi et al., 2013)
<i>Artemisia vulgaris</i>	Leaves/Combination of MeOH, PE, water (1:1:1)	In vivo	Erythrocytic	65.16 % inhibition of parasitemia at 500 mg/kg	(Kodippili et al., 2011)

MeOH = methanol; EtOH = ethanol; CF = chloroform; PE = petroleum ether; DCM = dichloromethane; EtOAc = ethyl acetate; W = crude extract prepared from *A. annua* grown under white LED light (445 and 554 nm); B = crude extract prepared from *A. annua* grown under blue LED light (445nm); R = crude extract prepared from *A. annua* grown under red LED light (660 nm); CQ = chloroquine.

Several clinical trials of *A. annua* either as infusions or as a whole-plant powder taken in the form of a capsule have also been reported, showing the effectiveness of this plant as a traditional remedy against malaria (Weathers et al., 2014; Gruessner et al., 2021). For example, a clinical study was conducted in the Democratic Republic of Congo using an infusion of high artemisinin (0.5–0.75%) cultivar at two doses (5 g of herb in 1 L of water and 9 g/L) in separate groups, and it indicated 77% and 70% cure rates, respectively, after seven days of treatment (Blanke et al., 2008). However, there were high levels of recrudescence (Mueller et al., 2004). Blanke et al. (2008), also reported a 70% cure rate with *A. annua* tea following a 7-day treatment, with a high level of recrudescence after day 28 in a small, randomized, double-blinded trial. The high level of recrudescence could be due to the rapid clearance of artemisinin, as it is metabolized by cytochrome P450 (CYPs) enzymes. Cytochrome P450 (CYPs) is a superfamily of hemeprotein enzymes present in the livers of humans, whose function is to metabolize xenobiotics. These enzymes are involved in the first-pass effect of many drugs, including artemisinin, which is actively metabolized by CYP2B6 and CYP3A4 enzymes (Kanne et al., 2022). Medicinal plants and or pure molecules with safety profiles that can inhibit cytochrome P450 enzymes, especially CYP2B6 and CYP3A4, which are responsible for the metabolism of artemisinin, can be utilized as a traditional remedy or as modern therapy in combination with artemisinin, respectively, to fight malaria. In an *in vitro* investigation of the effect of an infusion of *A. afra* and *A. annua* on human hepatic CYP2B6 and CYP3A4 enzymes, both plant extracts inhibited the activity of both enzymes (Afshar et al., 2011). It was also shown that *A. annua* is a potent inhibitor of CYP3A4 (Czechowski et al., 2018). This could be due to the presence of molecules other than artemisinin within the plant matrix. Despite it being devoid of artemisinin or having a negligible artemisinin content (Karl et al., 2011; Liu et al., 2009). *A. afra* has equally demonstrated good anti-plasmodial properties both when it is used in infusions or in crude extracts *in vitro*, *in vivo* and in clinical studies (Table 2). *A. afra* is native to South Africa and has a long history of use as a traditional remedy against coughs, colds, fever, and malaria (van Wyk, 2011). Infusions of *A. afra* are used to treat malaria traditionally (Liu et al., 2009). Crude extracts of *A. afra* (ethanol, hexane, and dichloromethane extracts) collected from 5 different countries in Africa demonstrated anti-plasmodial activity against

chloroquine-resistant *P. falciparum* strains (Kanne et al., 2019). Report on the antimalarial activities of *A. afra* both *in vitro*, and *in vivo* as well as clinical studies has been reviewed by du Toi and van der Kooy (du Toi and van der Kooy, 2019). However, there are recent investigations on the anti-plasmodial activities of tea infusions of *A. afra in vitro*. For example, infusions of *A. afra* cultivars with traces of artemisinin (19 nM artemisinin) and without detected levels of artemisinin demonstrated activity against the early-stage gametocytes associated with altered morphology of the gametes (Snider et al., 2020). This level of activity was shown to be higher in cultivars containing traces of artemisinin (Snider et al., 2020). In the same study, activity against the asexual forms of *P. falciparum* was reported (Snider et al., 2020). Furthermore, the *A. afra* tea infusion demonstrated antiplasmodial activity against the pre-erythrocytic and erythrocytic stages of *P. falciparum in vitro* in a dose-dependent manner (Mueller et al., 2009). Attention has been paid to *A. afra* and *A. annua* either in infusions or crude extracts for the investigation of anti-plasmodial properties over the years. However, there are several other *Artemisia spp.* for which a potential anti-malarial activity has been reported, either *in vitro* or *in vivo*. These include *Artemisia abyssinica*, *A. apiacea*, *A. gorgonum*, *A. absinthium*, *A. vulgaris*, *A. lancea*, *A. nilagirica*, *A. abrotanum*, *A. japonica*, *A. nilegarica*, *A. ciniformis*, *A. bienis*, *A. turanica*, *A. indica* and *A. maciverae*. An overview is given in **Table 2**.

During a malaria attack, xanthin oxidase (XO) is upregulated, leading to the production of reactive oxygen species (ROS), which trigger inflammation (Narsaria et al., 2011; Iwalokun et al., 2006; Guermonprez et al., 2013). Ty et al. (2019) showed a correlation between elevated plasma level activities of XO and cytokine production in malaria patients. Pro-inflammatory cytokines together with other cell mediators are responsible for the pathogenesis of inflammatory diseases. Medicinal plants and pure molecules that can inhibit the production of these pro-inflammatory cytokines can be utilized as a traditional remedy or as a modern therapy for the supportive treatment of these diseases. In addition to their anti-malarial properties, *A. annua* and *A. afra* have also been reported to exhibit anti-inflammatory properties. For instance, hot water extracts of *A. annua* prepared in a method similar to traditional ways demonstrated anti-inflammatory properties by modulating pro- and anti-inflammatory cytokines (de Magalhães et al., 2012; Song et al., 2020). At the molecular level, Abate et al. (2021) confirm the anti-

inflammatory potential of leaf extracts of *A. annua* with hydroethanol for the inhibition of TNF- α gene expression. Additionally, the anti-inflammatory potential of *A. afra* has been shown to significantly inhibit the production of nitric oxide (NO) and interleukin 6 (IL-6) in a dose-dependent fashion in several investigations (Muleya et al., 2014). There are other species of medicinal plants (**Figure 16**) that belong to the genus *Artemisia* with reported anti-inflammatory potentials, including *Artemisia vulgaris*, *A. absinthium*, *A. apiacea*, *A. dracunculus*, *A. campestris*, *A. vestita*, *A. inculta*, *A. A. montana*, *A. scoporia*, *A. vulgaris*, *A. sieversiana*, *A. frigida*, *A. copa*, *A. argyi* and *A. princeps*.



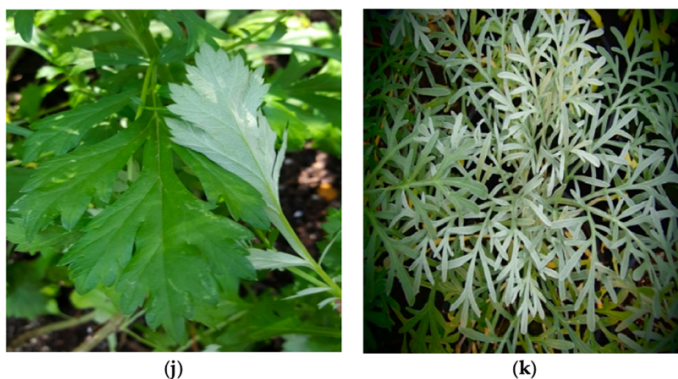


Figure 16. Field pictures of *Artemisia* spp. with reported anti-plasmodial properties: (a) *A. annua*, (b) *A. afra*, (c) *A. absinthium*, (d) *A. vulgaris*, (e) *A. indica*, (f) *A. lancea*, (g) *A. japonica*, (h) *A. abrotanum*, (i) *A. bienis*, (j) *A. nilagirica* and (k) *A. gorgonum*.

3.4. Anti-Malarial Properties, Anti-Inflammatory and Immunomodulating Effects of Secondary Metabolites

Following the isolation of almost 600 and 400 secondary metabolites from *A. annua* (Brown et al., 2010) and *A. afra* respectively (Liu et al., 2009), both plant species have served as a reservoir for bioactive molecules against different ailments, including malaria. Some of the isolated metabolites have been shown to have a direct anti-malarial property, while others act by increasing the bioavailability of active components against malaria. Artemisinin is one of the potent molecules with direct anti-malarial activity. However, following the emergence of resistance by the parasite against artemisinin, recent investigations on the biological activity of other secondary metabolites against malaria in the quest for the discovery of new molecules have gained attention in recent decades. The presence of these metabolites could potentialize artemisinin or reduce the risk of developing resistance. In this context, the presence of flavonoids may be a relevant key factor, broadening the pharmacological properties of Artemisia-based preparations. For instance, in an *in vitro* investigation of quercetin against malaria parasites, the authors showed that quercetin has moderate, direct anti-plasmodial activity, with an IC_{50} value of $19.31 \pm 1.26 \mu M$, and a high selectivity index (Ali et al., 2021). In the same study, anti-malarial properties of quercetin in *P. berghei*-infected mice demonstrated that it performs chemo-suppressive activity in a dose-dependent manner. Jansen et al. (2017), further confirmed the moderate anti-plasmodial properties of quercetin *in vitro*, with an IC_{50}

value of 9.5 µg/mL (31.4 µM). Additionally, three flavonoids; rutin, rhamnetin and quercetin, alongside derivatives of quercetin, inhibited the growth of laboratory strains and field isolates of *P. falciparum* in an *in vitro* investigation (Ganesh et al., 2012). Using an experimental set-up to determine the anti-malarial activity of flavonoids from *A. annua*, Willcox, et al. (2009) also showed that chrysosplenetin, chrysosplenol-D, cirsilineol and artemetin have direct anti-plasmodial activities *in vitro* (with an IC₅₀ range of 26–65 µmol/L) and that these molecules could also potentiate the effect of artemisinin on *P. falciparum*. Artenuic acid and artenuin B were also shown to have weak direct anti-malarial activity (Yin et al., 2008). In an *in vivo* study conducted to assess the anti-malarial properties of apigenin, a flavonoid common to both *A. afra* and *A. annua*, this compound was shown to significantly suppress the growth of *P. berghei* in infected mice in a dose-dependent way (Amiri et al., 2018). In addition, Fallatah and Georges, (2017) reported that active efflux of glutathione from mature erythrocytes was induced by apigenin through an erythrocytes ATP-binding cassette subfamily C member 1 (ABCC1)-mediated mechanism. This effect may lead to oxidative stress in *P. falciparum*-infected erythrocytes, and therefore, the inhibition of parasite proliferation, as confirmed in an *in vivo* study by Amiri et al. (2018). Additionally, kaempferol (a flavonoid) was shown to inhibit the growth of *P. berghei* in a dose-dependent manner (Somsak et al., 2018). These researchers could show that kaempferol has a curative, prophylactic and chemo-suppressive effect on malaria *in vivo*. However, Jansen et al. (2017), reported a lack of anti-plasmodial properties of kaempferol *in vitro*, although the researchers isolated the molecule from medicinal plants other than those of the Asteraceae family. Kaempferol also acts as an inhibitor of glycogen synthase kinase-3 (GSK3) of the malaria parasite (Sok et al., 2015); hence, blocking erythrocyte invasion by the parasite. Barlianna et al. (2014) confirmed the moderate anti-plasmodial properties of a derivative of kaempferol (kaempferol-3-O-rhamnoside) in *P. falciparum*, with a reported IC₅₀ value of 106 µM. **Figure 17-20** summarizes structures of compounds with direct or indirect anti-malaria properties.

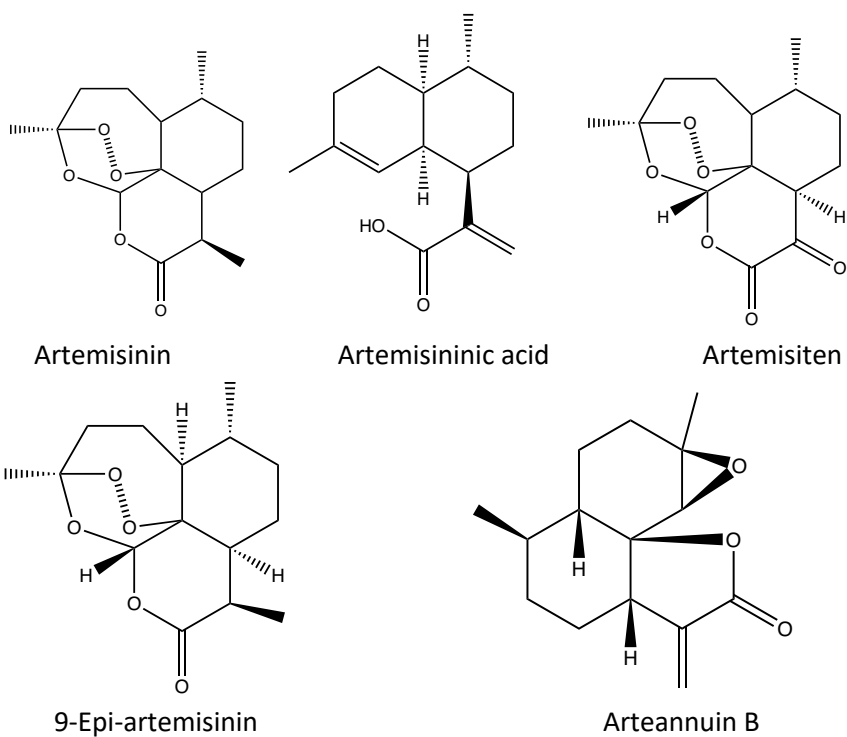


Figure 17: Sesquiterpenes with direct anti-plasmodial activity

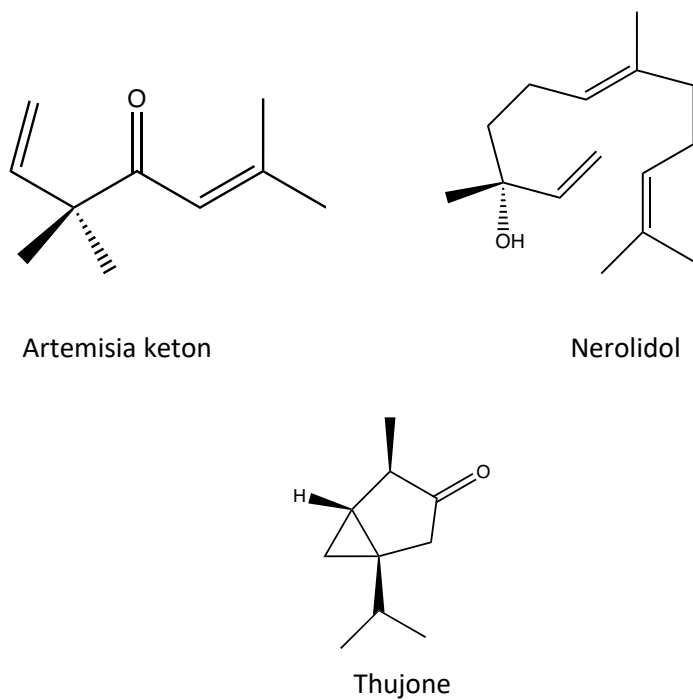


Figure 18: Monoterpenes with direct anti-plasmodial activity.

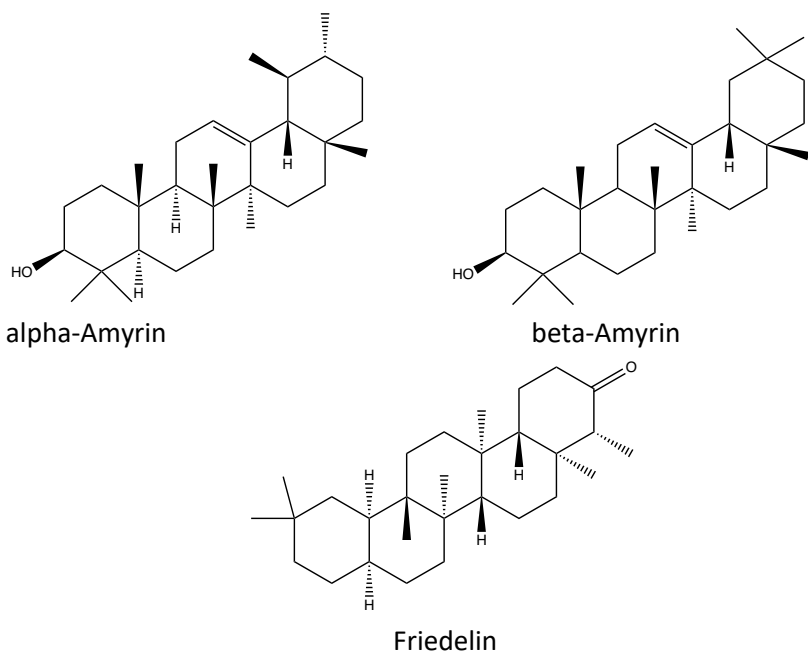
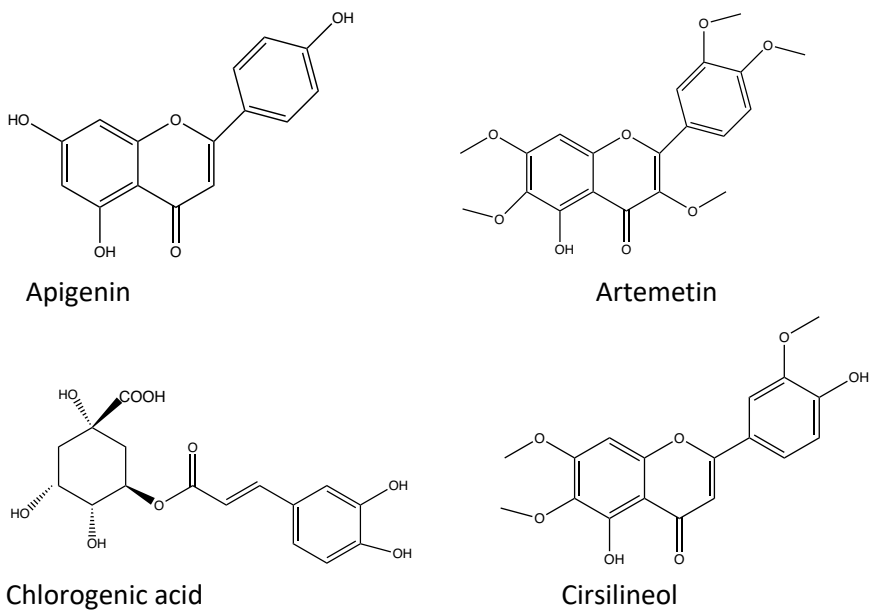
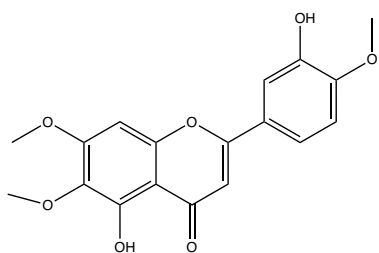
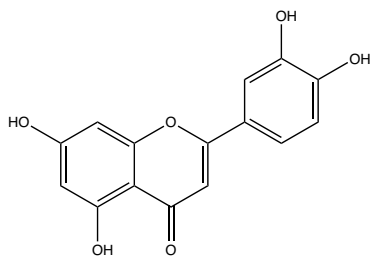


Figure 19: Diterpenes with direct anti-plasmodial activity

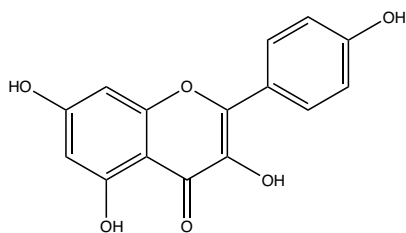




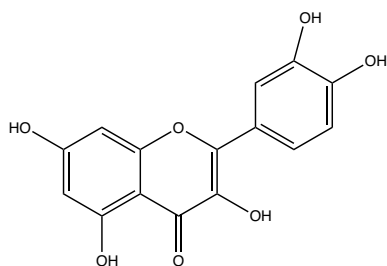
Eupatorin



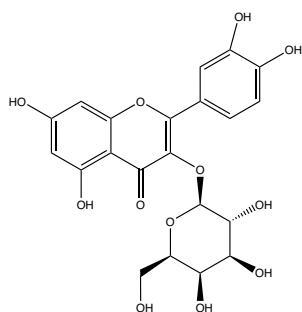
Luteolin



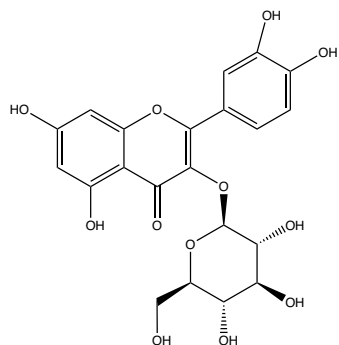
Kaempferol



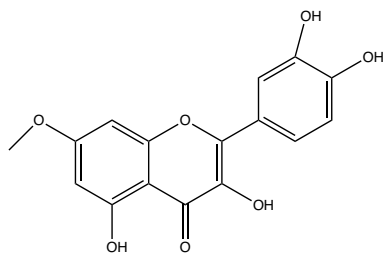
Quercetin



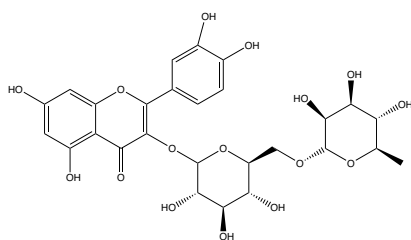
Quercetin-3-galactoside



Quercetin-3-glucoside



Rhamnetin



Rutin

Figure 20: Polyphenols with direct anti-plasmodial activity

While some components show a direct anti-malarial activity, others may act indirectly by synergistically increasing the bioavailability of active compounds. One mechanism by which this may be achieved is through the inhibition of cytochrome P450 enzymes, which are involved in the rapid metabolism of active compounds and have an immunomodulating effect. In an *in vivo* study carried out by [Li et al. \(2018\)](#) to investigate the effect of the interaction between artemisinin, artemisinin B, artemisinin acid and scopoletin on *P. yoelii*-infected mice, the researchers showed an increase in the level of exposure of artemisinin, which enhanced the mean plasma concentration and led to improved anti-malarial activity. In their findings, the potency of their combination (four-compound therapy) was almost four times higher compared to that of pure artemisinin. Artemisinin B, artemisinin acid and scopoletin were believed to have improved the bioavailability of artemisinin ([Chen et al., 2014](#)), which is highly metabolized by CYP450 enzymes, including CYP3A4 ([Chen et al., 2014](#)). Additionally, artemisinin B has been demonstrated to be a potent inhibitor of CYP3A4 ([Nibret et al., 2010](#)), and this effect could enhance the mean plasma concentration of artemisinin, leading to improved bioavailability, and therefore, an increase in activity. Thujone, norilodol and artemisia ketone, on the other hand, have been reported to be potent inhibitors of CYP2B6 ([Nibret et al., 2010](#); [Abass et al., 2010](#)). Several of these compounds with direct or indirect anti-malarial properties have also shown anti-inflammatory properties. In addition to its anti-malarial properties described above, kaempferol also has anti-inflammatory properties ([Devi et al., 2015](#)). In an investigation carried out by [Kim et al. \(2014\)](#) on the anti-inflammatory properties of artemisinin extracted from *A. annua* using different solvents, artemisinin was also found to have an inhibitory effect on lipopolysaccharide-induced nitric oxide (NO), prostaglandins and proinflammatory cytokine production, such as IL-1 β , IL-6 and IL-10. Additionally, artemisinin was found to down regulate pro-inflammatory cytokines, and hence, ameliorate inflammation ([Huai et al., 2020](#)) in an *in vivo* investigation. Furthermore, artemisinin was found to be an inhibitor of TNF- α expression ([Abass et al., 2010](#)); hence, this demonstrates its anti-inflammatory potential at the molecular scale. Additionally, artemisinin B isolated from *A. annua* has a strong inhibitory effect on the production of cytokines, such as IL-1 β , IL-6 and TNF- α ([Cai et al., 2017](#)). This could further explain the anti-inflammatory potential of *A. annua*. Table 3 summarizes

compounds isolated from *A. annua* and *A. afra* with direct or indirect anti-plasmodial and anti-inflammatory properties.

Table 3. Secondary metabolites with direct and indirect-antimalaria properties.

N	Compound	Biological Effect	Pharmacodynamics	Pharmacokinetics	Reference
1	α -Amaryn	-Immune-modulating -Direct antimalarial properties	N	N	(Kriel, 2010)
2	β -Amyrin	-Immune-modulating -Direct antimalarial	N	N	(Kriel, 2010)
3	Apigenin	- Direct antimalarial properties	-Efflux of GS from mature erythrocyte	N	(Amiri et al., 2018)
4	Arteanoides	-inhibition of TNF- α production -Anti-inflammatory	-Inhibition of PGE2 -Inhibition of NO production	N	(Qin et al., 2018)
5	Artemisinic acid	-Weak antimalarial	N	N	(Suberu et al., 2013)
6	Artemetin	-Weak antimalaria	N	N	(Willcox, 2009)
7	Artemisinin (Qinghaosu)	-Direct-antimalarial properties - Anti-inflammatory	-Inducer of CYP3A4 -Inhibition of TNF- α gene expression.	-Metabolized by 'P3A4, CYP2B6	(Abate et al., 2021; Xiong et al., 2010)

		-Inhibition of pro-inflammatory cytokines			
8	Artemisitene	-Direct-antimalarial properties	N	N	(Suberu et al., 2013)
9	Artennuin B	-Weak antimalarial -Anti-inflammatory -Inhibition of IL-1 β , IL-6, TNF- α production	- Inhibitor of CYP3A4	-	(Cai et al., 2017; Chen et al., 2014)
10	Artennuic acid	-Weak antimalarial -Anti-inflammatory	N	N	(Cheng et al., 2012)
11	Artemisia ketone	-Direct antimalarial properties	- Inhibition of CYP2A6 CYP2B6. -Inhibition of hemozoin crystallization	N	(Nibret & Wink, 2010)
12	Casticin	Indirect antimalarial	N	N	(Willcox, 2009)
13	Chlorogenic acid	-Anti-inflammatory -Inhibition of IL-8 and IL-6	N	N	(Melillo De Magalhães et al., 2012)
14	Chrysosplenetin	-Weak antimalarial	N	N	(Willcox, 2009)

15	Chrysosplenol-D	-Weak antimalaria	N	N	(Willcox, 2009)
16	Cirsilineol	-Weak antimalaria	N	N	(Willcox, 2009)
17	1 α ,4 α -Dihydroxybishopsolicepolide	-Direct-Antimalarial activity	N	N	(Moyo et al., 2019)
18	9-Epi-artemisinin	-Direct-antimalarial properties	N	N	(Suberu et al., 2013)
19	Eupalitin	-Anti-inflammatory	N	N	(Min et al., 2009)
20	Eupatorin	-Weak antimalarial	N	N	(Willcox, 2009)
21	Friedelin	Immune-modulating, Antimalarial activity	N	N	(Kriel, 2010)
22	Jasceolidin	-Anti-inflammatory	N	N	(Min et al., 2009)
23	Kaempferol	-Direct antimalarial properties -Anti-inflammatory	-Inhibition of GSK3 β	N	(Somsak et al., 2018)

24	Luteolin	-Direct antimalarial properties	N	N	(Cheng et al., 2012; Ferreira et al., 2005)
25	Mono caffeoylquinic acid	-Indirect antimalarial	N	N	(Suberu et al., 2013)
26	Nerolidol	-Direct-antimalarial properties -Anti-inflammatory	- Inhibition of CYP2A6 CYP2B6 -Inhibition of hemozoin crystallization	N	(Nibret & Wink, 2010)
27	Quercetin	-Direct antimalarial properties - Anti-inflammatory	-Modulation of pro/Anti-inflammatory cytokines -Inhibitor of thioredoxin reductase -Inhibition of GSK3 β	N	(Ali et al., 2021)
28	Quercetin-3- galactoside	- Direct antimalarial properties.	N	N	(De Magalhães et al., 2012)
29	Quercetin-3-glucoside	- Direct antimalarial properties	N	N	(Ganesh et al., 2012)
30	Rhamnetin	- Direct antimalarial properties	N	N	(Ganesh et al., 2012)

31	Rosmarinic acid	-Indirect antimalarial -Anti-inflammatory -inhibition of IL-8 and IL-6	N	N	(De Magalhães et al., 2012)
32	Rutin	- Direct antimalarial properties	N	N	(Ganesh et al., 2012)
33	Scopoletin	-Anti-inflammatory. -Indirect antimalarial	- Modulation of Pro/Anti-inflammatory cytokines	N	(Cheng et al., 2012; Malik et al., 2011)
34	Thujone	Direct-antimalarial properties	-Inhibition of CYP2A6 CYP2B6.	N	(Abass et al., 2011; Nibret & Wink, 2010)
35	Tri-caffeoylquinic acids,	-Indirect antimalarial	N	N	(Suberu et al., 2013)
36	Yomogiartemin	-Direct-Antimalarial activity	N	N	(Moyo et al., 2019)

N = No report. Compounds 1, 3, 4, 5, 6, 7, 8, 25, 32, 33, 34, 35, 30, 31, 27 and 28 isolated from *A. annua*. Compounds 2, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 26 and 29 isolated from both. Compounds 36 and 37 isolated from *A. afra*.

Quercetin showed a GSK3 β -mediated cytokine-modulating effect by regulating pro- and anti-inflammatory cytokine levels in *P. berghei*-infected mice (Ali et al., 2021). In this investigation, quercetin was also found to significantly reduce the levels of TNF- α and IFN- γ and raise the levels of IL-10 and IL-4 (Ali et al., 2021). In a study carried out by Min et al. (2009) to determine the anti-inflammatory effect of eupatilin and jasceosidin on carrageenan-induced inflammation in mice, both molecules were found to inhibit the expression and activation of cyclooxygenase (COX)-2 and nuclear factor kappa β , respectively. The same authors further reported a reduction in TNF- α and IL-1 β , as well as prostaglandin E2 (PGE2), levels in mice by eupatilin and jasceosidin. The researchers isolated these molecules from *Artemisia princeps*; however, these molecules have also been isolated from *A. annua* and *A. afra*, and they are also present in other *Artemisia* species. Qin et al. (2018), also reported the anti-inflammatory properties of two newly rearranged heterodimers of cadinene sesquiterpene and one phenylpropanoid unit (Arteanoids B and C).

3.5. Pharmacokinetics and Bioavailability of Secondary Metabolites of *A. annua* and *A. afra*

While pharmacokinetics describes the absorption, distribution, metabolism, and elimination of a drug or pharmakon, bioavailability, on the other hand, describes the fraction of the absorbed drug that reaches the systemic circulation, and it varies between compounds, both of which influence the observed therapeutic or biological responses (Ritter et al., 2018). The pharmacokinetics and bioavailability of artemisinin and derivatives have been more extensively studied either *in vitro*, *in vivo* or in clinical studies than other metabolites in this plant species. This is probably due to its excellent anti-malarial properties, and it is also a drug that is used in therapy. Several pharmacokinetic studies have been conducted on artemisinin administered as a single compound or in a plant matrix, such as in whole-plant powder or as an infusion, both of which may influence the pharmacokinetics of artemisinin. For example, in a pharmacokinetic study of artemisinin following the oral administration of a traditional preparation of *A. annua*, it was found that artemisinin is absorbed faster from herbal tea preparations than it is from capsules (from 30 min to 2.3h) (Räth et al., 2004). However, both formulations had a similar bioavailability. Desrosiers et al. (2020) reported increases in the serum levels of artemisinin when it is administered as a whole

plant as compared to that when the capsule of artemisinin is administered, indicating that the plant matrix has a positive impact on the bioavailability of artemisinin. In a multicomponent assessment of the pharmacokinetics parameters of *A. annua* in rats, [Fu et al. \(2022\)](#) showed that scopoline, scopoletin, rutin, chrysosplenol D, casticin and three sesquiterpenes (arteannuin B, dihydroartemisinic acid and artemisinic acid) were detected in rat's plasma post-oral administration. In the same study, the authors concluded that chrysosplenol D and casticin were rapidly absorbed with shorter half-lives ($t_{1/2}$, 2.68 ± 3.62 h and 0.33 ± 0.07 h, respectively) as compared to the speed of the absorption of scopoletin, which has a longer half-life ($t_{1/2}$, 6.53 ± 1.84 h). The long half-life of scopoletin *in vivo* could explain its effect on the modulation of cytochrome P450 enzymes and the modulation of pro- and anti-inflammatory cytokines. In the same study, scopoletin, casticin, artemisinin and chrysosplenol D were metabolized by phase II enzymes. Following the intravenous administration of eupatilin in mice, it was found that eupatilin was poorly adsorbed due to its low systemic exposure ([Wang et al., 2018](#)). Eupatilin was rapidly metabolized by phase II enzyme into eupatilin-7-glucuronide (E-7-G), and both molecules were distributed in the intestine, liver, and kidneys. It was confirmed by the authors that eupatilin has a shorter half-life ($t_{1/2}$, 0.29 h) as compared to that of its metabolite, E-7-G, which has a longer half-life ($t_{1/2}$, 4.15 h) and might be responsible for the potency of eupatilin *in vivo*. Poor absorption and a high biliary elimination rate limit the bioavailability of quercetin. However, the absorption of quercetin depends on the chemical structure of the molecule. Quercetin aglycon is mainly absorbed in the small intestine and stomach ([Crespy et al., 2001](#)), while quercetin rutinoside is absorbed in the colon ([Huai et al., 2020](#)). [Erlund et al. \(2000\)](#) also confirmed the absorption of quercetin aglycon in the gastrointestinal tract in clinical studies. The results from an *in vitro* incubation of human small microsomes with quercetin aglycon show that it is highly metabolized by phase II enzymes in the small intestine ([van der Woude et al., 2004](#)). Apigenin, a natural flavonoid common in the *Asteraceae* family and also found in other plant species, has a poor systemic availability due to its low solubility in water and lipids. After a single oral administration in rats, apigenin was found widely distributed throughout the body (liver, kidneys, RBCs, urine, and intestine) within 10 days post-administration, and it was excreted mainly in urine ([Gradolatto et al., 2004](#)).

Apigenin is extensively metabolized by phase I and phase II enzymes, making its bioavailability very low (Gradolatto et al., 2004; Tang et al., 2012). The results of the oral and intra-venous administration (400 mg/kg and 50 mg/kg, respectively) of casticin in rats showed rapid distribution and elimination with a longer half-life ($t_{1/2}$, 36.48 min $\pm$ 7.24) for the oral administration as compared to those of intravenous administration ($t_{1/2}$, 20.86 min $\pm$ 2.02) (Xu et al., 2012). The absolute bioavailability (45.5 $\pm$ 11.0%) was high, which is an important factor for its clinical application (Xu et al., 2012). Barve et al. (2009) reported a low bioavailability (approximately 2%) of kaempferol due to its extensive first pass-effect metabolism in rats after oral administration. In the same study, the plasma concentration of kaempferol indicated a rapid absorption (t_{max} , 1 – 2 h) with a half-life of 3 – 4 h and with a large distribution (8–12 L/h/kg) and a high rate of clearance (approximately 3 L/h/kg). Zabela et al. (2016) also reported the rapid clearance (4.40 – 6.44 L/h/kg) and extremely short half-life ($t_{1/2}$, 2.93 – 3.79 min) of kaempferol after intravenous administration in rats. In a study carried out to determine the effect of plant matrix on the uptake of luteolin from aqueous solutions of *A. afra*, luteolin aglycone and luteolin-7-O-glucoside were more rapidly absorbed than when they were administered as pure solutions in Caco-2 cells after 1 h exposure. These results suggest that plant matrix may have a positive effect on the bioavailability of the flavonoid and hence greater in vivo potency (Mukinda et al., 2010).

3.6. Toxicity of Crude Extracts of *A. annua* and *A. afra* and Their Secondary Metabolites

Pre-clinical toxicity is often required for new substances and new medicinal products, which contain herbs with no traditional history of use. According to the WHO, traditional use refers to documentary evidence that a substance has been used for a specific health related or medicinal purpose over three or more generations. The guidelines of the WHO state that if a herbal product has a long history of traditional use without signs of toxicity (harmful effects), it should not be restricted, and they maintain its stand that no pre-clinical toxicity investigation is required (World Health Organization, 2015). *Artemisia annua* and *A. afra* have a long history of use as a traditional remedy in Southeast Asia and South Africa, respectively, for the alleviation of fevers related to malaria, amongst other diseases, for generations. However, several toxicity studies (cytotoxicity, acute and chronic toxicity) have been conducted

to determine the safety of *A. annua* and *A. afra*, including a couple of their secondary metabolites. For example, [Motshudi et al. \(2021\)](#) showed that crude extracts of *A. afra* prepared using chloroform, ethanol and water were relatively non-toxic to vero cells, with LC_{50} values > 400 g/mL, as compared to that of the positive control (doxorubicin), which was cytotoxic, with an LC_{50} value of 57.83 ± 3.02 mg/mL. [Loggenberg et al. \(2022\)](#) also confirmed the non-cytotoxic effect of aqueous extract of *A. afra*, with $IC_{50} > 350$ μ g/mL. In the same study, *A. annua* was confirmed to be non-cytotoxic, with $IC_{50} > 550$ μ g/mL, to vero cells. In addition to *in vitro* toxicity assays used to assess the safety profiles of *A. annua* and *A. afra*, several *in vivo* studies (acute and chronic studies) have been conducted to confirm the claims observed in *in vitro* studies. For instance, in an investigation carried out by [Mekenon et al. \(2020\)](#) to evaluate the toxicity of aqueous extracts of *A. afra* leaves on brain, heart and suprarenal glands in Swiss albino mice via an acute and sub-acute test, the researchers showed that leaves extract of *A. afra* is relatively safe for mice, with an $LD_{50} > 5000$ mg/kg body weight. In the sub-acute study, the microscopic examination of brain, heart and suprarenal glands showed no signs of toxicity in all the treatment groups. [Kane et al. \(2019\)](#) also confirmed the safety of *A. afra* leave extracts *in vivo* by determining its pharmacotoxicological effects after acute oral administration in mice at doses of 1000, 2000 and 2500 mg/kg as per body weight. An $LD_{50} > 2500$ mg/kg body weight, no signs of toxicity and no effect on the levels of alanine transaminase (ALT) and aspartate transaminase (AST) were reported and compared to those of the control in the study. Additionally, following the oral administration of hydro-ethanolic plant extract of *A. annua* in Swiss mice, the results showed no lethality or toxic reactions, with $LD_{50} > 5000$ mg/kg body weight ([Siddiqui et al., 2018](#)). The absence of toxicity symptoms suggests that *A. annua* is non-toxic and is well tolerated ([Siddiqui et al., 2018](#)). According to the classification of Loomis and Haye, substances with LD_{50} values between 500 and 5000 and between 5000 and 15,000 mg/kg body weight are regarded as being slightly toxic and practically non-toxic, respectively. The safety profiles of *A. afra* was further confirmed by [Mukinda et al. \(2010\)](#) by using an aqueous extract of *A. afra* in acute and chronic tests on mice and rats. The pharmacotoxicological effects of the extract were determined after intraperitoneal and oral administration. The researchers reported LD_{50} values of 2450 and 8960 mg/kg body weight for intraperitoneal and oral administration, respectively.

Both methods of administration were safe as the LD₅₀ values fell within the cut-off mark, following the guidelines of Loomis and Haye ([Loomis and Hayes, 1996](#)). After a chronic investigation of rats administered oral doses of *A. afra* extract (0.1 or 1g/kg/day), the rats survived 3 months of dosing, and LD₅₀ > 1000 mg/kg body weight was reported. There were no significant changes in their general behavior and hematological and biochemical parameters experienced, except for a transient decrease in AST activity level. No significant changes were observed in organ weights, and the histopathological results showed a normal profile, suggesting no morphological alterations. The above findings clearly support the use of *A. annua* and *A. Afra* as a traditional remedy in the fight against infections. Several compounds isolated from *A. annua* and *A. afra* have demonstrated good biological activities against different ailments, including malaria, with several toxicity studies having been conducted to determine their safety profiles. For example, in an investigation to determine the cytotoxic effect of dietary flavones on normal trout liver cell lines, the authors of ([Tsuji et al., 2008](#)) showed that while methylated flavones (5,7-Dimethoxyflavone and 3',4'-dime-thoxyflavone at concentration 25 μ M) are non-cytotoxic even at higher concentrations after 24 h of exposure, the hydroxylated flavones (Chrysin, quercetin, apigenin and luteolin), especially chrysin, were cytotoxic, with a reported IC₅₀ value of 2 μ M. However, in a sub-chronic toxicity study carried out by [Yao et al. \(2019\)](#), following the daily oral administration of chrysin (1000 mg/kg), the compound significantly decrease body weight, liver weight was increased, there were significant alteration in the hematology, and an LD₅₀ value of 4350 mg/kg was reported. The results from a sub-chronic toxicity study of quercetin examined in male and female CD2F1 mice administered at 62, 125 and 250 mg/kg indicated that quercetin is safe to CD2F1 mice, as they showed no signs of toxicity, and no changes in hematological and histopathological parameters were observed ([Cunningham et al., 2022](#)). [Lucida et al. \(2019\)](#) also confirmed the safety of quercetin in an acute test, with an LD₅₀ value > 1600 mg/kg. Several studies have been conducted to investigate the toxicity of volatile components of *A. annua* and *A. afra*. For instance, in an investigation to evaluate the toxic effects of α -terpinene (a monoterpene common to both species) in the liver tissue of rats intraperitoneally administered at doses of 0.5, 0.75 and 1.0 mL/kg for 10 days. [Baldissera et al.\(2017\)](#) showed that α -terpinene induces

oxidative stress and has cytotoxic and genotoxic effects on the liver tissue, involving caspase activation. This was due to increased levels of reactive oxygen species, alanine aminotransferase, aspartate aminotransferase increased and glutathione S-transferase. There are several reviews that have detailed the toxicity of volatile components of both plants ([Abass et al., 2010](#); [Efferth and Kaina, 2010](#); [Wojtunik-Kulesza et al., 2022](#)).

3.7. Resistance to ACTs and Broad-Spectrum Pharmacology of Artemisia-Based Samples

With the persistence of malaria as a devastating disease with high burden and mortality rates, the WHO recommends “Artemisinin Combination Therapy (ACTs)” as the first line of treatment (artemether + lumefantrine, artesunate + amodiaquine, artesunate + mefloquine, dihydroartemisinin + piperaquine or artesunate + sulfadoxine–pyrimethamine) ([World health Organization, 2018](#)). However, there have been reported cases of resistance to ACTs, leading to treatment failures. Artemisinin resistance was documented in 2008 on the Thailand–Cambodia border after artesunate monotherapy ([Alker et al., 2007](#)). However, retrospective analysis indicated that artemisinin resistance likely emerged in 2001, before the widespread deployment of ACTs in Cambodia ([Dondrop et al., 2009](#)). The mechanism of resistance to artemisinin drugs is associated with point mutations in the Kelch 13 (K13) propeller gene. In Cameroon and other African countries (Central African Republic, Chad, Kenya, Madagascar, Malawi, Mali, Rwanda, etc.), non-synonymous K13 mutations have been reported, and the most frequent allele observed in Africa is A578S ([Isozumi et al., 2015](#)). Further studies revealed that four mutations (Y493H, R539T, I543T and C580Y) of the K13-propeller gene in *P. falciparum* are associated with artemisinin resistance ([Isozumi et al., 2015](#)). The emergence of resistance to ACTs has prompted the search for new strategies to fight against the disease. The optimal therapy for the management of the disease would consist of one that will target both gametocidal and clinical forms of the parasite. This will enable them to break the transmission cycle, and hence, it's possible eradication. The isolation of gametocidal compounds from *A. afra* ([Moyo et al., 2019](#)) and *in vitro* and *in vivo* investigations of the anti-plasmodial activity against the erythrocytic forms of malaria parasite ([Snider et al., 2020](#); [Mueller et al., 2004](#); [Zime-Diawara et al., 2015](#); [Liu et al., 2010](#)) as well as clinical results from trial studies, indicate that *A. afra* may be an important contributor in the fight

against malaria. The presence of the plant matrix, as reported, may play an important part, as it improves the pharmacokinetics of molecules with direct anti-plasmodial properties and those with indirect anti-plasmodial properties (Mukinda et al., 2010). This could be explained by the fact that other molecules present in the plant may limit the rate of the metabolism of compounds with direct anti-plasmodial properties by inhibiting the action of cytochrome P450 enzymes responsible for the metabolism of these compounds. This further demonstrates the potential of *A. afra* in the fight against malaria. Additionally, new drug combinations with molecules that could enhance the bioavailability of artemisinin, together with compounds that have direct anti-malarial properties, including those showing gametocidal effects, could function best in eliminating the disease. As an example, a drug combination or a standardized plant extract containing artemisinin (a potent anti-malarial molecule), artemunin B (a CYP3A4 inhibitor, but also showing a direct anti-malarial activity), quercetin (with direct anti-malarial activity and an inhibitor of thioredoxin reductase and anti-inflammatory), yomogiartemin (a gametocidal) and thujone (has a direct anti-malarial effect and a CYP2A6 and CYP2B6 inhibitor) could be of potential interest in treating malaria.

4. Conclusions

Based on *in vitro* and/or *in vivo* studies and limited clinical trials, there are more than twenty different species of *Artemisia* with the anti-malarial/anti-inflammatory properties investigated. Research over the years in the search for new molecules showing anti-malarial activities has been focused on erythrocytic forms of the malaria parasite, with limited work having been conducted on the pre-erythrocytic and gametocidal forms. In addition to artemisinin, several secondary metabolites present in different *Artemisia spp.* show direct anti-malarial activity. Other metabolites act indirectly by increasing the bioavailability of active components or by affecting other metabolic pathways that are important in sustaining the patient when they are fighting a malaria infection. A few of the isolated secondary metabolites with anti-plasmodial properties and immunomodulating effects, as well as anti-inflammatory properties, have been studied for their pharmacokinetics and pharmacodynamics properties in humans and their toxicological profiles. It would, therefore, be interesting to broaden the knowledge of *Artemisia*-based preparations by investigating their total pharmacological effectiveness

in clinical trials in relation to their chemical composition. The focus should, therefore, be put on investigating the presence of molecules in addition to artemisinin, resulting in a broad pharmacological activity and effectiveness during different stages of the malaria parasite, especially the gametocidal forms. This would enable the reduction of the link between human and insect vectors, and hence, reduce malaria transmission.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/metabo13050613/s1>. Table S1: Monoterpenes isolated from *A. annua* and *A. afra*. Table S2: Sesquiterpenes from *A. annua* and *A. afra*. Table S3: Polyphenols from *A. annua* and *A. afra*. Table S4: Triterpenes from *A. annua* and *A. afra*. Table S5: Coumarins from *A. annua* and *A. afra*. Table S6: Diterpenes from *A. annua* and *A. afra*. Table S7: Guaianolides from *A. annua* and *A. afra*. Table S8. Simple Aryl Ketones from *A. annua* and *A. afra*. Table S9: Glaucolides from *A. annua* and *A. afra*. Table S10: Long chain alkanes from *A. annua* and *A. afra*. Table S11: Other compounds from *A. annua* and *A. afra*. References cited in supplementary materials are [135–197].

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1.9 Context and Objectives

The research presented herein was part of an ARES-PRD-2020 research project whose major purpose was to eliminate and eradicate *Plasmodium* reservoirs in high transmission settings in Cameroon, utilising *Artemisia spp* as a proof-of-concept. Malaria, being a disease of public health importance with a high burden and mortality, has a devastating effect on the affected population. The approved use of Artemisinin-based Combination Therapy (ACTs) for the treatment of malaria face challenges due to the emergence and spread of resistance by the parasite, leading to treatment failure. Also, the use of ACT is limited in Low- and Middle-Income Countries (LMICs) such as Cameroon. Moreover, ACTs do not ensure a complete gametocyte clearance, the infective stage of *Plasmodium*. A major weakness of this control strategy is that they do not target asymptomatic *Plasmodium* carriers, who constitute the vast majority of the infected people in endemic regions and serve as reservoirs that perpetuate the disease transmission over time. Therefore, there is an urgent need to revise the current control strategy. In the ARES-PRD-2020 project, the focus was to target and empty human *Plasmodium* reservoirs to accelerate malaria elimination in high-transmission settings, using a couple of geographically distinct sites in a phase II clinical trial in Cameroon for a proof-of-concept.

Specifically, the research presented herein focused on developing, standardizing, and validating the antiplasmodial properties of crude extracts of *Artemisia spp*, as well as the selectivity and development of an active formulation for use in the above-mentioned clinical trial study.

The research began with a thorough literature review on the chemical composition and pharmacological properties of *A. afra* and *A. annua* and the objectives of the study segmented into various chapters;

- As presented in Chapter 2, we explore the phytochemical diversity of both plants and developed metabolite fingerprints that can be utilized for the authentication of these medicinal plants.
- After detailing the impact of geographical location and season of harvest, we focused on investigating the impact of post-harvest handling practices on the metabolite contents of both plants and correlated them to antiplasmodial property.
- In the subsequent chapters, the focus was to identify and isolate molecules responsible for the observed antiplasmodial activity,

including new gametocyte compounds from *A. afra*. Also, we investigated the synergistic interaction between *A. annua* and *A. afra* extracts.

- Additionally, we developed a formulation comprising *A. afra* and *Hibiscus sabdariffa* and validated its antiplasmodial properties both *in vitro* and *in vivo* and including toxicity studies. This herbal combination aimed to mask the bitterness of the active component (*A. afra*) while maintaining its efficacy and safety profile.
- Moreso, curiosity was how this plant extract interacts with *Plasmodium* parasites. Here, we predicted the antiplasmodial mode of action of extracts prepared from these medicinal plants through metabolomics studies, through a collaboration with Dr. Lucia Mamede and Pr. Manuel Llinás (Huck Center for Malaria Research, CmaR, Pennsylvania State University, USA).
- In the last chapter, we investigated the activity and safety profile of another medicinal plant (*Erigeron bonariensis*) of ethnopharmacological relevance used in the Cameroonian folk medicine for the management of malaria.

Chapter Two

Seasonal and geographical effect on the phytochemical composition of *Artemisia annua* and *Artemisia afra*: Antiplasmodial properties

2.1 Foreword

With the limitations faced by malaria control strategies especially the emergence and spread of resistance to the available malaria drugs, there have been increasing use of two well-known medicinal plants; *Artemisia afra* and *Artemisia annua* as culturally sound alternative therapeutics to manage malaria. This practice is mostly seen in malaria endemic regions such as Cameroon and several other African countries. These medicinal plants are mostly taken in the form of an infusion, decoction or as plant powders in capsulated forms. These plants are being cultivated in several regions in Cameroon like in other African countries, formulated as herbal tea and sold in local markets. While this practice generates incomes and improves the livelihood of local farmers, inconsistency in the phytochemical composition of these plants attributed to geographical origin of collection and season, leads to inconsistent efficacy of these plants in combating malaria. Additionally, the close morphological similarities that exist between these plants makes herbal tea authentication problematic. It was therefore important to explore the phytochemical diversity and the impact of both season and geographical origin of collection on the secondary metabolite composition and correlate to the observed antiparasmodial properties for optimal chemical composition and to ensure consistent efficacy. As described in this section, plant samples were collected from several regions and seasons and analysed using chromatographic techniques to obtain metabolite fingerprints.

2.2 Exploring the phytochemical diversity and antiplasmodial ptential of *A. annua* and *A. afra* from differen geographical locations in Cameroon.

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Exploring the Phytochemical Diversity and Anti-Plasmodial Potential of *Artemisia annua* and *Artemisia afra* from Different Geographical Locations in Cameroon

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Figure 21: Cover page of published article

Abstract

In Cameroon, like in other African countries, infusions of *Artemisia afra* and *Artemisia annua* are widely used for the management of health-related problems, including malaria. The secondary metabolite contents of medicinal plants vary between different

geographical regions and seasons, directly influencing their effectiveness in treating ailments. This study explores the phytochemical diversity and anti-plasmodial potential of *A. annua* and *A. afra* from distinct geographical locations within Cameroon, aiming to define the optimal chemical composition in terms of anti-plasmodial activity. Extracts were prepared from plants collected from diverse regions in Cameroon during both the rainy and dry seasons, and their metabolic contents were analyzed using Thin-Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), and Gas Chromatography (GC). Their anti-plasmodial potential was assessed on a chloroquine-sensitive 3D7 *Plasmodium falciparum* strain. Additionally, the environmental parameters of the collecting sites were retrieved from multispectral satellite imagery. The activity profiles of the samples were associated with their environment, with distinct phytochemical compositions observed for each sample based on its geographical origin and season. Traces of artemisinin were detected in some of the *A. afra* samples, but it was present in the *A. annua* samples at a significantly higher concentration, especially in the rainy season samples (highest concentration in the Adamawa region, at 8.9% m/m artemisinin in the dry extract). Both plants are active at different levels, with *A. annua* more active due to the presence of artemisinin and *A. afra* probably active due to the presence of polyphenols. Both season and geographical location influence both plants' metabolic contents and hence their antimalaria activity. These findings suggest that the selection of a suitable *Artemisia* sample for use as a potential antimalarial treatment should take into consideration its geographical origin and the period of collection.

Keywords: Artemisinin; malaria; *Artemisia annua*; *Artemisia afra*; *Plasmodium*.

I. Introduction

Malaria is a disease of public health importance caused by parasites of the *Plasmodium* family and transmitted by female Anopheles mosquitoes. In most countries where malaria is endemic, this disease disproportionately affects children under the age of five and pregnant women. Globally, over 250 million malaria cases and 608,000 deaths were reported in 2022 in 85 malaria-endemic countries, with an estimated additional 13.4 million increased cases between 2019 and 2021 (World Health Organization, 2022). Sub-Saharan African countries are the hardest-hit zones, with approximately 95% of the world's malaria burden and 96% of the death rate, with over 80% of these being children under the age of five and pregnant women (World Health Organization, 2022). According to WHO data, 70% of all malaria cases are concentrated in 11 countries, with Cameroon accounting for over 2.7 million cases (World Health Organization, 2022). This can be related to increasing antimalarial drug resistance to all of the chemotherapeutic measures recommended at present, including "Artemisinin-based Combination Therapy" (ACT) (World Health Organization, 2022). The situation is further aggravated not only by Histidine-Rich Protein-2 gene (HRP2 gene) deletion (Eyong et al., 2022) which makes a diagnosis problematic, but also by the widespread resistance of mosquito to insecticides, as well as the rapid spread of invasive vector species (*Anopheles stephensi*) from the Horn of Africa. The fight against this life-threatening disease is one of the goals of the World Health Organization's (WHO) Global Technical Strategy for malaria to reduce transmission and achieve a world free of malaria by 2030. Natural products have been investigated as potential medications for diseases including malaria (Newman and Cragg, 2020) and are used by 80% of the global population in primary health care (Barbara and Willem, 2024). *Artemisia afra* Jacq. Ex Willd and *Artemisia annua* L are annual herbs belonging to the Asteraceae family that have a long history of use against fevers related to malaria (van Wyk, 2011; Klayman, 1985). Due to their complex mixtures of different phytochemicals, both plant species have proven to be effective in the clinical management of diseases as traditional remedies. Several studies have been reported indicating the effectiveness of both plants against malaria. For instance, *in vitro* studies, including clinical investigations, have shown that *A. afra*, despite being devoid of artemisinin or having a negligible artemisinin content (Ashraf et al., 2022), can exhibit good anti-plasmodial

properties against erythrocytic forms (IC_{50} between 5 $\mu\text{g/mL}$ and 15 $\mu\text{g/mL}$) (Jansen et al., 2017), hepatic forms, and gametocidal stages of the malaria parasite (Ashraf et al., 2022; Snider et al., 2021; Liu et al., 2009; du Toit and van de Kooy, 2019; Moyo et al., 2019; Soni et al., 2022). The pharmacological properties of *A. afra* and *A. annua* were well documented in reviews by du Toit and van de Kooy (2019) and Soni et al. (2022), respectively. The isolation of artemisinin from *A. annua* (Jeremić et al., 1973) a potent antimalarial compound, has made the plant a subject of intensive phytochemical investigation in recent decades. Interestingly, phytochemical investigation has confirmed the isolation of almost 700 secondary metabolites from *A. annua* and almost 500 from *A. afra* (Shinyuy et al., 2023). The qualitative and quantitative contents of plant secondary metabolites are greatly influenced by temperature, humidity, soil type, soil micro-organisms, soil pH, and altitude (Yang et al., 2018). This has a direct influence on a medicinal plant's ability to effectively treat a well-diagnosed disease. In Cameroon, like in other African countries, *A. annua* and *A. afra* are cultivated in different geographical regions, formulated as herbal tea, and sold throughout several regions for the management of health-related problems, including malaria, but without pharmacological quality control. Additionally, given the close morphological similarity between the two *Artemisia* species, it is difficult to distinguish between the two medicinal plants physically (**Supplementary Figure S1**), and hence, discerning the authenticity of herbal tea is problematic. There is an absolute need to compare the metabolic contents and profiling of these plants obtained from different regions and during different cultivation periods and to develop a pharmaceutical monograph for plant authenticity and for quality control measures. This study explores the phytochemical diversity and anti-plasmodial potential of *A. annua* and *A. afra* sourced from distinct geographical locations in Cameroon. The chemical composition of these *Artemisia* species was investigated, aiming to discern potential variations in the phytochemical profiles attributable to geographic factors and correlated with observed activity, for plant authenticity, the optimal chemical composition, and quality control of these herbal products. To achieve this, both plant species were harvested in different seasons from different locations in Cameroon (**Figure 22**) and analyzed using TLC, HPLC, and GC. The environmental parameters of the collecting sites were retrieved from multispectral satellite imagery to monitor the health status of the vegetation

where these plants were collected. Additionally, their anti-plasmodial activities were assessed, and a correlation was made between the observed activity and their metabolic fingerprints. The results of these findings show that the activity profiles of *Artemisia* plants are associated with their environment, with distinct phytochemical compositions observed for each plant sample based on its geographical origin and season of collection. Higher Normalized Different Vegetation Index (NDVI) values were observed during the rainy season regardless of the location of collection, indicating healthier and denser vegetation, which correlated with a higher artemisinin content (trend) and was associated with stronger antimalaria activity in *A. annua*.

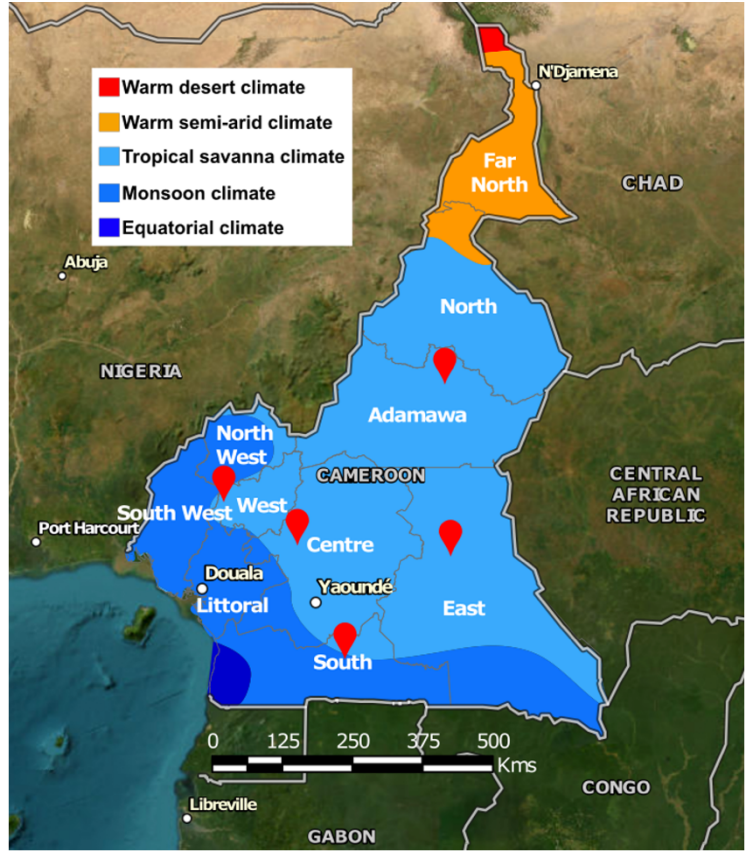


Figure 22: Location of the five collection sites. The map displays administrative regions of Cameroon, and the colors indicate the climate type (Beck et al., 2018).

II. Results

II.1 Comparison of TLC fingerprints of samples from different regions and seasons

Thin-Layer Chromatography (TLC) fingerprints show inter-species and intra-species variation in the chemical pattern of metabolites. For example, we observed the presence of a pink-colored compound in all of the *A. afra* samples with a retention factor (RF) of 0.78, while this was absent in all of the *A. annua* extracts (**Supplementary Figure S2a**). The chemical patterns from TLC also indicate the absence of artemisinin in all of the *A. afra* extracts but its presence in the *A. annua* samples (RF = 0.51) (**Supplementary Figure S2a**). Additionally, the TLC fingerprints of the *A. afra* samples from different locations show intra-species variation in the chemical patterns, indicating the influence of harvest location on the chemical profile. For example, the *A. afra* extracts from the Center, South, and Adamawa regions show the same chemical pattern of terpenes, while the extracts from the West and East are characterized by a blue spot with different RF values and which is absent in the other samples (**Supplementary Figure S2b**). The polyphenol fingerprints of both plants also show a distinct chemical pattern. For instance, luteolin and rutin are present in all *A. afra* samples but absent in the *A. annua* samples. Additionally, a region with black bands is detected in all *A. annua* samples though this is absent in *A. afra*. Samples of *A. afra* obtained from different regions show the same profile in terms of polyphenol composition (**Supplementary Figure S2c**). While seasonal variation was observed in the profile of terpene composition, there was no clear effect of season on polyphenol composition (**Supplementary Figure S2d**).

II.2. Influence of season and growing location on the natural abundance of major polyphenols

An HPLC analysis of polyphenols was performed on both series of samples harvested in the rainy and dry seasons. A reference calibration curve consisting of chlorogenic acid (0.1, 0.05, 0.025, and 0.0125 mg/mL in methanol) was made, and the polyphenol contents of 20 methanol crude extracts were estimated and expressed as % m/m equivalents of chlorogenic acid. Several polyphenols were identified in both samples through a comparison of the UV spectrum and retention time (Rt) of the analyte with those of standard reference compounds. These included rutin, chlorogenic

acid, luteolin, astragalin, and iso-chlorogenic acid A, B, and C. In addition, coumarins (scopoline and scopoletin) were detected. The results obtained show inter- and intra-species variation in the concentration of chlorogenic acid (CA) and its derivatives (**Figure 23**). For the *A. afra* samples, the highest concentration of CA and its derivatives was found in the rainy season samples from the Center region (9.61% m/m chlorogenic acid equivalents), with iso-chlorogenic acid A being the major metabolite (3.89% m/m chlorogenic acid equivalents). In the sample from the East region, no detectable amount of CA nor its derivatives was retrieved. Within the *A. annua* samples, the concentration of CA and its derivatives ranged from 0.18 to 3.61% m/m CA equivalents in the samples from the East and South regions, respectively, indicating the effect of location on the CA contents. Additionally, while iso-chlorogenic acid A, B, and C were present in all samples, cichoric acid was only found in both species obtained from the Center region in the rainy season.

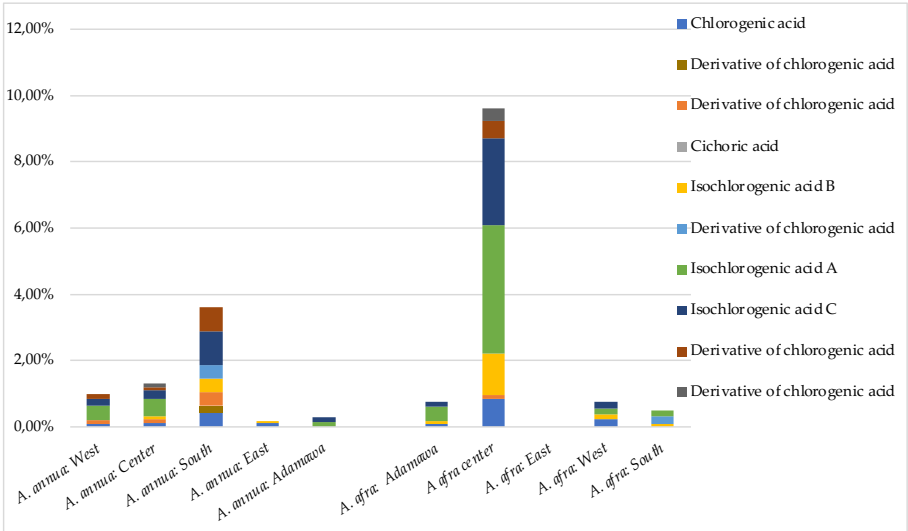


Figure 23. Contents of chlorogenic acid and its derivatives in *Artemisia* samples obtained in the rainy season expressed as % m/m equivalents of chlorogenic acid vary between different regions for both plants and inter-plant.

In addition to geographical variations, the results also show seasonal variations in the concentrations of CA and its derivatives. For example, while the *A. afra* samples obtained in the rainy season from the East region had no detectable amount of CA or its derivatives, the dry season samples presented high concentrations (1.73% m/m CA equivalents), with iso-chlorogenic acid B

being one of the major phenolic acids (**Figure 24**). Except for *A. afra* from the Center region, generally, higher contents of CA and its derivatives were retrieved in the dry season samples compared to the rainy season samples. No detectable contents of cichoric acid were found in the dry season samples from both species. As for the *A. annua* samples, generally, higher contents of CA and its derivatives were also observed in the dry season, except from in the samples from the South region.

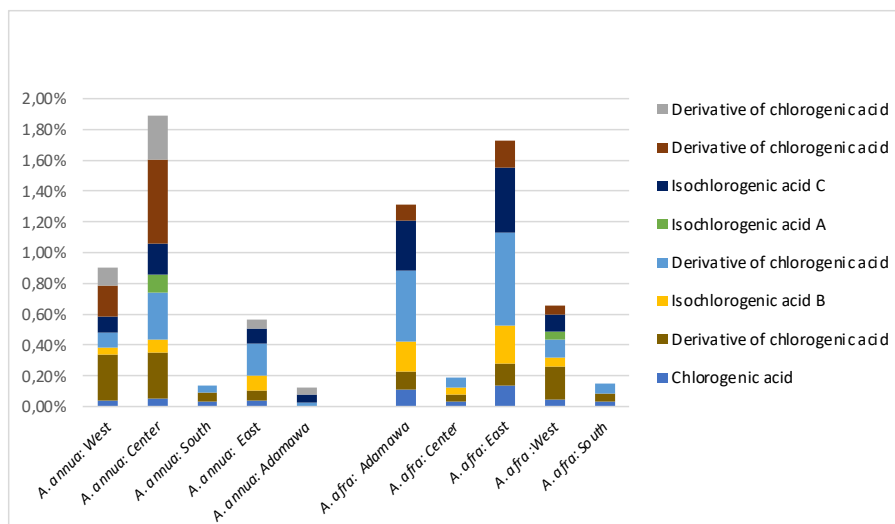


Figure 24. Effect of geographical location on the contents of chlorogenic acid and its derivatives in *Artemisia* samples obtained in the dry season expressed as % m/m equivalents of chlorogenic acid.

II. 3. Influence of season and growing location on the natural abundance of major semi-volatile components

A Gas Chromatography (GC) analysis of semi-volatile components was performed using the acetone extracts of both plants obtained from different locations and seasons. Again, both inter and intra-species variations in the chemical pattern could be observed. Hence, indicating the effect of geographical location on the composition and natural abundances of the semi-volatile components of both species. While α -thujone was only identified in the *A. annua* samples from the Adamawa region, camphor, α -pinene and linalool were some of the metabolites identified in all rainy season samples with varying relative composition (**Figure 25**).

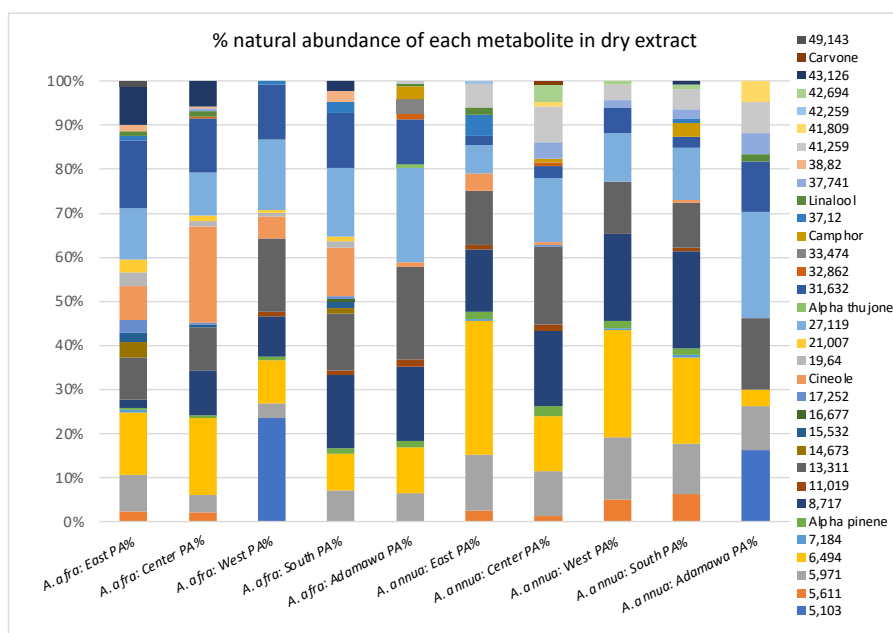


Figure 25. Influence of geographical location on the GC fingerprint and natural abundances of semi-volatile components in acetone extracts from both plants obtained from different regions in the rainy seasons. Each color indicates a metabolite, and the length gives an estimate of its relative occurrence.

The numbers represent the corresponding retention times (RTs); PA% = peak area percent. As for the samples obtained in the dry season, in addition to the locational variation, the results also show seasonal fluctuations in their composition. Carene-3-(+), terpineol, cineol, β -pinene, and camphor were some of the identified metabolites common to all of the dry season samples from both species but, again, with varying relative compositions (**Figure 26**).

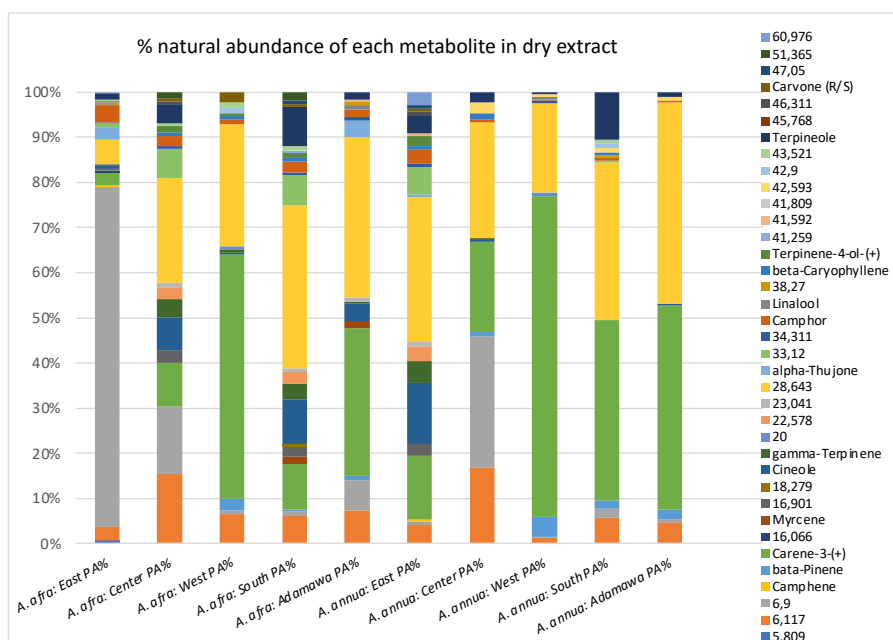


Figure 26. Variation in the GC fingerprint and natural abundances of semi-volatile components in acetone extracts from both plants obtained from different regions in the dry season. Each color indicates a metabolite, and the length gives an estimate of its relative occurrence. The numbers represent the corresponding retention times (RTs); PA% = peak area percent.

II.4. Influence of season and growing location on artemisinin content

Estimation of the artemisinin contents in the acetone and methanol crude extracts of both species from different regions and seasons indicated a higher concentration in the acetone extracts compared to the methanol extracts (Supplementary Figure S3). Chromatograms of artemisinin (as the standard) and the derivatized and underivatized samples following the HPLC analysis are shown in **Figure 27**.

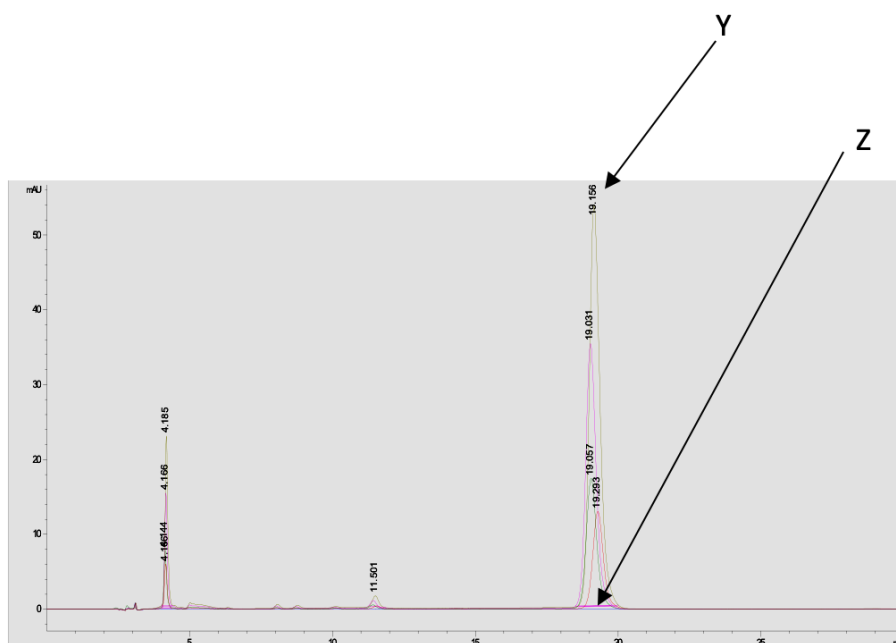


Figure 27. Chromatograms of artemisinin (standard) and derivatized and underivatized *A. annua* samples following HPLC analysis shows the presence of artemisinin in the *A. annua* samples. Y = chromatogram of derivatized samples and artemisinin, and Z = chromatogram of underivatized samples.

The presence of artemisinin was further confirmed using a Direct Infusion High Resolution Mass Spectrometry (DI–HRMS) analysis of the dry season samples of *A. annua* and *A. afra*, as derivatization can result in the production of artefacts. The spectrum for the artemisinin reference (Figure 7A) indicated the specific ions m/z 305.13550 ($[M+Na]^+$) and m/z 587.28107 ($[2M+Na]^+$), which were detected in the spectra of both the *A. annua* and *A. afra* samples using DI–HRMS (**Figure 28B and Figure 28C**), respectively.

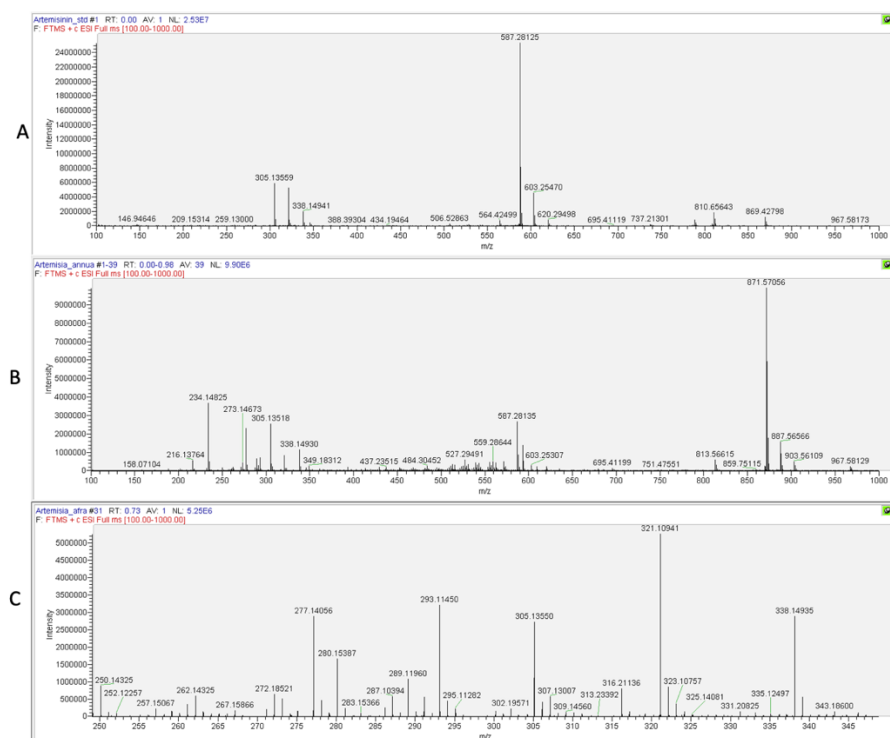


Figure 28. DI-¹H¹MS spectra of (A) artemisinin, (B) *A. annua*, and (C) *A. afra* to visualize artemisinin [M+Na]⁺ contents.

For the *A. afra* samples, only traces of artemisinin were found in some locations, while in other regions, no detectable amount could be found. As for *A. annua*, varying artemisinin contents were obtained as influenced by geographical and seasonal origin, with generally the highest contents detected in the rainy season samples (**Figure 29**). The *A. annua* samples from the Adamawa region showed the highest artemisinin content (8.88 and 2.71% for the rainy season and dry season samples, respectively), while the lowest contents were found in the samples from the East region.

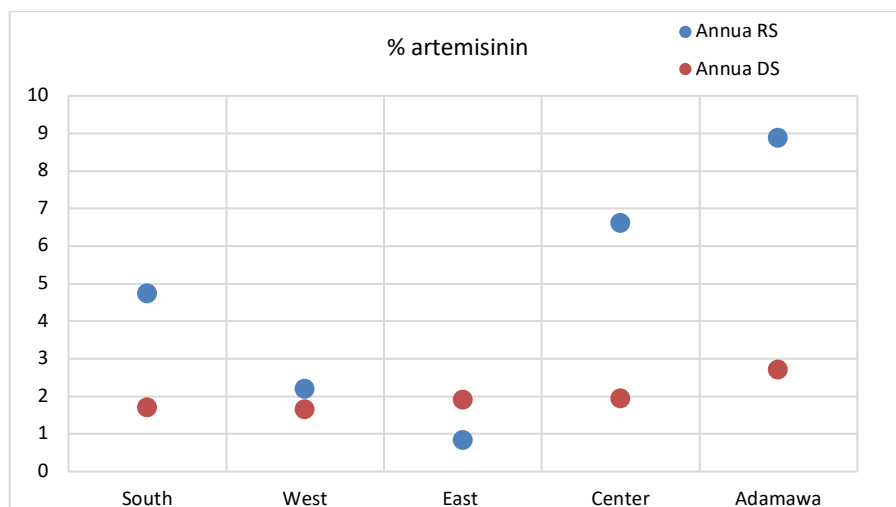


Figure 29. Effect of seasonal and geographical variation on artemisinin contents in *A. annua*. RS = rainy season samples, DS = dry season samples.

II.5. Seasonal and geographical variation in the total polyphenol contents

The total polyphenol contents (TPCs) were quantified in the extracts prepared using the infusion method (the traditional preparation), as well as the extracts obtained using the method described in the European Pharmacopoeia (heating and reflux) and in the methanol crude extracts. The TPCs were higher in the methanol crude extracts, followed by those obtained using the heating and reflux method, while infusion showed the lowest contents (**Supplementary Figure S4**). For the *A. annua* samples, only a relevant geographical fluctuation was ascertained (**Figure 30A**). However, for the *A. afra* samples, a seasonal effect was also observed, with the highest concentrations measured in the dry season samples, except for in the samples obtained from the Center region. The TPCs of *A. afra* ranged between 3.43 (in rainy season samples from the East region) and 8.43% m/m dry extract (in rainy season samples from the Center region) (**Figure 30B**). The values found within the *A. annua*-based extracts ranged between 3.29 and 5.92% m/m dry extract in the samples obtained from the Center region in the rainy and dry seasons, respectively (**Figure 30A**).

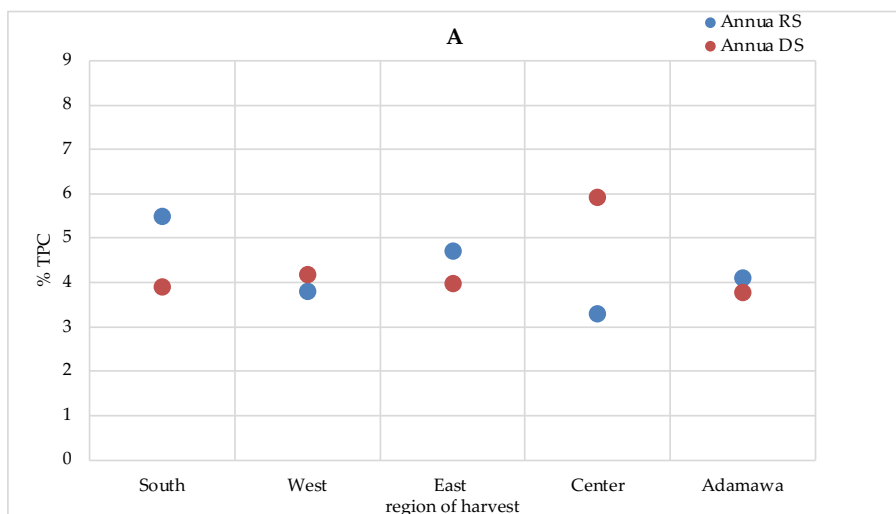


Figure 30A. TPCs expressed as % m/m polyphenol in dry extract in methanol extracts of (A) *A. annua* RS = rainy season samples; DS = dry season samples.

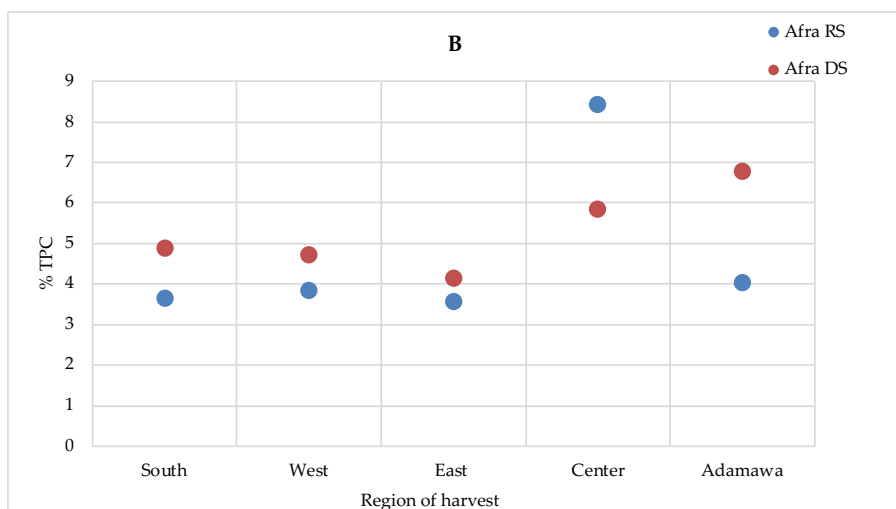


Figure 30B. TPCs expressed as % m/m polyphenol in dry extract in methanol extracts of *A. afra* obtained in the rainy and dry seasons. RS = rainy season samples; DS = dry season samples.

II.6. Antiplasmodial activity of the extracts and the correlation with chemical patterns

The anti-plasmodial activity of both plants was correlated with their metabolic fingerprints. Generally, the acetone extracts of both plants were more active than the methanol extracts (**Figure 31**). Both species were active against *Plasmodium* parasites. However, the *A. annua* extracts were more

active than the *A. afra* extracts, with the activities fluctuating between samples from different regions and seasons. The rainy season samples of the *A. annua* extracts were more active than the dry season samples. This activity was artemisinin-dependent and related to the higher contents observed in the rainy season samples. The IC₅₀ of artemisinin (standard drug) was 4.59 ± 1.15 ng/mL.

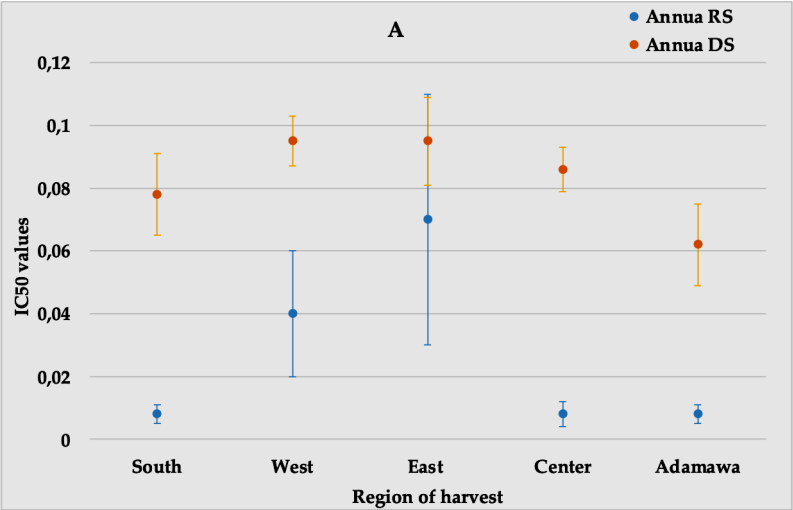


Figure 31A. IC₅₀ values (µg/mL) of acetone extracts of *A. annua* from different regions and seasons show variation between the different seasons and geographical location. RS = rainy season; DS = dry season.

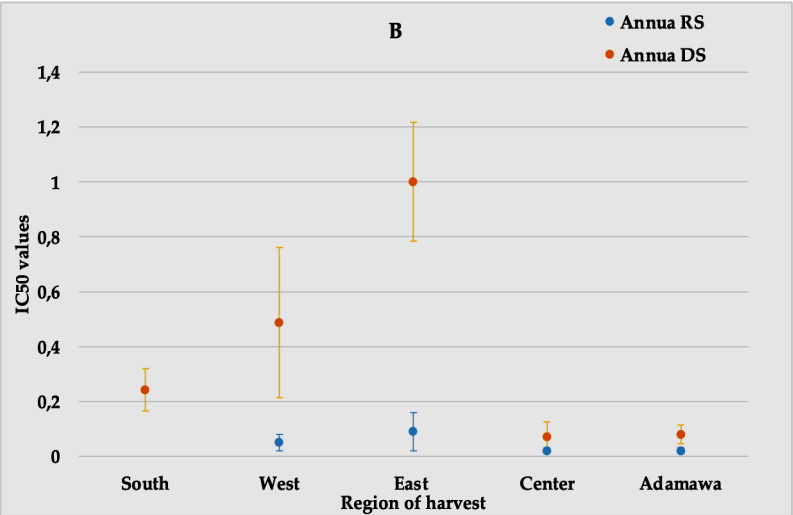


Figure 31B. IC₅₀ values (µg/mL) of methanol extracts of *A. annua* from different regions and seasons

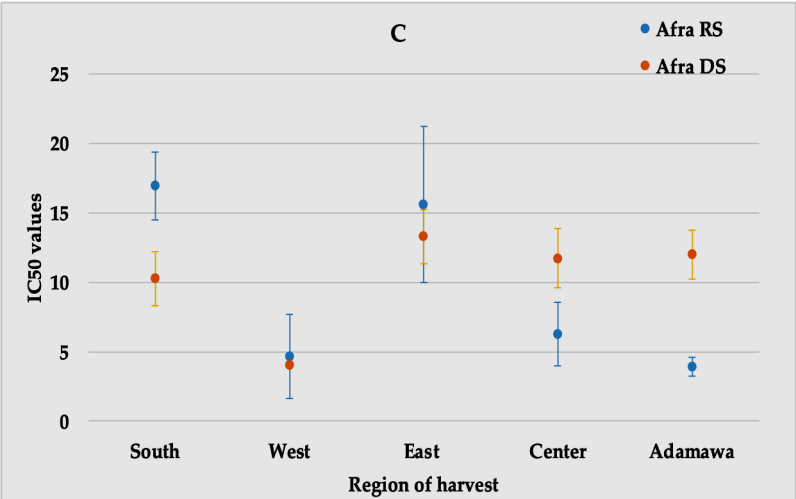


Figure 31C. IC₅₀ values (µg/mL) acetone extracts of *A. afra* from different regions and seasons. The activity varies between the different seasons and geographical location. RS = rainy season; DS = dry season.

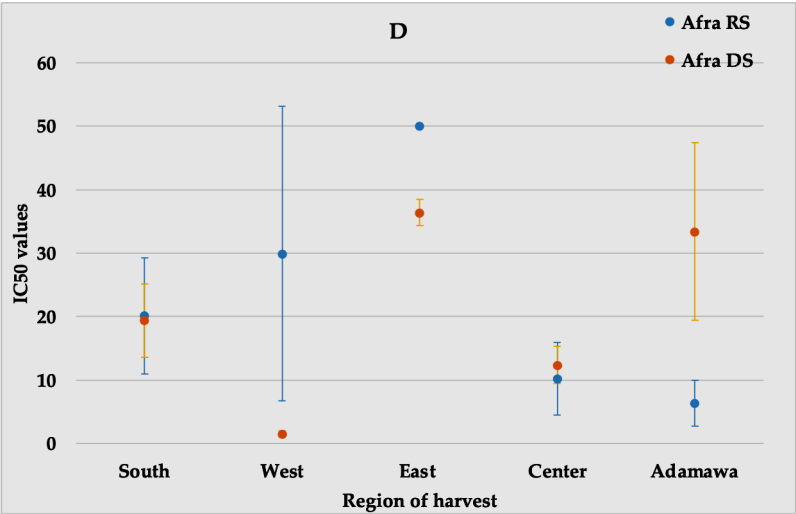


Figure 31D. IC₅₀ values (µg/mL) of methanol extracts of *A. afra* from different regions and seasons RS = rainy season; DS = dry season.

II.7. Correlation between environmental conditions and antiplasmodial properties

Anti-plasmodial activity was compared for both seasons with the health status of the environment from which the plants were collected. The Normalized Difference Vegetation Index (NDVI) values range from -1 to +1,

and values closer to +1 indicate denser and healthier vegetation. For each season, the NDVI values of the collection points were calculated. The results displayed in **Figure 32** indicate higher NDVI values during the rainy season regardless of the location of collection. This observation was, however, expected given that vegetation benefits from rainfall. A higher artemisinin content (trend) in the rainy season was associated with the stronger antimalaria activity for *A. annua*. For *A. afra*, its activity varied depending on the season and location of collection. The East collection point exhibited the lowest artemisinin content and had the least suitable vegetation environment, regardless of season.

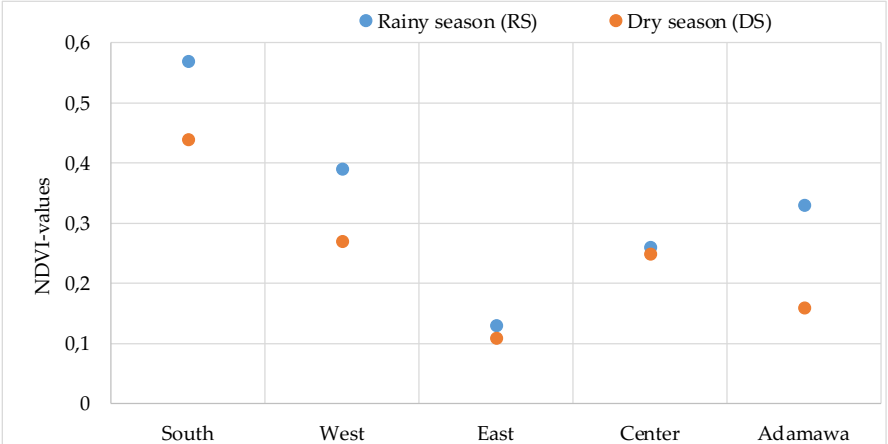


Figure 32. Variation in the NDVI values at the five collection sites and seasons.

III. Discussion

Plants’ metabolic composition is greatly influenced by geographical location and season, thus directly affecting their potential therapeutic applications (Olivier et al., 2023). In Cameroon, like in other African countries, *A. annua* and *A. afra* are cultivated throughout several regions and formulated as herbal tea for the management of malaria. However, this is without any pharmacological quality control. This problem is further aggravated by the close morphological similarity between the two *Artemisia* species, making herbal tea’s authenticity problematic. This work focuses its attention on the phytochemical diversity of both species, aiming to discern potential variations in their phytochemical profiles aributable to geographic factors and seasons and correlated with potential pharmacological quality.

Several analytical techniques, such as TLC, HPLC, and GC, were employed for the analysis of their metabolic contents, and by using both geographical and multispectral remote sensing data, correlations were made between the observed metabolic fingerprints and anti-plasmodial activity. The results from this study revealed notable variations in the phytochemical compositions of *A. annua* and *A. afra* among different geographical locations and seasons in Cameroon. These variations encompassed differences in the abundance of artemisinin and other secondary metabolites. *A. afra* and *A. annua* crude extracts are composed of complex mixtures of secondary metabolites, with varying biological activities. The distinctions in the chemical pattern of the semi volatile and polyphenol constituents observed using TLC, HPLC, and GC analyses between both species could serve as important tools for the correct identification of these medicinal products. For instance, the presence of rutin, astragalin, and luteolin in *A. afra* and their absence in *A. annua*, including the presence of the specific TLC bands in *A. afra*, could be used as a basis for the development of specific monographs for this plant species. Additionally, the TLC fingerprints show the presence of artemisinin specifically in *A. annua*. Intra-species variation in terms of the occurrence and relative composition of plant metabolites, especially in the semi-volatile components, result from the influence of different environmental factors, which vary between different regions and seasons.

Artemisinin is a well-known antimalaria compound with a sesquiterpene backbone originally isolated from *A. annua* (Jeremić et al., 1973). Also, traces of artemisinin have been found in some cultivars of *A. afra*, while other cultivars show no detectable content [9,15]. In this study, traces of artemisinin were detected in some *A. afra* samples, while others were devoid of this sesquiterpene lactone. The high artemisinin content in the *A. annua* samples obtained during the rainy season indicates the seasonal influence on the metabolic pathways involved in the synthesis of artemisinin. The rainy season is the growing season during which most of the plant's secondary metabolites are produced, resulting in alleviated concentrations. Regardless of the season, the artemisinin contents were high in the samples obtained from the Adamawa region, highlighting the effect of both season and geographical location on artemisinin content.

Three different sets of sample preparation were performed for the analysis of the total phenolic contents (TPCs): heating and reflux (using the protocol

of the European Pharmacopoeia), methanol crude extracts, and infusion (the traditional preparation). The TPC was greatly influenced by the sample preparation. The highest TPC was retrieved in the methanol extract, followed by the method from the pharmacopoeia, while infusion showed the lowest content. This may be explained by the fact that methanol is a good extraction solvent for most of the polyphenolic components. The higher TPC in the extracts obtained through heating and reflux of the plant powder compared to that in the infusions may have been due to the longer contact time involved in using the former. From this finding, the TPCs, including the contents of CA, tend to be higher in dry season samples of *A. afra* (with the exception of the samples from the Center region), again indicating the seasonal influence.

The acetone and methanol crude extracts prepared from the plant samples obtained both in the rainy and dry seasons from the five geographical regions were tested against a chloroquine-sensitive 3D7 *P. falciparum* strain. Three independent experiments were conducted, and the mean IC_{50} values were calculated and expressed in μg extract/mL. Based on the World Health Organization's guidelines ([Jansen et al., 2017](#)), anti-plasmodial activity levels are classified as follows: highly active ($IC_{50} \leq 5 \mu\text{g/mL}$), good activity (IC_{50} between $5 \mu\text{g/mL}$ and $15 \mu\text{g/mL}$), moderate activity (IC_{50} between $15 \mu\text{g/mL}$ and $30 \mu\text{g/mL}$), weak activity (IC_{50} between $30 \mu\text{g/mL}$ and $50 \mu\text{g/mL}$), and a lack of activity at $IC_{50} \geq 50 \mu\text{g/mL}$. The anti-plasmodial assays demonstrated varying degrees of efficacy in inhibiting *Plasmodium* parasites, with potential correlations with the distinct phytochemical profiles observed. The acetone extracts were more active than the methanol extracts. This may have been because of the higher artemisinin contents in the acetone extracts than that in the methanol extracts. Certain geographical locations exhibited higher anti-plasmodial activity, indicating a correlation between the phytochemical composition and anti-plasmodial properties. Both samples were active, but the *A. annua* extracts showed a significantly higher anti-plasmodial activity compared to that of *A. afra*. This activity was artemisinin dependent and stronger in the rainy season samples, being associated with a high artemisinin content. Additionally, the samples from the Adamawa region showed the highest artemisinin contents and were, together with the *A. annua* samples from the South and the East, the most active ones. These three types of samples showed comparable IC_{50} values, though the later yielded a lower artemisinin content. This indicates that the other components present in the

extract also affect its anti-plasmodial activities. Again, this indicates the importance of geographical location and season when cultivating an *Artemisia* spp. for use as a preventive or curative therapeutical agent against malaria. However, WHO has cautioned against the use of non-pharmaceutical sources of artemisinin because of the risk of delivering sub-therapeutic doses, which could exacerbate resistance to ACTs ([World Health Organization, 2019](#)), emphasizing the importance of species-specific differences. Despite the lack of relevant contents of artemisinin, *A. afra* showed promising activity against the Plasmodium parasites, suggesting its use without the above-mentioned threat to ACTs in emptying malaria reservoirs observed by WHO. The activity of *A. afra* was not affected by season or geographical location despite the relatively higher CA contents during the rainy season.

According to the findings of [Ferreira et al. \(1995\)](#), the best growing conditions for *Artemisia* are a humid and subtropical monsoon climate with average temperatures of 17.6 – 28.4 °C. Despite the different collecting sites being characterized by a similar climate, the retrieved NDVI values indicate significant variations in the environmental conditions between them. As analyzed using both geographical and multispectral remote sensing data, the rainy season presented healthier vegetation, which favored the artemisinin contents responsible for the greater activity in *A. annua* from this time point than that harvested in the dry season.

IV. Materials and Methods

IV.1. Chemicals and equipment

The reference substances used included artemisinin (Gibco, Fisher Scientific, Merelbeke, Belgium), 1, 8- cineole, α -thujone, β -thujone, rutin, chlorogenic acid, luteolin, astragalin, apigenin, caffeic acid, kaempferol, scopoletin, iso-chlorogenic acid, vitexin, fraxin, caftaric acid, 7-hydroxy coumaric acid, esculetin, quercetin, camphor, coumaric acid, and camphene (Merck, Darmstadt, Germany). The solvents applied—acetonitrile, methanol, formic acid, acetone, ethyl acetate, hexane, ethanol, and chloroform (Sigma-Aldrich, Overijse, Belgium)—were all HPLC-grade. Sulfuric acid, polyethylene glycol (PEG), vanillin powder, and diphenyl-borate diamino-ethanol (DPBAE) were obtained from Merck, Darmstadt, Germany.

IV.2. Description and collection of the plant material

Morphologically, both plants are similar in appearance, growing up to a height of at least 2 m. *A. afra* is a woody perennial herb with finely divided leaves that are oval and have an aromatic smell, are alternately arranged, and are silver-grey in the adaxial regions and light green in the axial regions [5]. *A. annua* is a large, annual, highly aromatic herb with a single stem covered with fine grey-green hairs and alternating aromatic leaves which are deeply dissected. The leaves and twigs of both plants were collected at the flowering stage from five regions (in both the rainy and dry seasons) in Cameroon: Ngaoundere in the Adamawa region (coordinates: 7.328 N, 13.585 E), Bertoua in the East region (coordinates: 4.579 N, 13.677 E), Sangmalema in the South region (coordinates: 2.937 N, 11.986 E), Bafia in the Center region (coordinates: 4.755 N, 11.224 E), and Dschang in the West region (coordinates: 5.446 N, 10.047 E). Voucher specimens were deposited at the Limbe botanical gardens, Cameroon. The samples were authenticated by a botanist and voucher numbers assigned (SCA 7137 and SCA 6340 for *A. annua* and *A. afra*, respectively).

IV.3. Preparation of the crude extracts

Two groups of plant materials were experimented with: rainy and dry season samples comprising acetone and methanolic crude extracts of four groups of 40 plant samples (20 *A. afra* and 20 *A. annua* samples obtained from five regions). Extracts were prepared following the protocol described by Mbah et al. (2012). Briefly, each plant sample was oven dried at 30 °C and ground into a fine powder, and each powder was divided into two equal masses and macerated at room temperature for 3 days using acetone or methanol. The macerated mixture was filtered through a funnel stuffed with Whatman filter paper No.1, and the filtrate was concentrated under a vacuum. Each crude extract was then dried and weighed and the yield calculated (Supplementary Table S2). All extracts were stored at 4 °C for subsequent use.

IV.4. Thin Layer Chromatography (TLC) analysis

Analytical Thin-Layer Chromatography (TLC) of the extracts was performed on precoated Si gel 60 F254 (Merck, Hohenbrunn, Germany) plates to determine the chemical patterns of the compounds in the extracts. A solvent system comprising n-hexane/ethyl acetate (70:30) was used to separate the semi-volatile compounds in the acetone extracts. After development, the TLC

plates were sprayed with sulfuric vanillin and heated for 10min at 100 °C, and the separated components were visualized in daylight. Separation of the polyphenol fraction in the methanol extracts was achieved using a solvent system comprising ethyl acetate, methanol, water, and formic acid (50:4:4:2.5). After development, the results were visualized through DPBAE/PEG spraying (prepared by dissolving 10 g and 50 g of DPBAE and PEG, respectively, in 1 L of methanol) and 366 nm illumination (UV light). The retention factor (RF) of the separated components was calculated and compared with the reference compounds.

IV.5. HPLC analysis

High-Performance Liquid Chromatography (HPLC) analyses of the polyphenols were performed on an Agilent 1260 Infinity II system using a protocol developed in the laboratory (Pharmacognosy, CIRM, University of Liège, Belgium). Briefly, the extracts were reconstituted at a concentration of 40 mg/10 mL of methanol, sonicated for 2 × 15min, and filtered through a 0.45 µm HPLC filter. A Luna PFP column (250 × 4.6 mm, 5 µm) was used, and the mobile phase consisted of a mixture of acetonitrile (A) and water/0.1% formic acid (B) in gradient conditions (A/B: 15/85, 18/82, 23/77, 30/70, 40/60, 50/50, 80/20, 100/0, 15/85, and 15/85 v/v). A volume of 10 µL was injected at a flow rate of 1 mL/min, and a run time of 87 min was set. A reference calibration curve consisting of chlorogenic acid (0.1, 0.05, 0.025, and 0.0125 mg/mL in methanol) was made. A Diode Array Detector (DAD) was used and set at 330 nm. The results were expressed as chlorogenic acid equivalents.

IV.6. Analysis of semi-volatile components using GC

A Gas Chromatography (GC) analysis of the semi-volatile components was performed on an Agilent Technologies Intuvo-9000 GC System using a method developed in the laboratory. Briefly, 100 mg of the samples was weighed and injected using the headspace method. The column used was a DB-WAX-UI (60 m × 250 µm × 0.25 µm). The analytical conditions included an oven temperature programmed from 45 °C to 250 °C at a rate of 2.615 °C/min, and the vial and transfer line temperatures were set at 130 °C and 150 °C, respectively. The chromatogram ran for 120 min, and detection was performed using a Flame Ionization Detector (FID) with a detection temperature of 280 °C. Identification of the different constituents was based on a comparison of the retention times with those of the standard samples.

IV.7. Quantification of artemisinin using the HPLC-UV method and derivatization

Detection and quantification of the artemisinin in the acetone and methanol crude extracts were performed using an HPLC-UV method after a pre-derivatization step, as described by [Diawara et al. \(2011\)](#) and [Zeyuan et al. \(2018\)](#). Before the pre-derivatization step, 50 mg of crude extract was dissolved in 5 mL of ethanol and submied to 2×15 min of ultrasonication. For all of the samples including the standards, 1 mL of solution was transferred into a 10 mL volumetric flask, followed by the addition of 4 mL solution of 0.2% (m/v) NaOH, leading to the formation of Q292 (**Figure 12**) after 30 min in a water bath at 50 °C. The mixture was cooled for 10 min, 1 mL of ethanol was added, and the flask was topped up to a mark (25 mL) with 0.2 N acetic acid, promoting the conversion of Q292 into Q260. **Figure 33** illustrates the derivatization reaction which produces Q260, as detected at 260 nm following the HPLC analysis. All of the samples were filtered using a 0.45 μm HPLC filter, and chromatographic separation was performed in reverse phase mode. A mobile phase consisting of a mixture of methanol and phosphate buffer (5.0 mM; pH: 6.3) (45/55 v/v) was used. The Luna PFP column (250 $\times$ 4.6 mm, 5 μm) was maintained at 35 °C. An injection volume of 20 μL and a flow rate of 1 mL/min with a run time of 25 min were set as the chromatographic conditions. A calibration curve consisting of 10, 20, 40, and 60 mg/mL of artemisinin was used to estimate the quantity of artemisinin in each extract.

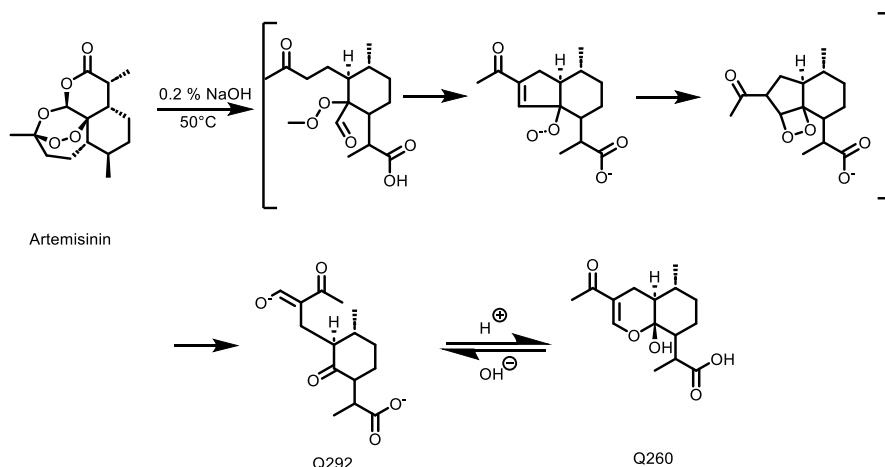


Figure 33. Schematic diagram of artemisinin's conversion into Q260 ([Zeyuan et al., 2018](#)).

IV.8. Quantification of polyphenols

The total polyphenol contents (TPCs) in the plant powder, infusions, and plant extracts (methanol extracts) were determined spectrophotometrically using the method described in the European Pharmacopeia. Briefly, 500 mg of plant powder was weighed in a round-boomed flask, and 100 mL of water R was added. The flask was heated and refluxed for 30 min and then cooled under running water and filtered into a 100 mL volumetric flask. The infusions were prepared by adding 5 g of plant powder into 1 L of water and left to extract for 15 min. Stock solutions of the methanol crude extracts were prepared by dissolving 20 mg of crude extract in 10 mL of methanol and ultrasonicated them for 15 min. For quantification, 2 mL of each solution was transferred into a 25 mL volumetric flask, and 1 mL of phosphomolybdotungstic reagent and 10 mL of water R were added and adjusted to 25 mL with a solution of sodium carbonate R. After 30 min in the dark, the absorbance was measured at 760 nm using a U-2900 spectrophotometer. Pyrogallol was used as a reference. A stock solution was prepared by dissolving 5.23 mg of pyrogallol in 100 mL of methanol and quantified as described above. The results were expressed as pyrogallol equivalents.

IV.9. Antiplasmodial activity

A chloroquine-sensitive 3D7 *P. falciparum* strain was obtained from the Malaria Research and Reference Reagent Resource Center, MR4, and maintained in vitro as described by [Trager and Jensen \(1976\)](#). The parasites were maintained at 3% hematocrit (human type A or O-positive red blood cells) in a Complete Culture Medium (CCM) prepared by supplementing RPMI 1640 (Gibco, Fisher Scientific, Merelbeke, Belgium) medium containing NaHCO₃ (32 mM), HEPES (25 mM), and L-glutamine with 10% heat-inactivated human plasma, 1.76 g/L of glucose (Sigma-Aldrich, Overijse, Belgium), 44 mg/mL of hypoxanthine (Sigma-Aldrich, Overijse, Belgium), and 100 mg/L of gentamycin (Gibco, Fisher Scientific, Merelbeke, Belgium). All of the cultures were placed in a humidified incubator at 37 °C with a standard gas mixture of 5% O₂, 5% CO₂, and 90% N₂. Parasitemia was verified through microscopy using Giemsa-stained thin films. A stock solution of the crude extracts was prepared by dissolving 5 mg of extract in 1 mL DMSO and diluting it with culture media. The anti-plasmodial test was performed in 96-well microtiter plates at an initial sample concentration of 50 µg/mL which was serially diluted to eight concentrations (50 to 0.391 µg/mL). Each sample was

tested in duplicate wells with the parasites at a final volume of 250 μ L, making the final DMSO concentration < 1%. Wells with parasites only served as the positive control, while the negative control consisted of wells with the culture medium only. A 1 μ g/mL stock solution of artemisinin (Gibco, Fisher Scientific, Merelbeke, Belgium) was prepared and serially diluted as above; this was used as the standard. After 48 h of incubation, parasite growth was estimated through determination of the plasmodial lactate dehydrogenase activity, as described by [Kenmogne et al. \(2006\)](#), and the IC₅₀ values were calculated through linear regression. All tests were repeated at least twice.

IV.10. Multispectral satellite imagery analysis of the health status of vegetation at the collection sites

The health status of the vegetation was monitored using Earth observation. The pair of Sentinel-2 satellites from the European Copernicus Programme (ECP) provides free images of the Earth's entire surface at a spatial resolution from 10 to 60 m. Each Sentinel-2 satellite carries a Multispectral Instrument (MSI) that captures images in 13 spectra bands, covering the visible, near-infrared, and shortwave infrared regions (443–2323 nm). Multispectral remote sensing data are widely used for land and ocean monitoring using spectral indices. To assess the vegetation's health and density, the Normalized Difference Vegetation Index (NDVI) was derived from the reflectance, i.e., the solar light reflected by the Earth's surface, in the red and near-infrared (NIR) bands using the following formula:

$$\text{NDVI} = (\text{NIR} - \text{Red}) / (\text{NIR} + \text{Red}).$$

The NDVI values range from -1 to +1. Vegetation absorbs most visible light for photosynthesis (implying a low reflectance in red) and reflects a significant amount of nearinfrared light (a high reflectance in NIR), leading to a positive NDVI. Denser and healthier vegetation presents an NDVI closer to +1. For each season, the NDVI values at the collection points were calculated using cloud-free Sentinel-2 images acquired on the dates closest to the collecting dates (**Supplementary Table S1**). Given the spatial resolution of Sentinel-2 images in the visible wavelengths (10 m), the NDVI was not retrieved at the plant level but at the level of the direct environment in which the plants grew.

V. Conclusions

The results show both intra-species and inter-species variations in the phytochemical constituents which fluctuate considerably with season and

geographical region. The artemisinin content was found to be high in the rainy season for *Artemisia annua*, while the polyphenol contents and chlorogenic acid and its derivatives were high in the dry season for *Artemisia afra*. The observed variations may have relevant implications for the anti-plasmodial potential of these plants, with specific geographic factors influencing their chemical composition, and highlight the importance of quality control in herbal medicine. The findings suggest that the selection of a suitable *Artemisia* sample for use as a potential antimalarial treatment must take into consideration its geographical origin, the period of collection, season, and remote sensing data. By understanding the environmental factors that influence the phytochemical composition of *Artemisia* samples using remote sensing data, this study may provide an insight into how natural products can be more effectively used as a preventative or curative measure against malaria. Further research is warranted to elucidate the major compounds responsible for the observed anti-plasmodial activities in *A. afra*.

Supplementary materials: The following supporting information can be downloaded at

www.mdpi.com/10.3390/molecules30030596/s1. **Figure S1:** Field pictures of (a) *A. annua* and (b) *A. afra*. **Figure S2:** TLC fingerprint: (a) Acetone extract of *A. afra* and *A. annua* obtained in the rainy season revealing variations in the terpene chemical composition. (b) Acetone extract of *A. afra* obtained from different regions showing similar chemical pattern of terpene composition, (c) Variation in polyphenol chemical pattern between both species (methanol extract of *A. afra* and *A. annua*) obtained in the rainy season, (d) Methanol extract of *A. afra* and *A. annua* obtained in the dry season revealing polyphenol chemical pattern. X= scopoletin, Y= artemisinin and Z= apigenin. **Figure S3:** Contents of ART in acetone and methanol extracts of samples from the Center and Adamawa regions. **Figure S4:** TPCs in the different methods of extract preparation (heating and reflux method proposed by European pharmacopoeia, tea infusion extract and methanol extracts. **Table S1:** List of the collected sites indicating the collection dates of the samples for both seasons and the dates of the closest satelite-2-images used for inferring the NDVI. **Table S2:** Yield of plant extracts.

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Conflicts of interest: The authors declare no conflict of interest

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Chapter Three

**Impact of post-harvest handling methods
on the chemical composition and
Antiplasmodial properties of *Artemisia
annua* and *Artemisia afra***

3.1 Foreword

Several post-harvest handling techniques have been used on herbs and medicinal plants either to increase the shelf life or for preservation and or to improve the therapeutic outcomes amongst others. However, in the context of medicinal plants, these practices can have an impact on the composition and variation in metabolite pattern. These variations can cause inconsistent therapeutic effect for the same batch of plant material. In this context, the secondary metabolic contents of *Artemisia afra* and *Artemisia annua* can be largely affected by post-harvest handling conditions that will affect the efficacy of these plants in the management of malaria. Some of these post-harvest handling practices include, fermentation, drying methods (sun drying, oven drying, freeze drying), storage conditions (temperature, humidity), and processing techniques (cutting, grinding), directly contributing to the effectiveness of the plant in treating malaria.

In this chapter, we explore the impact of some of these post-harvest handling conditions on the metabolite profile of *A. annua* and *A. afra* and analysed their antiplasmodial effect to propose or inform the best practice that would ensure optimal chemical composition and consistent efficacy. Plant samples were subjected to the post-harvest handling methods and infusions were prepared using the traditional preparation method and analysed through a combination of chromatographic techniques.

3.2 Post-harvest Handling Influences the Secondary Metabolite Contents and Antiplasmodial Properties of *Artemisia annua* and *Artemisia afra* (Submitted for publication)

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Abstract

Background: *Artemisia afra* and *Artemisia annua* are cultivated, processed, and used as a traditional remedy in malaria-endemic regions. However, batch-to-batch variation in the chemical composition resulting from post-harvest handling methods causes inconsistency in the efficacy and effectiveness of the plant preparation in treating malaria. This project aimed to investigate the impact of post-harvest handling on the secondary metabolic contents of infusions of *A. afra* and *A. annua* and correlate to antiplasmodial activity, for optimal chemical composition and consistent efficacy.

Methods: *A. afra* and *A. annua* samples were subjected to various post-harvest handling techniques, and infusions were prepared, lyophilized, and metabolic contents analyzed by High Performance Thin Layer Chromatography (HPTLC), High Performance Liquid Chromatography (HPLC), and Gas Chromatography (GC). Their antiplasmodial activity was determined using a chloroquine-sensitive 3D7 *Plasmodium falciparum* strain.

Results. Post-harvest handling affected the profiles of polyphenols and volatile compounds in both plant infusions and artemisinin content in *A. annua*. Non-fermented and sun-dried sample of *A. annua* had significant higher contents of artemisinin (9.86% m/m artemisinin in dry extract), total polyphenol contents (TPC) (2.85 % m/m in dry extract) and content of chlorogenic acid (CA) and its derivatives (0.18% m/m equivalent of CA) with improved antiplasmodial activity ($IC_{50} = 0.34 \pm 0.05 \mu\text{g/mL}$). Similarly, for the *A. afra* samples, non-fermented and sun-dried samples equally demonstrated the best phenolic profile with significantly higher content of CA (1.98% m/m equivalent of CA) and TPCs of 3.45% m/m in dry extract. In the same post-harvest handling condition, *A. annua* samples were more active than *A. afra* samples.

Conclusion: Regardless of the time of harvest, drying by sunlight improves the metabolite fingerprint of terpene and polyphenol composition. Artemisinin, chlorogenic acid, and total polyphenol contents were increased by sun-dried method and leading to improved pharmacological activity. This study supports the sun-dried method, providing more insight into how *A. annua* and *A. afra* can be effectively used for combating malaria. However, more studies are needed on other post-harvest handling methods to provide more efficient data for the quality and efficacy of these herbal products.

Keywords: Malaria, metabolite content, *Artemisia afra*, *Artemisia annua*, artemisinin.

I. Introduction

Today's culture uses medicinal plants for a wide range of purposes, including cosmetics, medicines, herbal foods, and beverages, and they are essential, particularly in the present era. Due to the effectiveness of natural products, especially medicinal plants, they have been in high demand and are valued for their therapeutic properties with at least 80% of the global population relying on them for some aspect of primary health care, especially in Low- and Middle-Income Countries (WHO, 2023). Natural products have served as potential treatments for diseases, including malaria, for decades (WHO, 2023). In particular, the traditional medicinal preparation of infusions or decoctions of *Artemisia afra* and *Artemisia annua* is widely practiced in Africa, where malaria is endemic (Weathers, 2025). Malaria, being one of the most severe parasitic disease in the world, affects mostly children under 5 years of age and pregnant women (Venkatesan, 2025). This makes malaria a disease of top priority for elimination (Li et al., 2025). Despite the approved use of Artemisinin-based Combination Therapies (ACTs) for combating malaria, the emergence and spread of resistance by the parasites, especially *Plasmodium falciparum*, which accounts for the majority of malaria cases in endemic regions, has compromised this effort (Venkatesan, 2025). This limitation, which is further compounded by other factors such as cultural impediments to Western medicines, has led to the increased use of traditional remedies, especially *A. annua* and *A. afra*, in the context of malaria among other ailments.

Native to South Africa and South East Asia, respectively, *A. afra* and *A. annua* are two species of the genus *Artemisia* known for their medicinal properties, particularly in treating malaria (Shinyuy et al., 2025). Previous phytochemical analysis reported that both plants contain a diversity of bioactive compounds, including monoterpenes, diterpenes, sesquiterpenes, flavonoids and their glycosides, coumarins, phenolic acids, and essential oils, among others (Shinyuy et al., 2023). Artemisinin (one of the major constituents of ACTs) belongs to the sesquiterpenoid class isolated from *A. annua*, with little or no content in *A. afra*. However, both plants have demonstrated biological activity against several diseases (anti-cancer, antibacterial, anti-

inflammatory, immunomodulating, anti-oxidant) and are known to be of ethnopharmacological relevance. Extracts as well as infusions of both plants have demonstrated promising antiplasmodial properties, including activity against *Plasmodium* gametocytes (Abdou et al., 2022; Weathers et al., 2014; Ogbole et al., 2014; Ashraf et al., 2022; Moyo et al., 2019; Snider and Weathers, 2021). This makes them good alternatives for both chemotherapy and transmission-blocking agents in the fight against malaria. With several *in vitro*, *in vivo* studies, and clinical trials, both plants are effective in combating malaria (Abdou et al., 2022; Weathers et al., 2014; Ogbole et al., 2014). For this reason and coupled with their safety profiles (Mukinda and Syce, 2007), both plants are being cultivated, harvested, processed, and sold in local markets, especially in malaria-endemic regions (Shinyuy et al., 2025).

In our previous studies, we found that different batches of both plants harvested from different regions and seasons demonstrated metabolite variation and inconsistent efficacy against malaria parasites. This batch-to-batch inconsistency in metabolite profile and variation in activity was ascribed to the impact of growing environment and season of collection (Shinyuy et al., 2025). In addition to the variability associated with the origin and season of harvest, the secondary metabolite content of medicinal plants is also strongly influenced by post-harvest handling conditions. These include fermentation, drying methods (such as sun-drying, oven-drying, shade-drying or freeze-drying), storage conditions (temperature and humidity), and processing techniques (cutting, grinding). Such factors directly affect the plant's therapeutic efficacy for a given condition (Su et al., 2019; Zhang et al., 2024; Zhu et al., 2018). While these impacts are well-documented in the context of Chinese medicine, they remain largely underexplored in Africa. In this regard, the metabolite profiles of *A. afra* and *A. annua* can be significantly altered by post-harvest handling practices, leading to batch-to-batch variability with direct implications in their antimalarial efficacy. It is therefore imperative to optimize these post-harvest handling conditions for quality control measures, optimal chemical composition, and to ensure consistent efficacy and quality of these herbal remedies, contributing to the broader exploration of herbal remedies as a sustainable and culturally relevant approach to combating malaria. This study aimed to investigate the impact of post-harvest handling techniques on the secondary metabolite composition of *A. afra* and *A. annua* infusions, and to correlate these

variations with their observed antimalarial activity. The goal is to identify optimal conditions for preserving chemical composition and to support the quality control of these herbal products. This could have a significant impact by informing best practices for post-harvest handling and optimizing the chemical composition of both medicinal plants, thereby ensuring consistent efficacy against malaria parasites. Plant samples were harvested in the rainy season at three time points from the same location and subjected to post-harvest handling methods. Infusions of both plants were prepared using the traditional preparation method, lyophilized, and metabolite contents were analyzed by chromatographic techniques, and antiplasmodial activity was assessed.

II. Materials and Methods

II.1. Plant collection and implementation of post-harvest handling techniques

Artemisia afra (AF) and *Artemisia annua* (AN) fresh leaves were harvested in the rainy season from the Adamawa region of Cameroon. Plant samples were sorted to get rid of debris, and voucher specimens were deposited at the Limbe Botanical Gardens for authentication, and a voucher number was assigned (SCA 6115 and SCA 7032 for *A. annua* and *A. afra* respectively). The same batch of plant harvested at three different time points (6:00 am, 12:00 pm, and 6:00 pm) from the same location (farm) was divided and subjected to various post-harvest handling methods. For instance, the batch of samples harvested at noon (12:00 pm) was divided into two groups. One group was allowed to ferment in a sealed bag for 7 days for *A. annua* and 10 days for *A. afra* samples. After this process, both fermented and non-fermented samples were further divided into three batches and subjected to different drying methods viz, sun-drying that lasted 4 weeks for *A. annua* and 5 weeks for *A. afra*. Drying under shade (by spreading the sample in an open environment devoid of direct sunlight) which took 8 weeks and 9 weeks respectively for *A. annua* and *A. afra* samples. On the other hand, oven-drying was done at 40 °C for 48 h for both plant samples. **Table 1** summarizes the different post-harvest handling techniques.

Table 4: Implementation of post-harvest techniques on *A. afra* (AF) and *A. annua* (AN) plant sample.

Sample code	Sample name	Period of harvest	F/NF	Method of drying
AN1	<i>A. annua</i>	Afternoon (12:00 pm)	F	Sun-dried
AN2	<i>A. annua</i>	Afternoon (12:00 pm)	F	Shaded-dried
AN3	<i>A. annua</i>	Afternoon (12:00 pm)	NF	Shaded-dried
AN4	<i>A. annua</i>	Afternoon (12:00 pm)	NF	Oven-dried
AN5	<i>A. annua</i>	Evening (6:00 pm)	NF	Shaded-dried
AN6	<i>A. annua</i>	Morning (6:00 am)	NF	Shaded-dried
AN7	<i>A. annua</i>	Afternoon (12:00 pm)	NF	Sun-dried
AN8	<i>A. annua</i>	Afternoon (12:00 pm)	F	Oven-dried
AF9	<i>A. afra</i>	Afternoon (12:00 pm)	F	Oven-dried
AF10	<i>A. afra</i>	Afternoon (12:00 pm)	NF	Shaded-dried
AF11	<i>A. afra</i>	Afternoon (12:00 pm)	NF	Sun-dried
AF12	<i>A. afra</i>	Morning (6:00 am)	NF	Shaded-dried
AF13	<i>A. afra</i>	Afternoon (12:00 pm)	NF	Oven-dried
AF14	<i>A. afra</i>	Evening (6:00 pm)	NF	Shaded-dried

Plant samples were harvested at three time periods: in the morning (6:00 am), afternoon (12:00 pm) and in the evening (6:00 pm). Harvesting was done at the same location for *A. afra* and *A. annua* and subjected to different conditions. F = Fermented and NF = Non-fermented plant sample, AF=*Artemisia afra* and AN = *Artemisia annua*

II.2. Preparation of infusions

Fourteen (14) infusion samples (**A1 – A14**) were prepared by adding 5g of each plant powder in 1 L of tap H₂O at 100°C, allowed to react for 15 min with occasional stirring, and filtered through a 0.45 µm filter paper. Additionally, AN15 was specially prepared using cold water (5g/L of tap H₂O, react for 15 min). All infusions were lyophilized (Christ Epsilon 2-4 LSCplus) to obtain dry extracts, weighed, and kept at 4 °C for subsequent analysis.

II.3. High Performance Thin Layer Chromatography (HPTLC) analysis

High Performance Thin layer Chromatography (HPTLC) analysis of infusion extracts was performed on a CAMAG system using precoated Si gel 60 F₂₅₄ (Merck, Hohenbrunn, Germany) plates. Stock solutions of extracts were prepared by dissolving 20 mg of extract in 1mL of methanol, ultrasonicated for 15 min, and filtered through a 0.45 µm filter. To obtain chemical pattern of volatile components in A1-A8 samples, a solvent system comprising n-hexane/ethyl acetate (70:30 v/v) was used and 10 µL of sample was applied (using Linomat 5) on Si gel 60 F₂₅₄ plate and after development in ADC2-system, plates were derivatized with a solution of vanillin-sulfuric acid (prepared by dissolving 1g of vanillin in 100 mL of methanol and with a dropwise addition of 2 mL sulfuric acid), heated for 5 min at 100 °C, and components were visualized in daylight. For the separation of polyphenol composition in all samples (A1-A14) alongside standard references, a solvent system comprising ethyl acetate, methanol, water, and formic acid (50:4:4:2.5) was used. After development, revelation was done by spraying with DPBAE/PEG (prepared by dissolving 2 g and 10 g of DPBAE and PEG, respectively, in 200 mL of methanol) and visualized at 366 nm (UV light).

II.4. High Performance Liquid Chromatography analysis

HPLC analysis was carried out on an Agilent 1260 Infinity II system following a modified method from [Shinyuy et al. \(2025\)](#). Infusion extracts (100 mg/5 mL in 60% methanol) were sonicated (2 × 15 min) and filtered (0.45 µm). Separation was performed on a LUNA PFP column (250 × 4.6 mm, 5 µm) using a gradient mobile phase of acetonitrile (A) and water with 0.1% formic acid (B) at a flow rate of 1 mL/min. The gradient profile (A/B, v/v) was: 15/85 → 100/0 → 15/85 over 87 min. A 20 µL injection volume was used, and detection

was done at 330 nm with a Diode Array Detector (DAD). Standard compounds (1 mg/mL) and a chlorogenic acid calibration curve (0.0125–0.1 mg/mL) was prepared for quantification.

II.5. Gas Chromatography analysis

The volatile compounds in all samples were analyzed using Gas Chromatography (GC) on an Agilent Technologies Intuvo-9000 GC System ([Shinyuy et al., 2025](#)). In brief, 100 mg of each sample was placed into vials and analyzed using the headspace injection method. The separation was carried out on a DB-WAX-UI column (60 m × 250 µm × 0.25 µm). The oven temperature was programmed to increase from 45 °C to 250 °C at a rate of 2.615 °C per minute. The vial and transfer line temperatures were maintained at 130 °C and 150 °C, respectively. The chromatographic run lasted 120 minutes. Detection was achieved using a Flame Ionization Detector (FID) set at 280 °C. Compounds were identified by comparing their retention times with those of known standards.

II.6. Quantification of total polyphenol contents

Using the method described in the European Pharmacopeia, total polyphenol contents (TPC) in all extracts were quantified by spectrophotometry. Briefly, stock solutions of samples composed of 50 mg of extract in 5 mL of methanol were ultrasonicated for 2 X 15 min and centrifuged at 3000 rpm for 10 min. Before spectrophotometric analysis, 2 mL of each solution was transferred into a 25 mL volumetric flask, 1 mL of phosphomolybdotungstic reagent was added followed by 10 mL of water R. This was adjusted to 25 mL mark with a solution of sodium carbonate R. After 30 min in the dark, the absorbance was measured at 760 nm using a U-2900 spectrophotometer. Pyrogallol was used as a reference. A stock solution was prepared by dissolving 5.23 mg of pyrogallol in 100 mL of methanol and quantified as described above. The results were expressed as pyrogallol equivalents.

II.7. Detection and quantification of artemisinin

Artemisinin was identified and measured in all AN infusion samples (A1–A8) using an HPLC-UV technique after a derivatization process, as previously outlined by [Zeyuan et al. \(2018\)](#). In summary, 50 mg of each extract was dissolved in 5 mL of ethanol, sonicated for 30 minutes, and centrifuged at 3000 rpm for 10 minutes. Then, 1 mL of the resulting solution and the

artemisinin standard were each placed into a 10 mL volumetric flask. To this, 4 mL of 0.2% (m/v) NaOH was added, and the mixture was heated in a water bath at 50 °C for 30 minutes, resulting in the formation of Q292 (**Figure 34**). After cooling for 10 minutes, 1 mL of ethanol was added, and the volume was adjusted to 10 mL with 0.2 N acetic acid, leading to the formation of Q260, a compound with a UV absorption maximum at 260 nm. **Figure 34** shows the derivatization reaction that yields Q260, which was detected at 260 nm during HPLC analysis. All samples were filtered through a 0.45 μm HPLC filter, and separation was carried out using reverse-phase chromatography. The mobile phase was a 45:55 (v/v) mixture of methanol and phosphate buffer (5.0 mM, pH 6.3). Chromatographic conditions included a Luna PFP column (250 $\times$ 4.6 mm, 5 μm) maintained at 35 °C, a 20 μL injection volume, a 1 mL/min flow rate, and a 25-minute run time. A calibration curve with artemisinin concentrations of 10, 20, 40, and 60 mg/mL was used to quantify artemisinin in each sample.

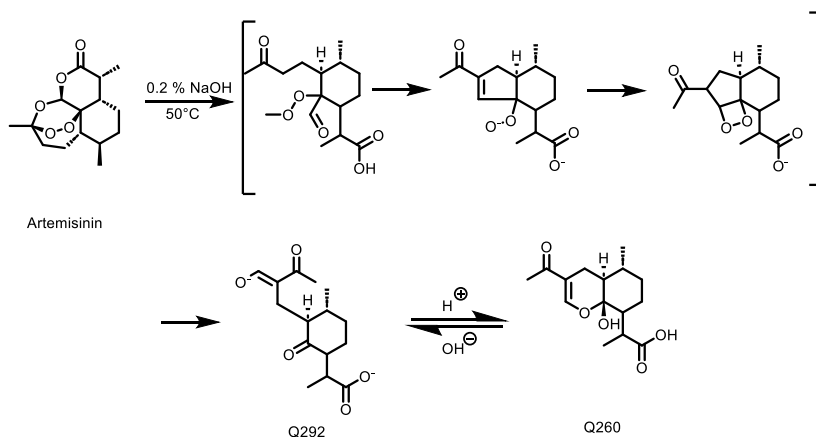


Figure 34: Quantitative reaction of artemisinin with dilute alkali. The condensation reaction of artemisinin in an alkaline solution and the subsequent chemical transformation to Q292 to Q260. The reaction conditions were 0.2% NaOH at 50°C, which selectively yields Q292 when artemisinin is treated with ethanol and 0.2% sodium hydroxide solution. Q292 and Q260 exhibit keto-enol tautomerism influenced by pH changes.

II.8. Antiplasmodial test

Antiplasmodial test of lyophilized extracts was carried out using a chloroquine-sensitive 3D7 *P. falciparum* strain (Malaria Research and Reference Reagent Resource Center, MR4). Parasites were maintained *in*

vitro (Trager and Jensen, 1976) in 3 % hematocrit (human type A or O-positive red blood cells), Complete Culture Medium (CCM) prepared by supplementing RPMI 1640 (Gibco, Fisher Scientific, Merelbeke, Belgium) medium containing NaHCO₃ (32 mM), HEPES (25 mM), and L-glutamine with 10 % heat-inactivated human plasma, 1.76 g/L of glucose (Sigma-Aldrich, Overijse, Belgium), 44 mg/mL of hypoxanthine (Sigma-Aldrich, Overijse, Belgium) and 100 mg/L of gentamycin (Gibco, Fisher Scientific, Merelbeke, Belgium). All cultures were placed in a humidified incubator at 37 °C with a standard gas mixture of 5 % O₂, 5 % CO₂, and 90 % N₂. Parasitemia was verified by microscopy using Giemsa-stained thin films. A 10 mg lyophilized extract was dissolved in 1 mL DMSO and diluted with culture media, and an anti-plasmodial test was performed in 96-well microtiter plates at an initial concentration of 100 µg/mL and serially diluted to eight concentrations (100 to 0.782 µg/mL). Each sample was tested in duplicate wells with the parasites at a final volume of 250 µL, making a final DMSO concentration of < 1 %. Negative control consisted of wells with culture medium only, while wells with parasites only served as the positive control. A 1 µg/mL stock solution of artemisinin (Gibco, Fisher Scientific, Merelbeke, Belgium) was prepared and serially diluted as above; this was used as a standard. After 48 h of incubation, parasite growth was estimated by determination of plasmodial lactate dehydrogenase activity as described by Kenmogne et al. (2006), and IC₅₀ was calculated by linear regression. All tests were repeated at least twice in technical duplicates.

III. Results

III.1. Yield of infusions

Generally, there was no observable pattern in the yield of extraction following the traditional preparation of both plant infusions. This suggests that post-harvest handling techniques do not affect the yield of extraction. However, for AN samples, the yield of extraction ranged from 165 mg (A3) to 241 mg (A8), while for the AF samples, the yield of extraction ranged from 136 mg (A10) to 229 mg (A13), **Figure 35**.

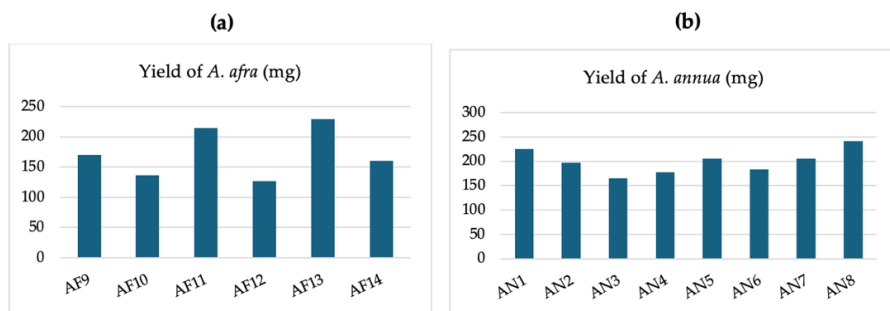


Figure 35: Yield of tea infusions (a) *A. annua* (AN1-AN8) and (b) *A. afra* (AF9, AF14). AN1, AN2 and AN8 (12:PM/fermented, sun, oven and shade-dried respectively), AN3, AN4, and AN7 (12:PM/non-fermented, shade, oven and sun-dried respectively), AN5 and AN6 (shaded dried 6: am and 6: pm time of harvest respectively), AF10, AF11 and AF13 (12:PM/non-fermented, shade, sun and oven-dried respectively), AF9, AF12 and AF14 (shaded dried 6: am and 6: pm time of harvest respectively) and A9 (12:PM/fermented and oven-dried).

III.2. HPTLC-fingerprint

High Performance Thin Layer Chromatography (HPTLC) fingerprint shows variation in the chemical composition of metabolites present in each sample. For the AN samples, post-harvest handling techniques impact both the polyphenol and volatile components (**Figure 36a, b and c**). For example, fermented samples (AN1, AN2, and AN8) show the absence of artemisinin (blue band) and of a green band, which were present in the non-fermented samples (AN3-AN7) (**Figure 36b**). Additionally, AN4 and AN7 had prominent bands than other samples (**Figure 36a**), indicating that the method of drying has an impact on both polyphenol and volatile chemical patterns. For the AF samples, post-harvest handling also influenced their chemical pattern. For instance, there was the absence of rutin, hyperoside, and chlorogenic acid in AF9, which were present in other samples (AF10-AF14). Also, AF9, AF10, AF12, and AF14 have faint bands compared to AF11 and AF13, which showed prominent bands (**Figure 36c**). This indicates the impact of post-harvest handling (PHH) techniques, especially fermentation and the method of drying, on the HPTLC-fingerprint.

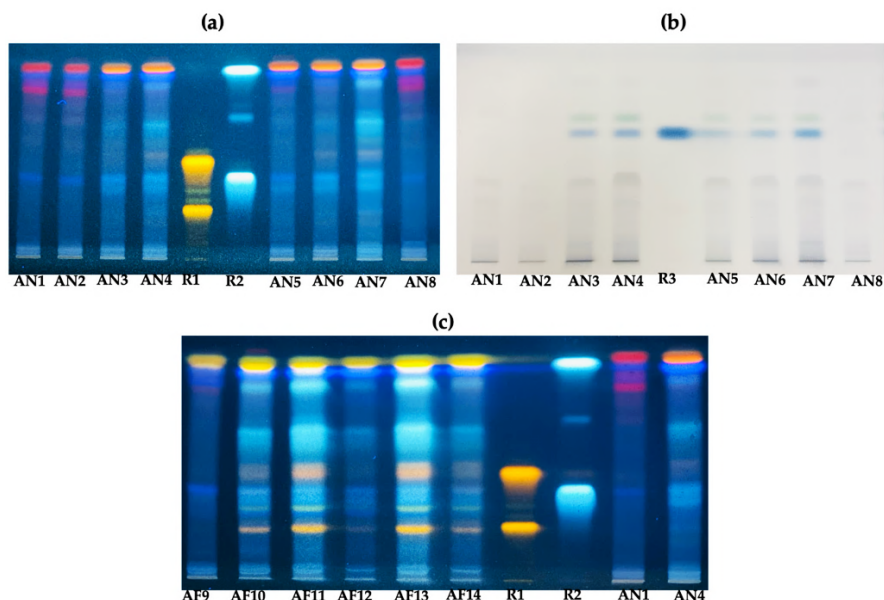


Figure 36: Effect of post-harvest handling on the HPTLC profile of (a) polyphenol chemical pattern of *A. annua*, (b) terpene composition of *A. annua*, and (c) polyphenol composition of *A. afra*. R1 (Rutin and hyperoside in order of increasing retention factor), R2 (chlorogenic acid and caffeic acid in order of increasing retention factor), R3 (artemisinin). AN1, AN2 and AN8 (12:00 pm/fermented, sun, oven and shade-dried respectively), AN3, AN4, and AN7 (12:00 pm/non-fermented, shade, oven and sun-dried respectively), AN5 and AN6 (shaded dried 6:00 am and 6:00 pm time of harvest respectively), AF10, AF11 and AF13 (12:00 pm/non-fermented, shade, sun and oven-dried respectively), AF9, AF12 and AF14 (shaded dried 6:00 am and 6:00 pm time of harvest respectively) and A9 (12:00 pm/fermented and oven-dried).

III.3. Impact of post-harvest handling methods on the UPLC fingerprint of infusions

UPLC analysis of all samples (AN1-AF14) shows the effect of PHH techniques on the metabolite contents of both plants. For both plants, fermentation negatively impacts the polyphenol profile while non-fermented samples show the best profile (**Figure 37**). However, regardless of whether fermented or not, the shade-drying method negatively impacted the polyphenol profile (**Figure 38 and 39**). The best profile was obtained for non-fermented and processed by either oven-drying (40°C) or the sun-drying method (**Figure 38**). It was also observed that the time of harvest did not affect the metabolite profile. By comparing the UV-spectrum and retention time (RT) of standard and those of analyte (A13), several polyphenols were identified, including

Chlorogenic acid, Caffeic acid, isochlorogenic acid A, B, and C, rutin, luteolin, hyperoside, and astragalin.

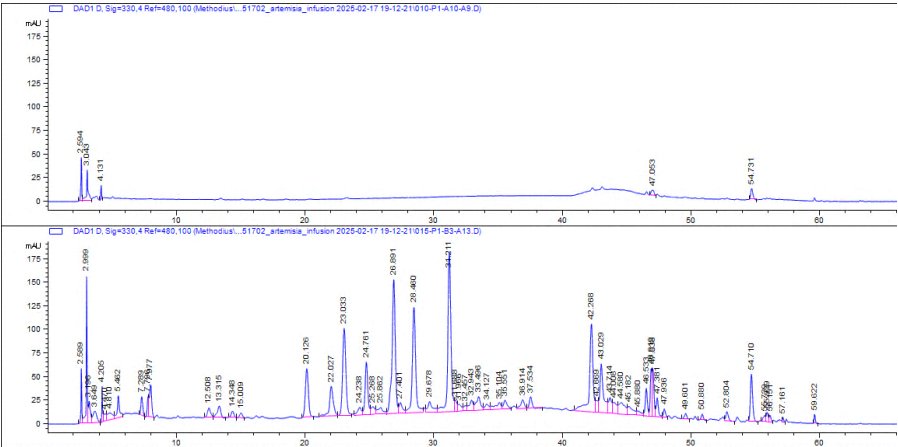


Figure 37: Impact of fermentation on the UPLC fingerprint of polyphenol composition of *A. afra*. AN9 = *A. afra* harvested at 12:00 pm, fermented and oven-dried (40°C), and A13 = *A. afra* harvested at 12:00 pm, non-fermented and oven-dried (40°C)

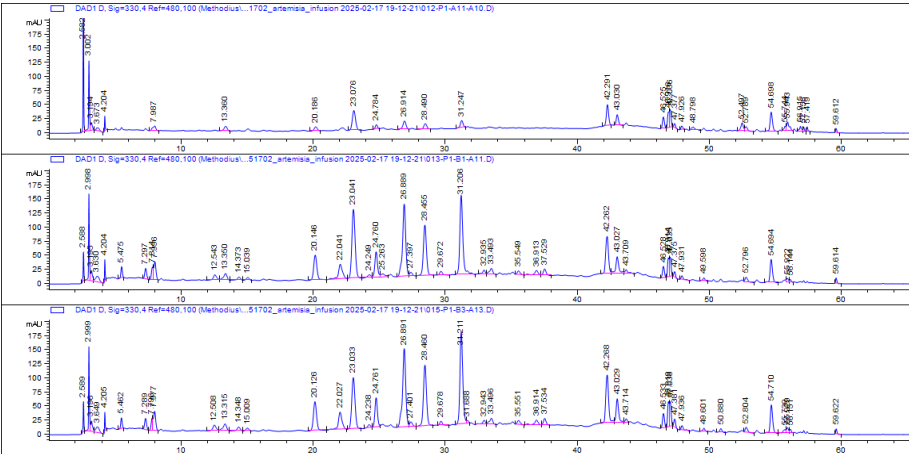


Figure 38: Effect of drying method on the UPLC-profile of polyphenol composition in *A. afra* infusions (A10, A11, and A13). AF10 = *A. afra* harvested at 12:00 pm, non-fermented and shade-dried, AF11 = *A. afra* harvested at 12:00 pm, non-fermented and sun-dried, and AF13 = *A. afra* harvested at 12:00 pm, non-fermented and oven-dried (40°C). Oven-drying and sun-drying methods show the best profile.

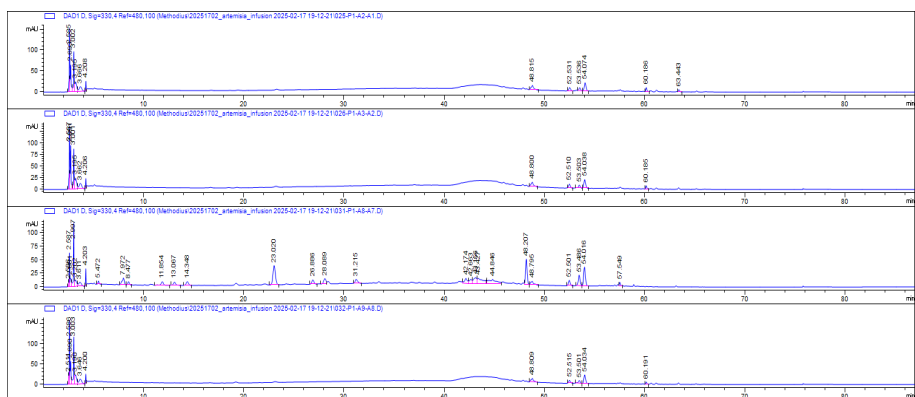


Figure 39: Impact of post-harvest handling techniques on the UPLC fingerprint of polyphenol composition of *A. annua*. AN1, AN2, and AN8 (12:00 pm/fermented, sun, oven, and shade-dried respectively) and AN7 (12:00 pm/non-fermented and sun-dried). Shade-drying method and fermentation negatively impact the polyphenol profile in *A. annua*, whereas sun-drying gives the best profile.

III.4. Percentage m/m equivalent of chlorogenic acid and its derivatives in the infusions

In addition to the metabolite fingerprint obtained using UPLC analysis, the contents of chlorogenic acid (CA) and derivatives in the infusions of all the samples were quantified. Standard chlorogenic acid at concentrations mentioned above was injected in the same sequence with the samples, and a calibration curve was generated and used to estimate the quantity of CA, and its derivatives, in the infusions and expressed as m/m equivalent of CA. The results presented in **Figure 40** show variations in the quantity of CA and its derivatives in infusions affected by post-harvest handling methods. For instance, except for AN4 and AN7, no content of CA nor its derivatives was seen in the *A. annua* samples, with A7 having the highest concentration (0.18% m/m equivalent of CA). For the *A. afra* samples, a high content of CA was observed as compared to *A. annua* samples. However, there were variations in the quantity of CA in the different samples, influenced by the post-harvest handling techniques. For example, while no content of CA was seen in the AF9 sample, AF10 - AF14 showed the presence of CA and its derivatives, with AF11 and AF13 showing the highest concentrations, respectively, of 1.98 to 2.20% m/m equivalent of CA (**Figure 40**).

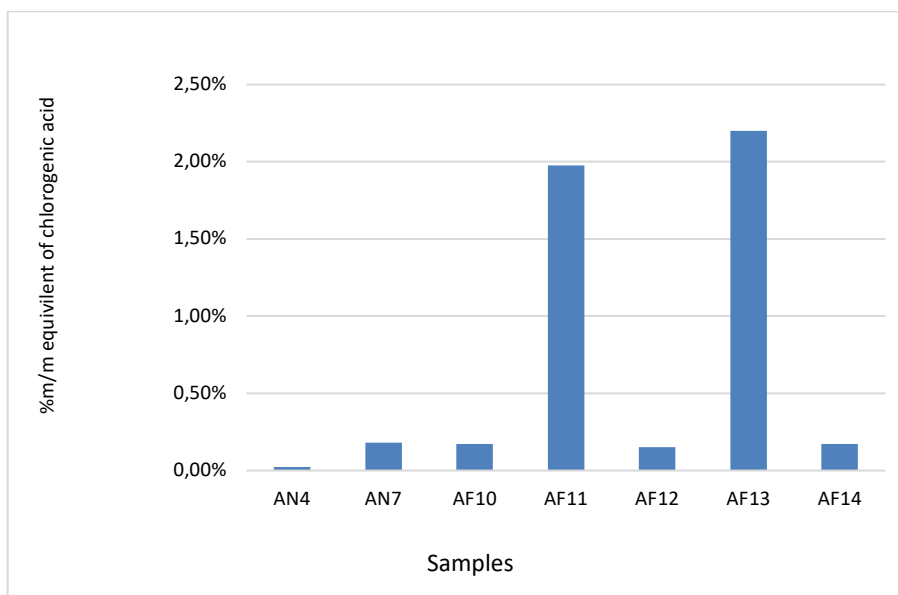


Figure 40: Post-harvest handling greatly impacts the concentration of CA and derivatives in infusion samples. AN4, and AN7 (12:00 pm/non-fermented, oven and sun-dried respectively), AF10, AF11 and AF13 (12:00 pm/non-fermented, shade, sun and oven-dried respectively), AF9, AF12 and AF14 (shade-dried 6:00 am and 6:00 pm time of harvest respectively). AN1, AN2, AN3, AN5, AN6, AN8 and AF9 had zero CA content. Method of drying had an influence on the concentration of CA and its derivatives, with oven and sun-drying presenting the highest concentrations.

III.5. Influence of post-harvest handling techniques on total polyphenol content in infusions

Total polyphenol contents (TPCs) in all samples were quantified as described in the European Pharmacopeia and expressed in pyrogallol equivalents. For the *A. annua* samples, TPCs ranged from 1.08 % to 2.85 % m/m in dry extract. Sample AN7 had the highest TPCs, which agrees with the prominent bands observed in the HPTLC fingerprint, while AN6 had the lowest (**Figure 41**). Sample AN15 (cold water infusion prepared using plant powder of sample AN7) had a lower TPC of 1.23% compared to AN7, 2.85 % indicating that hot water infusion extracted more polyphenols. Concerning the *A. afra* samples, the highest TPCs were seen in the AF11 sample (3.45% m/m in dry extract), whereas the least was found in AF14 (1.13% m/m in dry extract). Also, the high TPCs in AF11 align with the prominent bands observed following HPTLC fingerprint and UPLC analysis.

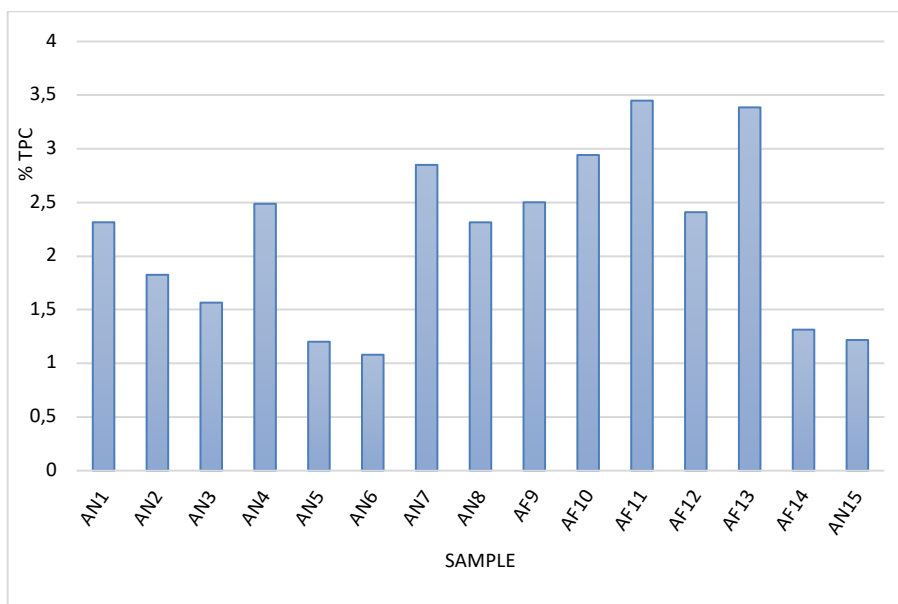


Figure 41. Impact of post-harvest handling practices on the total polyphenol contents (TPCs) expressed as % m/m polyphenol in dry extract. The sun-dried method showed high TPCs in both *A. annua* (AN7) and *A. afra* infusions (AF11). AN1, AN2 and AN8 (12:00 pm/fermented sun, oven and shade-dried respectively), AN3, AN4, and AN7 (12:00 pm/non-fermented, shaded, oven and sun-dried respectively), AN5 and AN6 (shaded dried 6:00 am and 6:00 pm time of harvest respectively), AF10, AF11 and AF13 (12:00 pm/non-fermented, shade, sun and oven-dried respectively), AF12, AF14 (non-fermented, shade-dried 6:00 am and 6:00 pm time of harvest respectively) and AF9 (12:00 pm/fermented and oven-dried) and AN15 (cold water infusion prepared using plant powder of sample AN7).

III.6. GC-FID profile of infusions

Gas chromatography analysis of lyophilized infusion samples was performed on an Agilent Intuva GC system by the heat space method to obtain the chemical profile of volatile compounds present in the infusion of both plants subjected to post-harvest handling methods. The first observation there was variation that existed between both plants, with cineole being one of the major components in the *A. afra* samples (**Figure 42**), whereas camphor (data not shown) was the main volatile compound in the infusions of *A. annua* (**Figure 42**). The results show that shaded and sun drying methods favor the concentration of volatile compounds, with the highest concentrations as compared to other drying methods. Several volatile compounds were identified by comparing the retention times of analytes to those of standard

reference compounds, including cineole (1), thujone (2), camphor (3), and terpineol (4) (**Figure 42**).

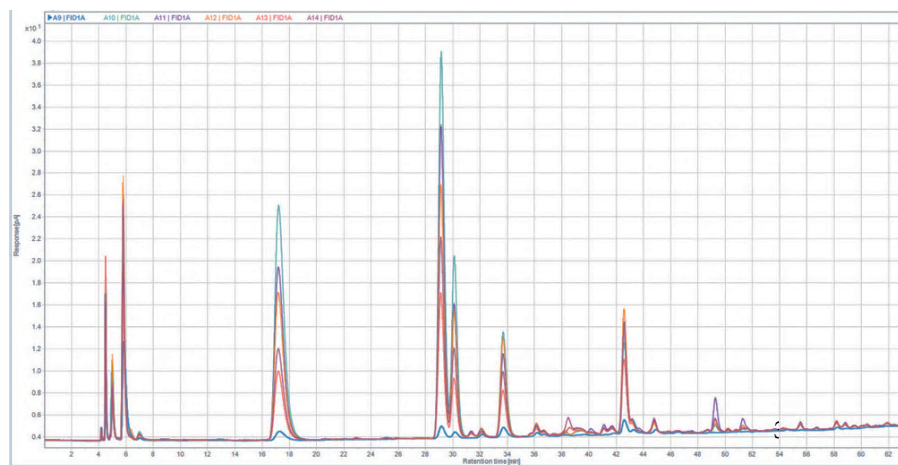


Figure 42: Effect of post-harvest handling techniques on the GC profile of *A. afra* infusions. Fermentation and the method of drying negatively impact the volatile components in AF infusions. Cineole (1), thujone (2), camphor (3), and terpineol (4). Legend on the top left of the figure in order: blue = AF9 (12:00 pm/fermented and oven-dried), green = AF10 (12:00 pm/non-fermented shade-dried), purple = AF11 (12:00 pm/non-fermented sun-dried), yellow = AF12 (non-fermented, shaded-dried, 6:00 am time of harvest), red = AF13 (12:00 pm/non-fermented oven-dried) and light purple = AF14 (non-fermented, shade-dried, 6:00 pm time of harvest).

III.7. Impact of post-harvest handling methods on artemisinin contents in *A. annua* infusions

Following the previously described derivatization procedure, the quantity of artemisinin in the *A. annua* samples (AN1 to AN8 and AN15) was estimated using the UPLC-UV approach. **Figure 43** displays UPLC chromatograms of *A. annua* infusion samples after derivatization, indicating sample AN7 (blue chromatogram) with the highest peak intensity for artemisinin at retention time 15.78). Standard reference artemisinin was used at various concentrations (0, 10, 20, 40, and 60 µg/mL) to develop a calibration curve needed to estimate the quantity of artemisinin in the infusions.

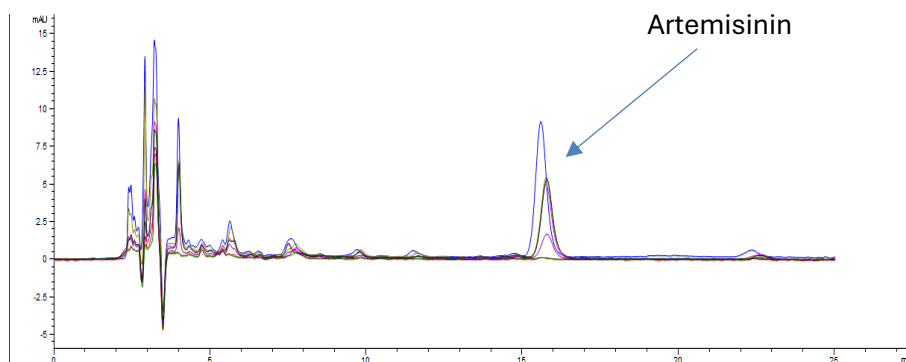


Figure 43: UPLC chromatograms of derivatized *A. annua* infusion samples. Sample AN7 (blue chromatogram) had the highest peak intensity demonstrating sun-dried as the method to dry *A. annua* samples.

The results show variations in the concentration of artemisinin, which is greatly influenced by post-harvest handling techniques, especially the method of drying and fermentation. The highest concentrations of artemisinin were seen in AN7 (9.86% m/m artemisinin in dry extract) compared to other samples (**Figure 44**) in line with the highest peak intensity in **Figure 43**. Fermented samples (AN1, AN2, and AN8) had zero artemisinin content (**Figure 44**), which is consistent with the HPTLC profile, regardless of the drying technique. Together with the infusions made as previously mentioned, sample AN15 was specially made by cold water infusion, which likewise revealed notable amounts of artemisinin (6.15% m/m artemisinin in dry extract), demonstrating that the sesquiterpene molecule is extracted using this technique.

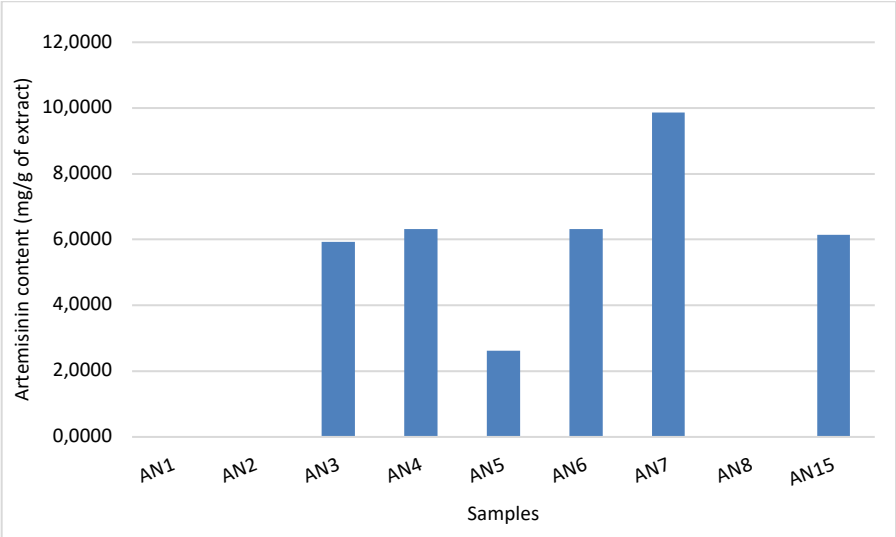


Figure 44: Impact of post-harvest handling conditions on the concentration of artemisinin in *A. annua* infusions. The sun-drying procedure presents the ideal conditions to guarantee a high concentration of artemisinin; however, both fermentation and drying have the largest influence. AN1, AN2, and AN8 (12: 00 pm/fermented sun, oven, and shade-dried respectively), AN3, AN4, and AN7 (12: 00 pm/non-fermented shaded, oven, and sun-dried respectively), AN5 and AN6 (shaded dried 6: 00 am and 6: 00 pm time of harvest respectively) and AN15 = Cold water infusion of AN7.

III.8. *In vitro* antiplasmodial activity of infusion

Antiplasmodial activity of all samples was performed on chloroquine-sensitive 3D7 *P. falciparum* parasites. Results in **Table 2** show inconsistency in antiplasmodial activity of the samples subjected to different post-harvest handling methods. Except for AN1, AN2, and AN8, which showed weak activity, all *A. annua* had strong antiplasmodial activity ($IC_{50} < 5\mu g/mL$). The non-fermented oven-dried sample (AN7) showed the best activity with an IC_{50} value of $0.34 \pm 0.13\mu g/mL$. Regarding the *A. afra* samples, AF9 was not active at the highest concentration tested, whereas samples AF11 and AF13 had weak antiplasmodial activity. These activities were, however, better for the *A. afra* samples. Nonetheless, the observed antiplasmodial activity was impacted by the way the plant sample was handled after harvest. On the other hand, time of harvest did not affect activity.

Table 5: *In vitro* antiplasmodial activity of infusion (n=3).

Sample code/standard drug	IC ₅₀ (µg/mL) ± SD
AN1	52.25 ± 2.05
AN2	47.18 ± 14.31
AN3	0.89 ± 0.26
AN4	0.92 ± 0.10
AN5	1.46 ± 0.40
AN6	0.99 ± 0.55
AN7	0.34 ± 0.05
AN8	66.06 ± 14.66
AF9	NA
AF10	62.42 ± 14.07
AF11	46.81 ± 2.12
AF12	67.38 ± 7.23
AF13	45.73 ± 3.73
AF14	77.33 ± 5.49
AN15	0.56 ± 0.13
Artemisinin	0.006 ± 0.002

AN1, AN2 and AN8 (12:00 pm/fermented, sun, oven and shade-dried respectively), AN3, AN4, and AN7 (12:00 pm/non-fermented, shade, oven and sun-dried respectively), AN5 and AN6 (shade-dried 6:00 am and 6:00 pm time of harvest respectively), AF10, AF11 and AF13 (12:00 pm/non-fermented, shade, sun and oven-dried respectively), AF9, AF12, AF14 (shade-dried 6:00 am and 6:00 pm time of harvest respectively) and A9 (12:00 pm/fermented and oven-dried and AN15 (cold water infusion prepared using sample AN7), NA = Not active.

IV. Discussion

Artemisia afra and *Artemisia annua*, known for their ethnopharmacological and ethnomedicinal importance, are cultivated, processed, and sold in local markets for the treatment of diseases, and in particular malaria. Despite their widespread use, numerous reports show that herbal products available to consumers are of variable quality. This disparity in quality of herbal preparations can be attributed to the fact that their production is complex (Tanko et al., 2005). In addition to variations in the phytochemical profile of AN and AF, which were attributed to both seasonal and geographical origin of harvest (Shinyuy et al., 2025), both plants' secondary metabolites can be influenced by post-harvest handling practices. In this context, batch-to-batch

variations resulting from these practices (including the method of drying) can cause inconsistency and or effectiveness of the medicinal plants in treating malaria. Based on these observations, the study aimed to investigate the impact of post-harvest handling conditions on the metabolite contents of AN and AF infusions and their efficacy against malaria parasites. This is to develop and optimize the best post-harvest handling practices that will ensure both batch-to-batch consistency in their chemical composition and hence consistent and improved pharmacological effect. To achieve this objective, plant samples were harvested as described above, subjected to various post-harvest handling practices, and the metabolite content of infusions of all samples was analyzed by chromatography techniques. Additionally, antiplasmodial tests were conducted on a chloroquine-sensitive Pf3D7 strain and correlated to the observed chemical profile.

In the present study, there was variation in the metabolite contents of both plants subjected to different post-harvest handling practices, resulting in inconsistent antiplasmodial activity. For example, the HPTLC profile shows a complete loss of artemisinin and some volatile compounds in the fermented samples of AN infusions (**Figure 36b** and **Figure 43**). The loss of the sesquiterpene molecule could be due to enzymatic oxidation during the fermentation process and fungal contamination, leading to degradation, potentially reducing the artemisinin levels ([Laughlin, 2002](#)).

For the non-fermented and dried by shade, oven, and sun samples, artemisinin was spotted in the HPTLC profile (**Figure 36b**), and quantification indicated high concentrations in the sun drying method (AN7: 9.86% m/m artemisinin in dry extract) while shaded and oven-drying methods had comparable content (**Figure 43**). This is consistent with the findings of [Ferreira and Luthria \(2010\)](#), who showed an average bioconversion rate of 43% for oven, shade-dried and 94% for sun-dried leaves of dihydroartemisinic acid to artemisinin. This points to the fact that sun-drying improves this bioconversion and hence high concentrations of artemisinin as seen in this study. Dihydroartemisinic acid (the main biosynthetic precursor of artemisinin) undergoes a process called photooxidation to artemisinin, catalyzed by sunlight, resulting in high artemisinin content of AN dried by sunlight ([Sy and Brown, 2002](#)). In contrast, shade-drying is a long-term process, and this prolonged drying method, especially in regions with high

tropical humidity, may cause fungal contamination of leaves and potentially reduce artemisinin levels (Laughlin, 2002).

Processing, including fermentation, enzymatic treatment, thermal, and non-thermal treatments, has a significant influence on the yield of phenolic compounds (Chen et al., 2013). The findings of Chen et al. (2013) point out that phenolic content decreases during processing, especially fermentation and the method of drying, probably through microbial degradation and hydrolysis of glycosidic bonds (Zhao et al., 2025). Generally, AF infusions showed better HPTLC (Figure 36c) and UPLC profiles with several phenolic acids compared to AN samples in this study. It was observed that both fermentation and shade-drying methods negatively impacted the profile of both plants with reduced concentrations of CA and its derivatives (Figure 40). There was a total loss of rutin, hyperoside, and chlorogenic acid in the HPTLC profile of fermented AF samples (Figure 36c). Estimation of the content of CA and its derivatives indicated higher concentrations in the non-fermented sun-dried samples for both plants (AN7 and AF11) (Figure 40). Long-term drying methods may cause beneficial metabolites to oxidize and degrade, which would reduce their content, as in the case of the shade-drying method (Ryu et al., 2025). The observed increase in chlorogenic acid and its derivatives in the non-fermented sun-dried samples could be because plant tissue undergo stress when exposed to UV-light and in response triggers photo-protective mechanisms that lead to increased production of phenolic compounds such as chlorogenic acid and its derivatives (Moyo et al., 2022).

For volatile compounds in both AN and AF infusions, the profile of AF infusions indicated the presence of several volatile compounds, with cineole as one of the major compounds (Figure 42), whereas camphor was the major volatile compound in AN infusions. The profiles of both plant samples were impacted by the method of drying. However, fermented samples showed a profile with an important loss of compounds compared to non-fermented samples, as seen both in the HPTLC (Figure 36b) and GC fingerprints (Figure 42). In contrast to other compounds (artemisinin, chlorogenic acid, and other polyphenolic compounds), shade-drying showed a better profile of volatile compounds in comparison with the oven-drying method. Farmanpour Kalalagh et al. (2023) also reported that shade-drying favors the concentration of volatile components while the oven-drying method leads to a decrease in the intensity of peaks in the GC-FID profile of both plant samples

(Khangholil and Rezaeinodehi, 2008). This is mainly due to the increase in oxidation processes induced by high temperature, leading to breakdown and reduced concentration (Bita and Gerats, 2013).

The observed variations in the content of metabolites, especially the content of artemisinin, chlorogenic acid, and their derivatives, among others, resulted in changes in the pharmacological effect of infusions of these medicinal plants. This study pointed out that, except for the fermented samples, all AN infusions showed strong antiplasmodial activity with IC_{50} values $< 5 \mu\text{g/mL}$. Ashraf et al. (2022); Snider and Weathers (2021) also reported the *in vitro* antiplasmodial properties of infusions made with *A. annua*, including activity against *Plasmodium* gametocytes. For the AN samples, AN1, AN2, and AN8 had weak antiplasmodial activity (IC_{50} values $> 45 \mu\text{g/mL}$), whereas samples AN3 to AN7 had strong antiplasmodial activity, which correlates with the observed metabolite profile. The weak antiplasmodial activity in AN1, AN2, and AN8 samples is largely due to the loss of artemisinin contents and other polyphenolic compounds (Figure 39 and 43). Nonetheless, the strong activity in AN3 to AN7 samples supports the hypothesis that artemisinin is a potent antimalarial compound in *A. annua* (Ashraf et al., 2022). In this study, AN7 demonstrated the best antiplasmodial activity with the lowest IC_{50} value (Table 2), which correlates with the highest artemisinin content (Figure 44) and the most concentrated phenolic profile, emphasizing the importance of the sun-drying method. Also, we showed that cold water infusions (AN15) extracted artemisinin from *A. annua* (Figure 44) and showed strong antiplasmodial effects compared to other AN samples (Table 2). Generally, we observed weak *in vitro* antiplasmodial properties for the *A. afra* samples compared to *A. annua* samples that had a strong antiplasmodial effect. However, samples AF11 and A13 (non-fermented and dried by sun and oven, respectively) had better antiplasmodial activity, which correlates with the highest CA and polyphenol content (Figure 40), including a better phenolic profile as compared to other *A. afra* samples (Figure 38). Phenolic compounds can interfere with the malaria parasite's life cycle and reduce its ability to cause disease by triggering oxidative stress (Mamede et al., 2020). Moreover, quercetin and its analogues, luteolin, rutin, kaempferol, and scopoletin (a coumarin), naturally found in *A. afra* (Shinyuy et al., 2025), have been reported to inhibit the development of *Plasmodium falciparum* (Nchang et al., 2023). Coumarins content has been shown to significantly increase by the

sun-drying method (Ba et al., 2016). Sample AF9 was not active at the highest concentration tested (IC_{50} values > 100 $\mu\text{g/mL}$), which is in line with its zero content of CA and poorer phenolic profile (Figure 38 and 40). This demonstrates the effect of post-harvest handling practices of infusions of *A. annua* and *A. afra* herbal products as evidenced by the metabolite profile and the inconsistent antiplasmodial activities.

V. Conclusion

The drying of medicinal plants is essential for their preservation. However, drying protocols must be carefully optimized to prevent any reduction in phytochemical concentrations. The findings of this study demonstrate that post-harvest handling practices significantly influence the secondary metabolite profiles of *Artemisia annua* and *Artemisia afra*. Regardless of the harvest period, sun-drying was found to enhance the metabolite fingerprints, particularly concerning terpene and polyphenol composition. Notably, the contents of artemisinin, chlorogenic acid, and total polyphenols increased following sun-drying, which in turn correlated with improved pharmacological activity. These results support sun-drying as an effective method for preserving and enhancing the therapeutic potential of *A. afra* and *A. annua* in the fight against malaria. Nevertheless, further investigations into other post-harvest handling techniques are warranted to generate more comprehensive data for optimizing the quality and efficacy of herbal medicinal products.

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Chapter Four

Synergistic interactions between *Artemisia afra* and *Artemisia annua*

4.1 Foreword

Growing evidence indicates that extracts from *Artemisia afra* and *Artemisia annua* exert antiplasmodial activity through distinct and potentially complementary mechanisms. While *Artemisia annua* activity is primarily attributed to artemisinin-mediated oxidative damage, *Artemisia afra*, which lacks artemisinin, has been proposed to interfere with parasite redox balance, mitochondrial function, and stress-response pathways ([Mamede et al., 2025](#)). These mechanistic differences provide a strong biological rationale for investigating whether combinations of the two extracts can result in synergistic interactions. Synergistic drug interactions are of particular interest in antimalarial therapy, as they may enable dose reduction, enhance efficacy, and limit the emergence or spread of resistance. Importantly, synergy should be distinguished from simple dose additivity or maximal inhibition. Synergistic interactions frequently emerge at intermediate effect levels and reflect genuine pharmacological interactions between compounds rather than the dominance of a single agent at high concentration ([Tang et al., 2015](#)).

4.2 Synergistic interactions between *Artemisia afra* and *Artemisia annua*: Rationale for combining *Artemisia afra* and *Artemisia annua*

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Abstract

Background: Accumulating evidence indicates that *A. afra* and *A. annua* exert antiplasmodial effects through mechanistically distinct, yet potentially complementary, biological pathways. Whereas the activity of *A. annua* is largely attributed to artemisinin-driven oxidative damage to parasite biomolecules, *A. afra* which contains no artemisinin has been proposed to modulate parasite survival by disrupting redox homeostasis, impairing mitochondrial function, and altering stress-response mechanisms.

Aim: The primary objective of this work was to systematically evaluate the interaction between a methanolic extract of *Artemisia afra* and an acetone extract of *Artemisia annua* against *Plasmodium* parasites using a quantitative synergy framework.

Methods: Methanol and acetone extracts of *Artemisia afra* and *Artemisia annua*, respectively were prepared by maceration. By applying multiple reference models implemented in SynergyFinder Plus, the synergistic landscape was characterized against a chloroquine-sensitive *Plasmodium falciparum* 3D7 strain, across a broad concentration matrix and identify combinations of biological and translational relevance.

Result: A clear global synergistic signal was observed across all four models, with positive mean synergy scores and highly significant p-values. Mean synergy scores reached 19.51 for ZIP, 11.85 for HSA, 11.97 for Loewe, and 19.63 for Bliss. The magnitude of synergy varied between blocks; Block 1 consistently displayed the strongest and most coherent synergistic pattern.

Several combinations combining strong synergy with intermediate inhibition were identified. Notably, the combination of 15 µg/mL *A. afra* and 0.05 µg/mL *A. annua* produced 53.73% inhibition while maintaining a high Bliss synergy score of 48.94

Conclusion: This study highlights key biologically relevant interaction windows that hold significant implications for resistance management and future translational research. Collectively, these insights establish a strong foundation for follow-up investigations employing herbal preparations, resistance-oriented assays, and *in vivo* experimental models.

I. Introduction

Malaria remains a major global health threat, with an estimated 263 million cases and more than half a million deaths recorded in 2023. Sub-Saharan Africa bears the overwhelming majority of this burden, accounting for over 95% of global cases and 96% of malaria-related deaths ([World Health Organisation, 2024](#)). The disease disproportionately affects vulnerable populations, particularly children under five, pregnant women, and migrant workers in endemic regions ([Venkatesan, 2025](#)). To improve treatment outcomes and curb resistance, the World Health Organization recommends Artemisinin-based Combination Therapies (ACTs) as the first-line regimen. ACTs pair a fast-acting artemisinin derivative with a second drug of a different mechanism, thereby lowering the risk of resistance and treatment failure by forcing the parasite to overcome multiple evolutionary barriers. However, the emergence and spread of drug resistant strain of the parasite to the current therapy poses a major draw back in malaria control strategy especially in Sub-Saharan African countries ([Venkatesan, 2025](#)). This set back amongst other factors have increase the use of traditional medicinal herbs especially *Artemisia annua* and *Artemisia afra* as therapeutic alternatives against malaria ([Shinyuy et al., 2025](#)). These medicinal plants are increasingly cultivated in malaria endemic regions and use in the treatment of malaria among other ailments. Despite their potentials in the management of malaria, these medicinal plants face challenges and have been controversially advocated by many researchers for use in the management of malaria. The primary reason is centered around the delivery of sub-therapeutic doses of artemisinin in the traditional preparations of *A. annua* for management of malaria ([Liu et al., 2006](#); [World Health Organisation, 2012](#)). Nonetheless,

there have been debates in the past decade regarding the role of artemisinin and chemical composition of a traditional preparation of both plants in the fight against malaria (Mamede et al., 2025). However, recent findings show variations in the chemical profile of both plants with no content of artemisinin in *A. afra* compared to the significantly higher concentrations in *A. annua* (Shinyuy et al., 2025). Additional investigations shows that the profile of *A. afra* is rich in polyphenols compared to *A. annua* and appears to modulate lipid-related precursors and signaling mechanisms in the parasite metabolic path ways compared to *A. annua* whose antiplasmodial activity is predominantly driven by its artemisinin content, consistent with this compound's known impact on hemoglobin digestion and redox-related metabolic pathways (Mamede et al., 2025). These divergent modes of action provide a compelling rationale for exploring whether combining the two extracts can yield synergistic interactions rather than simple additive effects. Synergy is of particular importance in antimalarial pharmacology because it can enhance efficacy, reduce the doses required for therapeutic effect, and help delay or prevent the emergence of drug resistance. Crucially, synergistic interactions must be distinguished from dose additivity or maximal inhibition achieved by a dominant compound. True pharmacological synergy typically emerges at intermediate effect levels, where combined agents interact in a manner that produces a biological response greater than the sum of their independent effects (Tang et al., 2015). Such interactions reflect genuine mechanistic complementarity rather than mere potency overlap, underscoring the value of rigorously evaluating combination therapies involving *A. afra* and *A. annua*.

The primary objective of this work was to systematically evaluate the interaction between a methanolic extract of *Artemisia afra* and an acetone extract of *Artemisia annua* using a quantitative synergy framework. By applying multiple reference models implemented in SynergyFinder Plus, we aimed to characterize the synergy landscape across a broad concentration matrix and to identify combinations of biological and translational relevance. Beyond establishing the presence of synergy, this study sought to determine whether such interactions could support an artemisinin-paring effect, thereby reducing reliance on high concentrations of *A. annua*. The identification of synergistic combinations producing intermediate levels of inhibition was also intended to inform future mechanistic investigations and

resistance-focused assays, including evaluation against artemisinin-partially resistant parasites.

II. Materials and Methods

II.1. Preparation of crude extract

Methanolic and acetone crude extracts of *Artemisia afra* and *Artemisia annua*, respectively were prepared by maceration (Mbah et al., 2012) from plants harvested in the West region of Cameroon.

II.2. Preparation of extract combinations and experimental design

Drug combination assays were conducted using two plant extracts: a methanolic extract of *Artemisia afra* (extract A) and an acetone extract of *Artemisia annua* (extract B). Combination experiments followed a fixed experimental design based on serial dilutions. Stock solutions were prepared at defined concentrations and further diluted to generate intermediate and working solutions. Working solutions were adjusted to ensure accurate final concentrations in assay wells while maintaining constant solvent conditions across all treatments. Final concentrations were obtained by combining working solutions of extracts A and B according to a predefined concentration matrix. This matrix included single-extract conditions as well as combination treatments and was designed to span a broad range of concentrations for both extracts, allowing robust evaluation of dose–response relationships and extract–extract interactions. Following the initial formulation, minor adjustments were introduced by adding supplementary dose levels to improve coverage of the concentration space and ensure compatibility with downstream synergy analysis. Final concentrations tested for each extract are summarized in **Table 6**.

Table 6. Final concentrations of *Artemisia* extracts tested in single-agent and combination assays.

<i>Artemisia afra</i> methanolic extract (µg/mL)	<i>Artemisia annua</i> acetone extract (µg/mL)
0.0	0.000
0.5	0.001
1.5	0.003
5.0	0.010
15.0	0.030

Combination conditions were distributed across multi-well plates according to predefined layouts to minimize positional effects. Each plate included vehicle controls, single-extract treatments, and combination conditions. All experimental conditions were tested in three independent biological replicates ($n = 3$).

II.3. Assessment of parasite growth inhibition

A chloroquine-sensitive 3D7 *P. falciparum* strain was maintained *in vitro* in 3 % hematocrit (human type A or O-positive red blood cells), described by [Trager & Jensen \(1976\)](#), and parasite growth inhibition was quantified using the APAD colorimetric assay ([Kenmogne et al., 2006](#)), performed according to standard procedures routinely applied throughout this thesis. Inhibition percentages were calculated relative to vehicle controls.

II.4. Data cleaning and outlier removal

Raw inhibition data were obtained from three independent biological replicates for each experimental condition. Prior to analysis, data were subjected to a systematic cleaning procedure to minimize the influence of aberrant measurements. For each condition, outlier detection was performed on the three individual inhibition values using an interquartile range (IQR)-based approach. Values lying outside the interval defined by $Q1 - 1.5 \times IQR$ and $Q3 + 1.5 \times IQR$ were considered outliers and excluded from further analysis. Following outlier removal, inhibition percentages were recalculated as the mean of the remaining replicates. This procedure was applied uniformly across all datasets.

II.5. Synergy analysis

Synergistic interactions were evaluated using SynergyFinder Plus ([Zheng et al., 2022](#)). Synergy scores were calculated using four complementary reference models: Bliss independence, Loewe additivity, highest single agent (HSA), and zero interaction potency (ZIP). Each model classifies interactions as synergistic, additive, or antagonistic by comparing experimentally observed responses with model-specific expected effects. Synergy scores were interpreted using commonly accepted thresholds: values below -10 indicate antagonism, values between -10 and $+10$ indicate additive effects, and values above $+10$ indicate synergistic interactions. Multiple models were

applied to account for the complexity of potential mechanisms of action, as no single model can fully capture all types of extract–extract interactions.

III. Results

III.1. Synergy analysis of *Artemisia afra* and *Artemisia annua* combinations

The interactions between *A. afra* and *A. annua* extracts were evaluated across three independent experimental blocks using SynergyFinder Plus. Synergy was quantified using four reference models (ZIP, HSA, Loewe, and Bliss). As the two extracts were previously shown to act through distinct mechanisms ([Mamede et al., 2025](#)), the Bliss independence model was selected for detailed interpretation.

III.2. Overall synergy trends A clear global synergistic signal was observed across all four models, with positive mean synergy scores and highly significant p-values. Mean synergy scores reached 19.51 for ZIP, 11.85 for HSA, 11.97 for Loewe, and 19.63 for Bliss (**Figure 45**). Although the magnitude of synergy varied between blocks, Block 1 consistently displayed the strongest and most coherent synergistic pattern and was therefore used as the primary dataset for detailed analysis.

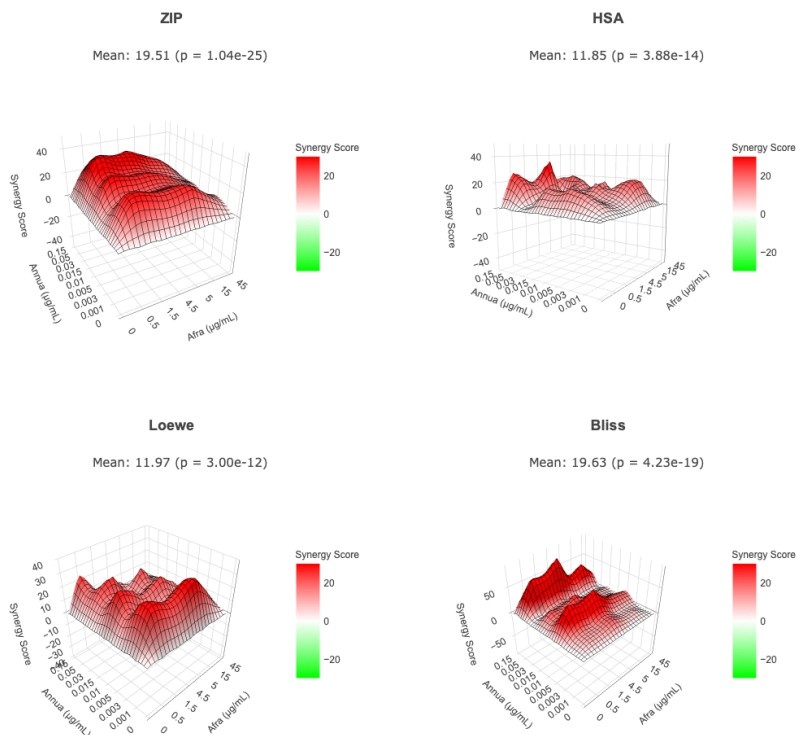


Figure 45. Mean synergy scores obtained for *A. afra*–*A. annua* combinations using ZIP, HSA, Loewe, and Bliss models across experimental blocks.

III.3. Relationship between synergy and growth inhibition

Analysis of the Bliss synergy landscape revealed that the highest synergy scores did not coincide with maximal growth inhibition. The strongest Bliss synergy score (81.31) was observed for the combination of 4.5 $\mu\text{g/mL}$ *A. afra* and 0.05 $\mu\text{g/mL}$ *A. annua*, which resulted in only 23.28% inhibition (**Figure 46**). This demonstrates a clear dissociation between synergy magnitude and inhibitory efficacy.



Figure 46. Bliss synergy landscape and corresponding inhibition matrix for *A. afra* - *A. annua* combinations.

Conversely, near-maximal inhibition (>70–80%) was primarily associated with high concentrations of one or both extracts, reflecting intrinsic dose-dependent effects rather than optimized synergistic interactions.

III.4. Identification of biologically relevant synergistic combinations

Several combinations combining strong synergy with intermediate inhibition were identified. Notably, the combination of 15 µg/mL *A. afra* and 0.05 µg/mL *A. annua* produced 53.73% inhibition while maintaining a high Bliss synergy score of 48.94. In addition, combinations involving high *A. afra* concentrations with very low *A. annua* concentrations consistently produced inhibition levels comparable to those achieved with much higher *A. annua* doses, indicating a potentiation effect.

III.5. Model-dependent behavior of synergy scores

While Bliss, ZIP, and HSA models revealed broadly consistent synergistic regions, the Loewe model yielded lower and more conservative scores. This divergence is consistent with the assumptions underlying the Loewe model and further supports the relevance of the Bliss-based analysis for these extracts.

V. Discussion

The present study demonstrates that the methanolic extract of *Artemisia afra* can synergistically potentiate the antiparasmodial activity of the acetone extract of *Artemisia annua* across a broad range of concentrations. Using multiple synergy reference models, a consistent synergistic signal was observed, with the Bliss independence model revealing particularly robust and biologically meaningful interaction patterns. This is in line with previous evidence showing that *A. afra* and *A. annua* act through distinct and complementary mechanisms ([Mamede et al., 2025](#)), thereby fulfilling the key assumptions underlying the Bliss model.

A central finding of this work is the clear dissociation between synergy scores and absolute levels of parasite growth inhibition. The highest synergy score was detected at moderate inhibition levels, whereas near-complete inhibition was mainly achieved through high concentrations of one or both extracts. This observation highlights an important conceptual distinction: synergy reflects an interaction between compounds that exceeds additivity, rather than the magnitude of the biological response itself. Similar observations have been reported in other drug combination studies, where maximal synergy frequently occurs at intermediate effect levels rather than at saturation ([Tang et al., 2015](#); [Yadav et al., 2015](#)). This distinction is particularly relevant in the context of antimalarial drug development, where dose escalation alone is neither a sustainable nor a desirable strategy. Instead, combinations that achieve meaningful inhibition at reduced doses are of greater translational value, especially when toxicity, cost, and resistance selection are considered.

One of the most striking outcomes of this study is the identification of combinations in which low concentrations of *A. annua* extract, when combined with *A. afra*, produce inhibition levels comparable to those obtained with much higher *A. annua* doses. Given that artemisinin and its

derivatives are the principal active compounds in *A. annua*, these findings strongly suggest an artemisinin-pairing effect mediated by components of *A. afra*. Such an effect is highly relevant in the current context of emerging artemisinin partial resistance in *Plasmodium falciparum*, particularly in Southeast Asia and increasingly in Africa (Dondorp et al., 2009; Ashley et al., 2014). Resistance is characterized by delayed parasite clearance and reduced susceptibility at the early ring stage, phenomena that are not adequately addressed by simply increasing artemisinin exposure. Combination strategies that enhance efficacy without increasing artemisinin pressure may therefore represent a valuable complementary approach.

A plausible contributor to the potentiation observed in *Afra*–*Annua* combinations is the flavonoid-rich phytochemical background of *A. afra*. *Artemisia* species contain diverse flavonoids (including luteolin, apigenin, quercetin and kaempferol-derivatives), several of which display biological activities that may modulate antiplasmodial responses and stress pathways. Importantly, increasing evidence indicates that artemisinin delivered within a plant matrix can be more effective than the purified compound, and that the co-occurrence of artemisinin with flavonoids in *A. annua* dried leaves may contribute to enhanced efficacy and/or improved bioavailability. In support of this concept, flavonoids have been reported to act synergistically with artemisinin derivatives in experimental malaria models, and mechanistic work suggests that plant phytochemicals can increase systemic exposure to artemisinin, at least in part through inhibition of CYP450-mediated first-pass metabolism. In addition, the plant matrix itself can influence flavonoid uptake; for example, studies discussed in recent reviews indicate that luteolin forms from *A. afra* may be absorbed more efficiently when presented within an aqueous plant extract than as isolated standards, supporting the idea that *A. afra* could contribute bioactive modulators beyond direct antiplasmodial activity. Collectively, these observations provide a biologically credible framework to investigate whether *A. afra* flavonoids participate in the observed interaction landscape by enhancing artemisinin-related efficacy (pharmacodynamic potentiation) and/or by modulating exposure (pharmacokinetic effects), which could be directly tested using standardized *A. afra* tea preparations combined with purified artemisinin.

Another important outcome is the identification of combinations displaying strong synergy at intermediate inhibition levels (approximately 50%). From a mechanistic standpoint, such conditions are particularly informative. At near-complete inhibition, cellular pathways may be broadly disrupted, obscuring specific molecular interactions. In contrast, partial inhibition allows finer dissection of parasite stress responses, redox imbalance, metabolic interference, or signaling perturbations induced by the combination. This approach aligns with current recommendations in antimalarial research, which emphasize the importance of studying drug effects under sublethal pressure to better understand mechanisms of action and resistance (Witkowski et al., 2013; Mok et al., 2015).

Building on these results, several perspectives emerge. First, replacing laboratory extracts with lyophilized herbal teas of *A. afra* represents a logical next step. Infusions are the most widely used and culturally relevant form of *Artemisia*-based treatments, and previous studies have shown that *A. annua* teas can exhibit antiplasmodial activity that cannot be fully explained by artemisinin content alone (Suberu et al., 2013; Elfawal et al., 2015). Testing *A. afra* tea in combination with purified artemisinin would directly address whether the observed synergy can be reproduced in a more realistic and translational formulation. Secondly, the most promising combinations identified in this study should be evaluated against artemisinin-partially resistant parasites using the ring-stage survival assay (RSA). The RSA has become the gold standard for assessing artemisinin resistance phenotypes (Witkowski et al., 2013) and applying it to selected *Afra*/artemisinin combinations would provide critical insight into whether synergy can overcome or mitigate resistance-associated survival mechanisms. Finally, the most biologically and pharmacologically relevant combinations identified *in vitro* warrant evaluation *in vivo*, particularly in murine malaria models. *In vivo* testing would allow assessment of pharmacokinetics, host–parasite interactions, and potential immunomodulatory effects of *A. afra*, which have been suggested but remain insufficiently explored. Importantly, *in vivo* validation is essential to determine whether the synergistic effects observed *in vitro* translate into improved therapeutic outcomes.

V. Conclusion

This chapter provides evidence that *Artemisia afra* extracts can synergistically enhance the antiparasitic activity of *A. annua* at reduced doses, supporting the concept of artemisinin-paring combinations. By emphasizing synergy rather than maximal inhibition, this study identifies biologically meaningful interaction windows with clear relevance for resistance management and future translational applications. Together, these findings lay the groundwork for subsequent studies using herbal preparations, resistance-focused assays, and *in vivo* models.

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Chapter Five

Isolation and Characterisation of Antiplasmodial and Gametocytocidal Compounds from *Artemisia afra*

5.1 Foreword

Malaria continues to exert a serious public health and socio-economic burden on the affected communities. This is largely due to increasing resistance of *Plasmodium* to all currently available antimalaria treatments including ACTs and the widespread of mosquito's resistance to insecticides. However, one of the most critical challenges in malaria elimination remains the absence of any approved drug specifically targeting gametocytes, the transmissible stage of the parasite. This gap in treatment is particularly concerning because asymptomatic carriers, who harbour the parasites without experiencing symptoms, serve as silent reservoirs of transmission, unknowingly spreading the disease without seeking treatment. Effective gametocidal agents are therefore crucial to breaking the transmission cycle and achieving malaria elimination.

In addition to its activity against asexual *Plasmodium* parasite, *Artemisia afra* has equally demonstrated significant anti-gametocytocidal properties ([Moyo et al., 2019](#)). Due to this observation, we sought to isolate and characterise molecules from *A. afra* with dual stage antiplasmodial activity. A bioassay-guided fractionation approach was employed, and the isolated molecules were characterised by the analysis of spectral data and comparison with literature.

5.2 Isolation and Characterisation of Antiplasmodial and Gametocidal Compounds from *Artemisia afra*

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Abstract

Background: Natural products continue to be an important source of compounds used for the development of modern therapeutics. *Artemisia afra* belongs to the Asteraceae family has a long history of use for the management of ailments particularly, malaria. The aim of this study was to isolate and characterize antiplasmodial and gametocidal compounds from *A. afra* through a bioassay-guided fractionation approach.

Materials and methods: Acetone extract of *A. afra* was fractionated using open column chromatography and preparative thin layer chromatography. The fractions were tested on a chloroquine-sensitive *Plasmodium falciparum* 3D7 strain and NF-54S *Plasmodium* gametocytes. Structure elucidation of the isolated molecules was achieved through a combination of techniques (NMR, LC-MS, UPLC-QDA, and IR).

Results: The results revealed potent antimalarial activity for specific compounds derived from *Artemisia afra*. Out of the four compounds isolated from *A. afra*, compound **(Y1)** and **(82C)** demonstrated promising dual stage antiplasmodial activities on both *Plasmodium* NF54S gametocytes and *Pf3D7* asexual parasites ($IC_{50} < 1 \mu\text{g/mL}$ for **Y1** and $IC_{50} < 5 \mu\text{g/mL}$ for **82C**). Compound **(Y1)** was hypothesized to be a derivative of sitosterol, while **(82C)** is hypothesized to be a derivative of ellagic acid. Compound **(R1)** was not active but new in the genus *Artemisia* while **(66A)** is identified in *A. afra* for the first time.

Conclusion: This study provides valuable insights into the bioactive constituents of *Artemisia afra* with dual stage antiplasmodial potential. The findings describe two molecules in *A. afra* with significant antiplasmodial properties including activity against gametocytes and may serve as promising candidates for further development into antimalarial drugs. To the best of our knowledge, these findings describe one molecule new in the genus *Artemisia* and one molecule new in *A. afra*.

Keywords: Malaria, *Plasmodium*, *Artemisia afra*, Molecules, Phytochemistry.

I. Introduction

Malaria, caused by *Plasmodium* parasites, remains a global health concern, with over 263 million estimated cases and killing over half a million people globally in 2023, (Venkatesan, 2025). The Sub-Saharan African countries shoulder a disproportionately high share of the global malaria burden with over 95% burden and 96% deaths (World Health Organization, 2024). The impact of the disease is especially severe among the most at-risk groups, including children below 5 years old, pregnant women, and migrant laborers traveling to endemic areas (Alehegn et al., 2020; Isiko et al., 2024). The World Health Organization propose the use of Artemisinin-based Combination Therapy as the firstline treatment regimen for malaria. ACTs consist of a minimum of two mechanistically distinct medicines, including one rapid-acting artemisinin derivative, which reduces the likelihood of resistance and recrudescence by necessitating the parasite to overcome two evolutionary challenges (Haldar et al., 2018). Despite the use of ACTs and other control strategies (vector control measures), malaria continues to post a significant global threat especially in endemic regions. This is largely due to the rapid development and spread of resistance to ACTs and to other antimalarial drugs

necessitating the need for continuous exploration for novel antimalarial agents. This problem is further aggravated by lack of safer transmission blocking agents that could target the *Plasmodium* gametocytes (the form of the parasite responsible for disease transmission).

Several strategies have been employed over the years to develop modern therapeutics including synthetic chemistry, molecular modeling, modification of existing molecules, natural products among others. Interestingly, over 50 % of modern therapeutics stem from natural products and it has been a subject of importance over the past years. In the context of malaria, quinine and artemisinin are two examples of medicines derived from the bark of *Cinchona* tree and leaves of *Artemisia annua* respectively (Siqueira-Neto et al., 2023).

Artemisia annua is a member of the Asteraceae family, one of the largest genera with over 500 species distributed across the globe (Shinyuy et al., 2023). The isolation of artemisinin from this plant family makes it to gain international recognition and has been a subject of interest over the past decades in search of molecules with biological activity including antimalarials (Shinyuy et al., 2023). In this regards, other species within this family have been investigated for novel antimalaria molecules among others. One of the members of the Asteraceae family, *Artemisia afra*, a closely related species to *Artemisia annua* has attracted attention in recent years due to its high patronage in the traditional management of malaria. Native to South Africa, *A. afra* serve as a reservoir of bioactive molecules with over 500 secondary metabolites (Shinyuy et al., 2023). In addition to its antimalarial properties, *A. afra* has equally demonstrated biological activity to a couple of ailments including antibacterial, anti-fungal, anti-inflammatory, anti-cancer, anti-diabetic properties. In the context of malaria, recent findings have shown the effectiveness of *A. afra* against *Plasmodium* parasites including significant activity against *Plasmodium* gametocytes (Snider and Weathers, 2020; Moyo et al., 2019). This property makes *A. afra* an important tool in the fight against malaria indicating the presence of molecules with dual stage antiplasmodial properties. *A. afra* is rich in flavonoids, phenolic acids, coumarins, monoterpenes, diterpenes, sesquiterpenes making it a source of novel compounds of therapeutic importance.

Due to these observations, the aim of the study was to isolate and characterize molecules from *A. afra* with significant activity against the sexual

and asexual forms of *Plasmodium falciparum*. To achieve the aim of this study, a bioassay-guided fractionation approach was employed, and the active molecules were isolated by a combination of techniques including open column chromatography and preparative thin layer chromatography. Structural elucidation of isolated molecules was performed using spectra data obtained from Nuclear Magnetic Resonance (NMR), Liquid Chromatography-Mass Spectrometry (LC-MS), ultra-Performance Liquid Chromatography-QDA analysis and Infra-red spectroscopy (IR). The study identified two new molecules (**R1** and **66A**) described in *A. afra* for the first time and one (**R1**) new in the genus *Artemisia*. Additionally, two compounds (**Y1**) and (**82C**) isolated from *A. afra* (pending identification), demonstrated significant antiplasmodial and gametocytocidal properties *in vitro*.

II. Material and methods

II.1. Collection of plant material and preparation of crude extract

Fresh leaves of *Artemisia afra* samples were obtained from Dschang, West region of Cameroon and a voucher specimen was deposited at the Limbe Botanical Gardens for authentication. Plant sample was sorted to get rid of foreign material, oven dried at 40°C for two days powdered. Maceration was done for 72 hr using acetone, filtered through a whattman filter paper and concentrated by rotatory evaporation (Mbah et al., 2012). The recovered extract was store at 4°C for subsequent use.

II.2. Fractionation by column chromatography.

Column chromatography was carried out with glass columns using LiChroprep Si 60 (15-25 µm), (Merck 9336, Darmstadt, Germany) as a stationary phase. An aliquot of acetone extract prepared as described above was subjected to normal phase silica gel column chromatography. Briefly, 350 mg of extract was mixed with silate and loaded to a column containing silica gel and eluted with a gradient mobile phase beginning with hexane/ethyl acetate (95:5 v/v). Several fractions (260 fractions) were collected into test tubes (approximately 150 mL per tube). The fractions were combined based on their similar TLC profiles with n-hexane and ethyl acetate (70:30 v/v) as mobile phase. These fractions were further purified by preparative thin layer chromatography. All solvents used were analytical grade (Merck).

II.3. Preparative thin layer chromatography

Preparative thin layer chromatography was performed using precoated Si gel 60 F₂₅₄ (1.25 mm thick, 20 × 40 cm) gel plates to purify the fractions resulting from column chromatography. Hexane/ethyl acetate (60:40 v/v) was used in TLC chamber to achieve separation of metabolites in the various fractions. Each metabolite was scraped-off from the plate together with silica after development in a TLC tank and dissolved in dichloromethane/methanol (95:5 v/v). Ultrasonicated for 10 min, pressured filtered and concentrated by rotatory evaporation. This procedure was repeated several times for the different fractions to yield compound **R1** (6.13 mg), **Y1** (6.55 mg), **82C** (6.25 mg) and **66A** (2.50 mg).

II.4. Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR analyses were performed on a Bruker Avance III HD 500 MHz spectrometer equipped with a helium-cooled cryoprobe (Bruker BioSpin GmbH, Germany) to ensure high sensitivity and resolution. The entire isolated mass was dissolved in 200 µL of deuterated methanol (CD₃OD, d₄, containing 0.03% TMS as an internal standard) and transferred into 3 mm NMR tubes supplied by CortecNet (Les Ulis, France). A minimal solvent volume was used to maximize sample concentration and improve signal quality. The following experiments were acquired using standard Bruker pulse sequences and parameters: ¹H NMR: 256 scans for proton chemical shift analysis, COSY: 8 scans for proton-proton correlation, ¹³C NMR: 5120 scans for carbon chemical shift determination, HSQC: 32 scans for heteronuclear single quantum coherence, HMBC: 128 scans for long-range heteronuclear correlations and TOCSY: 8 scans for spin system connectivity. Spectral processing and interpretation were carried out using MestReNova software (Version 15.0.1), enabling detailed structural elucidation of the compound.

II.5. Liquid Chromatography-Mass Spectrometry Analysis (LC-MS)

Samples were dissolved in 0.1% methanol and filtered through 0.45 µm HPLC filter and DI-HRMS was performed on a Thermo Scientific LTQ XL Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Data were acquired in positive and or negative ion mode using full-scan (MSⁿ: MS¹ and MS²) with a mass range of 100–1000 m/z. The orbitrap operated at 30,000 resolution (FWHM definition). All experimental data were acquired following daily external calibration. A flow rate, 100 µl min⁻¹; spray voltage, 5

kV; sheath gas (N₂) flow rate, 20 arbitrary units; auxiliary gas (N₂) flow rate, 10 arbitrary units; capillary temperature, 275°C; and capillary voltage, 45 V, were set as electrospray inlet conditions.

II.6. UPLC-QDA analysis

The Acquity UPLC-QDa (Quadrupole) system was used (Antwerp, Belgium). The system comprised the following components: Sample Manager FTN (SN M19FTP099G, Ver. 1.71.395), Quaternary Solvent Manager (SN A20QSP242A, Ver. 1.72.415), column compartment, QDa mass spectrometer (SN KBD5552, Ver. V), and PDA detector (SN C20UPD080A, Ver. 1.70.63.67). Separation was performed using a Kinetex F5 column from Phenomenex (Utrecht, Netherlands). The mobile phase consisted of Milli-Q water with 0.1% formic acid (A) and UPLC-MS/MS grade methanol (B) with a gradient program summarised in **Table 7**. The flow rate was 0.2 mL/min, with detection wavelengths set at 210, 254, 280, and 300 nm. The injection volume was 5 µL. System cleaning involved 50:50 and 10:90 (v/v) acetonitrile-water mixtures, and post-analysis, the column was rinsed with a 65:35 methanol-water mixture for 1 hour at 0.2 mL/min.

For QDa analysis, both MS Scan (full scan, generating TIC signals for all masses) and SIR (Selected Ion Recording, detecting specific masses) modes were employed. Data acquisition spanned 0.15–35 min over an m/z range of 100–1,200 in positive ionization mode. The cone voltage was set at 30 V, with source and probe temperatures maintained at 120°C and 74°C, respectively.

Table 7: Gradient program for mobile phase used in UPLC-QDA analysis.

Times (min)	Acetonitrile (%)	Water + 0.1% Formic Acid (%)
0.00	5.0	95.0
25.01	65.0	35.0
25.43	65.0	35.0
25.84	5.0	95.0
35.00	5.0	95.0

II.7. Antiplasmodial test

II.7.1. *Plasmodium falciparum* 3D7 *in vitro* culture and viability test

As described by [Trager & Jensen \(1976\)](#), a chloroquine-sensitive 3D7 *P. falciparum* strain was maintained *in vitro* in 3 % hematocrit (human type A or O-positive red blood cells). Complete Culture Medium (CCM) used in the assay was prepared by supplementing RPMI 1640 (Gibco, Fisher Scientific, Merelbeke, Belgium) medium containing NaHCO₃ (32 mM), HEPES (25 mM), and L-glutamine with 10 % heat-inactivated human plasma, 1.76 g/L of glucose (Sigma-Aldrich, Overijse, Belgium), 44 mg/mL of hypoxanthine (Sigma-Aldrich, Overijse, Belgium), and 100 mg/L of gentamycin (Gibco, Fisher Scientific, Merelbeke, Belgium). Parasitemia was verified by microscopy using Giemsa-stained thin films. All cultures were placed in a humidified incubator at 37 °C with a standard gas mixture of 5 % O₂, 5 % CO₂, and 90 % N₂. A stock solution of crude extracts was prepared by dissolving 10 mg of extract in 1 mL DMSO, and an antiplasmodial assay was conducted using an initial extract concentration of 100 µg/mL, which was subsequently serially diluted to eight concentrations (100 to 0.782 µg/mL). Each extract was tested in duplicate wells with the parasites at a final volume of 250 µL, making a final DMSO concentration of < 1%. The positive control consisted of wells with parasites only, while the negative control consisted of wells with only culture medium. As a standard, a stock solution of artemisinin (1µg/mL) (Gibco, Fisher Scientific, Merelbeke, Belgium) was made and serially diluted as described above. During a 48-hour period of incubation, plasmodial lactate dehydrogenase activity was estimated to assess parasite growth, as previously reported ([Kenmogne et al., 2006](#)), and linear regression was used to determine the IC₅₀ (concentration that kills 50% of parasites). All tests were repeated at least twice.

II.7.2. Induction of gametocytogenesis, culture, and viability assay

Gametocyte induction and culture was performed as previously described ([Gebru et al., 2017](#)). Prior to the start of gametocyte culture, the asexual chloroquine-sensitive NF-54 *P. falciparum* strain was synchronised with D-sorbitol (5% w/v) and magnetically separated using LD-MACS. Starting with a 0.3% ring-stage parasitaemia, cultures were maintained in a complete culture medium at 6% haematocrit that was later reduced to 3% when parasitaemia of 3% was achieved. There was a daily medium change and N-

acetyl-D-glucosamine was added to a final concentration of 50 mM between days 10 and 14 to remove all parasite forms other than late-stage gametocytes (stage IV and V), which were then subjected to in vitro viability tests. Working concentrations were prepared by serially diluting the stock solution of extracts (prepared as previously described) in at least 1:500 (DMSO < 0.4 %). Methylene blue (50 μ M) prepared in sterile distilled H₂O was used as a gametocytocidal drug control. Medium (80 μ L) was introduced in each well, and 40 μ L of the extract/drug was added in well A and serially diluted (1:3 dilution) to well G. The last wells were used as growth control (well H). A column for the gametocyte dilution control was also prepared. Gametocyte cultures were purified with Nycoprep and MACS separation ([Held et al., 2014](#)), and an improved Neubauer counting chamber was used to quantify the parasites. 20 μ L of parasite suspension containing about 50000 gametocytes was added to each well. Plates were incubated for 48 h at 37°C, 5% CO₂, and 5% O₂. A luminescent assay based on the quantitation of ATP produced by the parasites (BacTiter-Glo) was used following the protocol described by [Lelièvre et al. \(2012\)](#) to assess the viability of gametocytes, and data were analyzed using R and the dcr-package to obtain IC₅₀ values. The assay was repeated at least twice in technical duplicate.

III. Results

III.1. Open column

350 mg of acetone extract of *A. afra* were fractionated as described above using open column chromatography. A total of 260 fractions were collected in test tubes (above 10mL per tube) that were subsequently consolidated into 26 groups based on TLC chromatographic profiles. Fractions F4, F10, and F20 were the most interesting based on the yield and on the activity. These fractions were further fractionated using preparative thin layer chromatography to compounds **R1** from F4, **Y1** and **82C** from F10 and **66A** from F20.

III.2. TCL profile of the isolated metabolites

Thin layer chromatographic analysis of compound (**R1**) gave a violet colour, (**Y1**), a purple color, **82C** a red color while **66A** gave a blue coloration with vanilin sulfuric reagent (**Figure 2**).

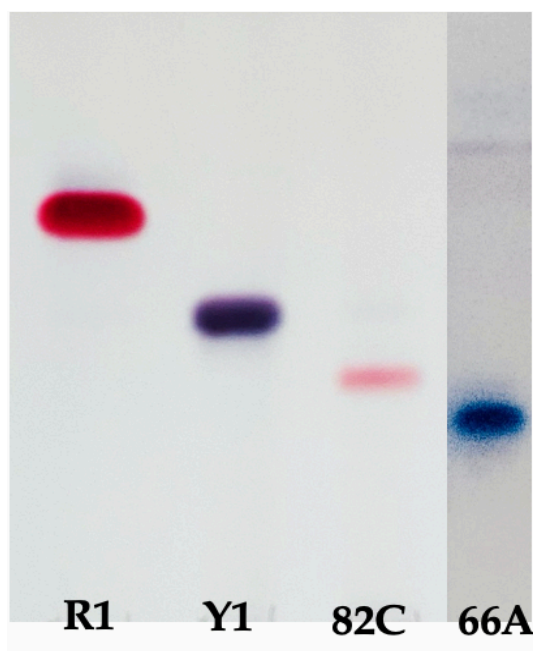


Figure 47: Thin layer chromatography of isolated metabolites

III.3. Structural determination

The acquired NMR spectra, including the proton (^1H), HSQC, and carbon (^{13}C) spectra for compound **R1**, are displayed in **Figures 4, 5, and 6**, respectively, providing detailed insights into the compound's structural features. Additional supporting spectra, such as COSY and TOCSY, including the spectra data for Compounds **66A** are available in the Supplementary material. Compound **R1** was identified a 3-methoxy-5-heneicosylphenol (**Figure 48**) with molecular weight of 418.70 g and with a molecular formula $\text{C}_{28}\text{H}_{50}\text{O}_2$ (CAS: 98168-58-2) while **66A** is a 4-ethenyl-2,5-dimethyl-5-hexene-2,3-diol (**Figure 49**) with a molecular formula $\text{C}_{10}\text{H}_{18}\text{O}_2$ and molecular weight of 193 g/mol. The LC-MS, IR and UPLC-QDA data are in supporting material. For compounds **Y1** and **82C**, the spectra data is pending analysis as the purity was not sufficient to determine the structures.

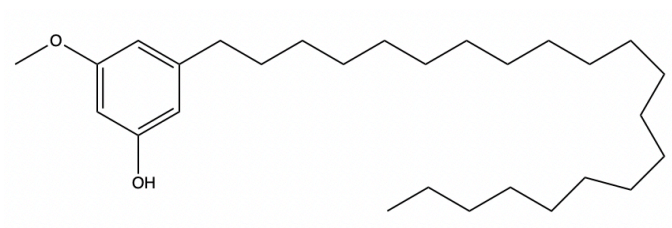


Figure 48: 3-methoxy-5-heneicosylphenol (**R1**)

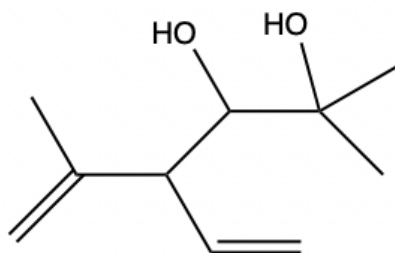


Figure 49: 4-ethenyl-2,5-dimethyl-5-hexene-2,3-diol (**66A**)

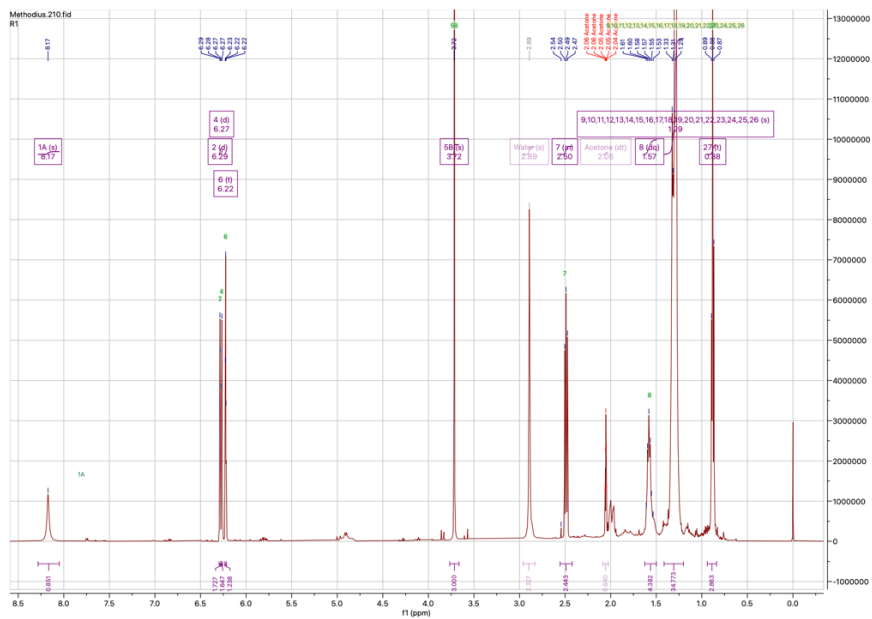


Figure 50: ¹H spectrum of the compound R

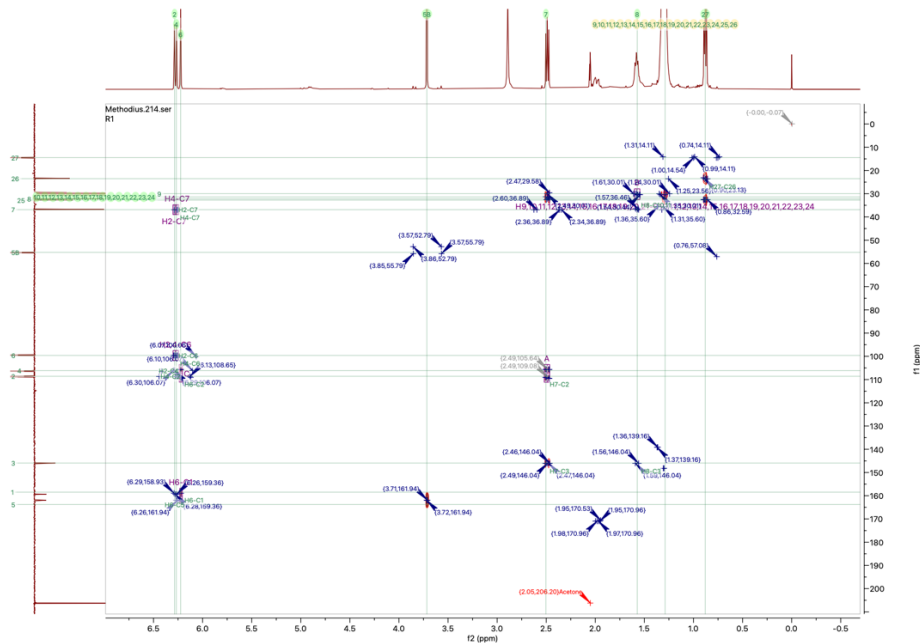


Figure 51: HMBC spectrum of the compound R1



III.4. Inhibition of *P. falciparum* asexual and sexual-blood stages

The anti-plasmodial activity was evaluated by exposing asexual chloroquine-sensitive (*Pf3D7*) and sexual NF-54 strains of *P. falciparum* to the isolated compounds and acetone extracts. The acetone extracts demonstrated strong gametocytocidal and anti-plasmodial activity with IC₅₀ values < 5 µg/mL (**Table 8**). Compound (**Y1**) was the most active against both the gametocytes and asexual parasites with IC₅₀ values < 1 µg/mL (**Table 8**).

Table 8: Dual stage drug sensitivity assays.

Sample /Standard drug	IC ₅₀ (µg/mL) ± SD	
	Gametocytes	Asexual parasites
Acetone extract	1.795 ± 0.220	17.651 ± 0.873
Y1	0.429 ± 0.056	0.035 ± 0.002
82C	0.619± 0.143	1.366 ± 0.358
R1	NA	NA
66A	NT	NT
Artemisinin	—	0.004 ± 001
Methylene blue	0.12 46	—

NA = Not active, NT = Not tested

IV. Discussion

Natural products have contributed significantly and constitute a large proportion of pharmaceutical drugs used in the treatment of various ailments ([Habibi et al., 2022](#)). In the context of malaria, well known examples of drugs that stemmed from natural product include quinine from the bark of *Cinchona* tree and artemisinin from *Artemisia annua*. Artemisinin constitutes part of the combination therapy known as Artemisinin Combination Therapy (ACT) used for the treatment of malaria for more than a decade. Although the use of ACT and other malaria control measures have achieved significant progress in reducing the burden and mortality, the emergence and spread of resistance to ACT has complicated the effort in curbing the disease especially in Sub-Saharan Africa where the burden and mortality are the highest ([WHO, 2022](#)). This limitation is further aggravated by the lack of safer transmission blocking drugs to target the gametocytes.

The form of the parasite responsible for the disease transmission. Therefore, there is the need for safer agents that could target both stages of the parasite (asexual and sexual forms) and hence reducing the link between human and insect vectors while also alleviating the symptoms of the disease.

Over the years, they have been growing interest in the search for new molecules with antiplasmodial activities from *A. annua* and from other members of the Asteraceae family including *Artemisia afra*. Several studies have shown that *A. annua* and *A. afra* have a rich phytochemical composition with activity against both asexual and sexual forms of *Plasmodium falciparum* (Snider and Weathers, 2020). In a study carried out by Moyo et al. (2019), two quaianolides isolated from *A. afra* showed significant activity against *Plasmodium* gametocytes. In line with the continuous search for molecules with significant antiplasmodial activity including activity against gametocytes, this study aimed to isolate and characterise compounds from *A. afra* with dual stage antiplasmodial activity through a bioassay-guided fractionation approach.

In this study, four compounds were isolated from *A. afra* through open column chromatography and preparative thin layer chromatography. These molecules were characterized by analysing spectral data obtained from NMR, LC-MS, UPLC-QDA and IR and by comparing with published data. Out of these molecules, two compounds (**Y1** and **82C**) showed significant activity against the asexual and sexual *Plasmodium* parasites (Table 7). The structural elucidation of these molecules is in progress. Nonetheless, from analysis of some spectra data, compound **Y1** yield a purple color with vanilline sulfuric reagent and was hypothesized to be a derivative of sitosterol. Compounds of this class especially beta-sitosterol has shown good antiplasmodial with IC₅₀ of 5.51 μ M (Philip et al., 2019). In our study, compound **Y1** demonstrated significant antiplasmodial activity against both the asexual and sexual forms of *Plasmodium* (Table 7). This pronounced activity may be attributed to the presence of an additional oxygen atom in **Y1** compared to β -sitosterol, which could influence its interaction with parasite targets.

Meanwhile, compound **82C** produced a red coloration with vanillin–sulfuric reagent and was hypothesized to be an ellagic acid derivative exhibiting notable antiplasmodial activity. Ellagic acid is well-documented for its ability to inhibit the growth of intraerythrocytic stages of *P. falciparum* by

interfering with β -haematin formation, a critical step in haem detoxification (Simões-Pires et al., 2009; Dell’Agli et al., 2003).

Compound **R1** was identified as 3—methoxy-5-heneicosylphenol (**Figure 46**). It gave a pink coloration upon reaction with vanillin-sulfuric (**Figure 45**). The compound has a molecular weight of 418.70 g/mol with a molecular formula $C_{28}H_{50}O_2$. Although **R1** showed no antiplasmodial activity, it is specific to *Artemisia afra* and absent in *A. annua*. This chemotaxonomic marker can therefore be used to distinguish these medicinal plants through TLC analysis, which is both cost-effective and easy to perform.

In contrast, compound **66A** was identified as 4-ethenyl-2,5-dimethyl-5-hexene-2,3-diol (**Figure 47**), a member of the monoterpene class, with a molecular formula $C_{10}H_{18}O_2$ and molecular weight of 193 g/mol. It produced a blue coloration with vanillin—sulfuric reagent (**Figure 45**). This compound was obtained in limited quantity, and therefore antiplasmodial testing was not performed. However, **66A** has previously been reported in other *Artemisia* species, such as *A. inculta* and *A. herba-alba* (Metwally et al., 2001), but this is the first report of its presence in *A. afra*.

V. Conclusion

This study successfully identified two compounds exhibiting significant activity against both asexual and sexual stages of *Plasmodium falciparum*. These findings highlight their potential as candidates for chemotherapy and transmission-blocking interventions, pending further optimization and drug development. In addition, the study reports, for the first time, the presence of a molecule to the genus *Artemisia* and specific to *Artemisia afra*. This compound could serve as a chemotaxonomic marker for authenticating *A. afra*, an important medicinal plant. Furthermore, we describe a previously unreported molecule in *A. afra*, which, although known in other *Artemisia* species, is documented here for the first time in this species, expanding the phytochemical profile of *A. afra* and contributing to its pharmacognostic characterization.

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Chapter Six

**Sensory Analysis, Phytochemical Profile,
Toxicity and Antimalarial Properties of a
Formulation of *Artemisia afra* and *Hibiscus
sabdariffa***

6.1 Foreword

Artemisia annua and *Artemisia afra* have consistently demonstrated potent antiplasmodial activity across *in vitro*, *in vivo*, and clinical studies, underscoring their therapeutic potential against malaria. Both species are relatively easy to cultivate and hold significant ethnopharmacological relevance, having been traditionally employed to manage various ailments, particularly malaria. Beyond their efficacy against asexual blood stages of *Plasmodium*, these plants have also shown promising activity against gametocytes, suggesting their potential role as transmission-blocking agents. However, a major limitation to their widespread use as herbal remedies lies in their pronounced bitterness, which negatively impacts patient compliance—especially among asymptomatic carriers who represent the majority in endemic regions. These individuals unknowingly harbour the parasite and contribute to the ongoing transmission. To address this challenge, developing a dosage form that preserves or enhances the antimalarial efficacy and safety profile of *A. annua* and/or *A. afra*, while improving taste, could significantly increase adherence. In this context, we explored a formulation combining *A. afra* with *Hibiscus sabdariffa*, aiming to mask the bitterness of *A. afra*. *H. sabdariffa*, widely cultivated in West, North, and Central Africa, is renowned for its vibrant red calyces, the most utilized part in culinary and beverage preparations (e.g., teas, jams, sauces, syrups, wines). Its characteristic acidic-sweet taste makes it an ideal candidate for improving the palatability of *A. afra*-based remedies, thereby enhancing acceptability and compliance in malaria management.

6.2 Sensory Analysis, Phytochemical Profile, Toxicity and Antimalarial Properties of a Formulation of *Artemisia afra* and *Hibiscus sabdariffa* (Article in preparation)

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Abstract

Background: *Artemisia afra* has been traditionally used for its antimalarial properties. However, due to its bitter taste, there is poor or lack of compliance with the herbal medicine, especially by asymptomatic malaria patients who constitute a vast majority of people in endemic areas.

Aim: This study aims to develop a formulation comprising *A. afra* and *Hibiscus sabdariffa* with an improved acceptance level, and to validate its antiparasmodial properties, safety profiles, and phytochemical composition.

Methods: Infusions of *A. afra* and *H. sabdariffa* were analyzed by HPTLC and HPLC. Sensory analysis was done through a hedonic rating test. Additionally, *in vitro* antiparasmodial properties were assessed on a chloroquine-sensitive 3D7 *Plasmodium falciparum*, while *in vivo* antimalarial test was assessed on *Plasmodium berghei*-infected mice. Also, acute and sub-chronic oral toxicity was assessed using an appropriate animal model.

Results: Equal combination of *A. afra* and *H. sabdariffa* (ARTEFOL; 5g *A. afra* and 5g *H. sabdariffa* in 1 L of water) infusion masks the bitterness of the former with an overall acceptance level of 7.2 on a 9.0 scale hedonic score. Phytochemical profile of ARTEFOL reveals the presence of phenolic acids (chlorogenic acid, Isochlorogenic acid A, B, and C, caffeic acid), coumarins (scopoletin and scopoline), and flavonoids (luteolin, rutin). ARTEFOL was found to be active with an IC_{50} of $46.81 \pm 2.12 \mu\text{g/mL}$ as compared to *A. afra* infusion (IC_{50} , $45.81 \pm 5.92 \mu\text{g/mL}$). There was a significant suppression of parasitaemia and prevention of packed cell Volume reduction by ARTEFOL compared to the control in a dose-dependent manner. Toxicity tests revealed no signs of toxicity with $LD_{50} > 5,000 \text{ mg/kg BW}$. Biochemical and hematological analysis reveal no signs of toxicity.

Conclusion: The study contributes to the broader exploration of herbal medicine as a sustainable and culturally relevant approach to combating malaria, with potential implications for the development of alternative antimalarial therapies needed to drive malaria elimination efforts.

Keywords: Malaria, *Artemisia afra*, *Hibiscus sabdariffa*, *Plasmodium*, Resistance.

I. Introduction

Malaria is an endemic vector-borne parasitic disease caused by a protozoan parasite of the genus *Plasmodium*, common in tropical and subtropical countries in which children and pregnant women are high-risk groups (Siqueira-Neto et al., 2022). Malaria is an important public health threat in Cameroon, with the whole country exposed to the risk of *Plasmodium* transmission, with suspected cases in healthcare facilities varying between 3.3 – 3.7 million per year (Antonio-Nkondjio et al., 2019). Despite the approved control and treatment measures by the WHO, conventional antimalarial drugs, including Artemisinin-based Combination Therapy (ACTs), face challenges such as the emergence and spread of resistance (World Health Organization, 2024; Balikagala et al., 2021). A situation aggravated by the lack of safer agents against the gametocytes, the infective stage of the *Plasmodium*. In addition to the widespread development of resistance to ACTs, limited access to modern therapeutics, including ACTs to treat malaria, because of financial, geographical, and/or cultural impediments, makes the treatment of the disease problematic. However, improved knowledge of plants from traditional pharmacopeias and validated traditional medications in improved traditional medicine can result in access to effective, standardized, ever-present, moderately priced therapeutics for management of malaria by local populations (Fikadu et al, 2023).

According to the WHO, about 80% population of the world relies on traditional medicinal products for some aspects of primary healthcare. The potential of medicinal plants, known for their diverse bioactive compounds, has become a focal point in the search for novel antimalarial agents. Belonging to the Astereceae family, *Artemisia afra* (AF) is one of the most widely used herbs as a traditional remedy for malaria (Shinyuy et al, 2023). Several *in vitro* and *in vivo* reports of AF show its potency against malaria parasites, including activity against the gametocytes (Moyo et al, 2019; du Toi and Van de Kooy, Weathers, Snider et al., 2021, Kane). Despite the absence or vestigial amount of artemisinin contents, AF is active against *Plasmodium* parasites (Shinyuy et al, 2025). Nonetheless, AF has a characteristic bitter taste when taken in the form of an infusion, and this could have an implication in the overall acceptance or compliance with the herbal remedy despite its potency against malaria parasites (Abenwie et al, 2023). As such, there is a need to mask the bitterness of these medicinal

plants to increase patient compliance in the fight against malaria, an important factor in the case of asymptomatic malaria patients. Asymptomatic malaria patients constitute a vast majority of people in endemic areas and perpetuate the disease transmission. Therefore, the study aims to mask the bitterness of the herbal product by supplementing it with *Hibiscus sabdariffa* (Malvaceae family), which has a characteristic, acidic-sweet taste.

Commonly known as folere in Cameroon, Bissap in Senegal or Ivory Coast, Jamaica flower in Mexico and roselle in Jamaica, Indian sorrel ([Montalvo-González et al; 2022](#)), *H. sabdariffa* (HS), is known for its red calyces, the most utilized part in food or drink preparations (hot or cold drinks, tea, jellies, jams, sauces, wines, syrups, ice cream). HS calyces have been used medicinally as antimicrobial, antiparasitic, antioxidant, antispasmodic, hepatoprotective, anti-anemic, and anti-inflammatory ([Izquierdo-Vega et al, 2019](#)). In general, the therapeutic effects of HS have been associated with the presence of bioactive compounds such as phenolic acids and flavonoids ([Sahaili and Mashoor, 2022](#)). The characteristic sweet taste of HS makes it a good supplement to mask the bitterness of *A. afra*, with an overall goal of increasing patients' compliance with the herbal product. Additionally, the anti-inflammatory properties of HS could offer additional benefits to malaria patients who suffer from hyperinflammation resulting from aberrant cytokine production. Therefore, this study aims to develop a formulation comprising AA and HS with an improved acceptance level and to validate its anti-plasmodial properties, safety profiles, and analyze its phytochemical composition. The outcomes of this research could provide valuable insights into the development of herbal combinations as alternative antimalarial therapies, offering a sustainable and culturally relevant approach to combat malaria, contributing to the global efforts in malaria elimination.

II. Methods

II.1. Preparation of extracts

A. afra and *H. sabdariffa* were harvested from the West region of Cameroon; voucher specimens were deposited at the Limbe Botanical Gardens, Cameroon, and a voucher number was assigned. Leaves and twigs of AA and the calyces of HS were oven-dried at 40°C and infusions prepared using the traditional preparation method as follow; **(A11)** 5g of AA/1L, **(A17)** 5g of

HS/1L, **(A18)** 5g of AA + 5g of HS in 1L, allowed to react for 15 mins with occasional stirring and filtered through a 0.45 μ M filter-paper. An equal combination of infusions of A11 and A17 **(A19)** was made, and all were evaporated to dryness by lyophilisation. In addition to the infusion extracts, ethanol extracts of both plants were prepared by maceration ([Mbah et al., 2012](#)). All extracts were kept at 4°C for further analysis.

II.2. High Performance Thin Layer Chromatography (HPTLC) analysis of infusions

High-performance thin-layer Chromatography (HPTLC) infusion extracts were performed on precoated Si gel 60 F₂₅₄ (1.25 mm thick, 20 × 40 cm) gel plates (Merck, Hohenbrunn, Germany) plates to determine the chemical composition of metabolites in the extract. Two solvent systems were used to achieve the separation of polyphenol composition. The first solvent system, comprising ethyl acetate, methanol, water, and formic acid (50:4:4:2.5), separated fewer polar components, while a second solvent system consisting of Chloroform, ethyl acetate, and formic acid (5:4:1) achieved separation of metabolites with high polarity. After development, revelation was done by spraying with DPBAE/PEG (prepared by dissolving 10 g and 50 g of DPBAE and PEG, respectively, in 1 L of methanol) and visualized at 366 nm (UV light).

II.3. Ultra Performance Liquid Chromatography (UPLC) analysis of infusions

UPLC analyses were performed on an Agilent 1260 Infinity II system as described elsewhere ([Shinyuy et al., 2025](#)) with slight modifications. Infusion extracts were reconstituted at a concentration of 100 mg/5mL of 60 % methanol, sonicated for 2 x 15 min, and filtered through a 0.45 μ m HPLC filter. A LUNA PFP column (250 x 4.6 mm, 5 μ m). A mobile phase consisting of a mixture of acetonitrile (A) and water/0.1 % formic acid (B) in gradient condition (A/B; 15/85, 18/82, 23/77, 30/70, 40/60, 50/50, 80/20, 100/0, 15/85, 15/85 v/v) was used. A volume of 20 μ L was injected at a flow rate of 1 mL/min, and a run time of 87 min was set. A Diode Array Detector (DAD) was used and set at 330nm.

II.4. Ethical consideration

Ethical clearance for work with animals was granted by the University of Buea Institutional Animal and Use Committee (UB-IACUC), Cameroon, with a permission number: UB-IACUC N°23/2024.

II.5. Sensory analysis

Sensory analysis of infusions was done through the assessment of the hedonic rating of the food product. Four teas were prepared as described above, except for lyophilisation. Sensory cards (**Supplementary I**) were administered to 100 participants who tested the infusions (A11, A17, A19, and a positive control, lipton; a tea locally consume by Cameroonian population) and rated them on a scale of 9 based on the following parameters; bitterness, color, taste, consistency, flavour and overall acceptability.

II.6. *In vitro* antiplasmodial properties of infusions

Antiplasmodial test of infusion extracts was carried out using a chloroquine-sensitive 3D7 *P. falciparum* strain (Malaria Research and Reference Reagent Resource Center, MR4). Parasites were maintained *in vitro* (Trager and Jensen, 1976) in 3 % hematocrit (human type A or O-positive red blood cells), Complete Culture Medium (CCM) prepared by supplementing RPMI 1640 (Gibco, Fisher Scientific, Merelbeke, Belgium) medium containing NaHCO₃ (32 mM), HEPES (25 mM), and L-glutamine with 10 % heat-inactivated human plasma, 1.76 g/L of glucose (Sigma-Aldrich, Overijse, Belgium), 44 mg/mL of hypoxanthine (Sigma-Aldrich, Overijse, Belgium) and 100 mg/L of gentamycin (Gibco, Fisher Scientific, Merelbeke, Belgium). All cultures were placed in a humidified incubator at 37 °C with a standard gas mixture of 5 % O₂, 5 % CO₂, and 90 % N₂. Parasitemia was verified by microscopy using Giemsa-stained thin films. A 10 mg of extract was dissolved in 1 mL DMSO and diluted with culture media, and an anti-plasmodial test was performed in 96-well microtitre plates at an initial concentration of 100 µg/mL and serially diluted to eight concentrations (100 to 0.782 µg/mL). Each sample was tested in duplicate wells with the parasites at a final volume of 250 µL, making a final DMSO concentration of < 1 %. Negative control consisted of wells with culture medium only, while wells with parasites only served as the positive control. A 1 µg/mL stock solution of artemisinin (Gibco, Fisher Scientific, Merelbeke, Belgium) was prepared and serially diluted as above; this was used as a standard. After 48 h of incubation, parasite growth was estimated by determination of plasmodial lactate dehydrogenase activity as described by Kenmogne *et al* (2016), and IC₅₀ was calculated by linear regression. All tests were repeated at least twice in technical duplicate.

II.7. Acclimatization of the animals

Swiss albino mice weighing 20-28g and aged at least 7 weeks of both sexes were obtained from the Faculty of Medicine and Pharmaceutical Sciences, University of Douala, Cameroon. They were put in cages and acclimatized in the laboratory for one week before the study. The mice were kept at room temperature and exposed to a 12-h light/dark cycle, allowing free access to food (pellets) and water in standard cages

II.8. *In vivo* antimalarial activity of ethanol extracts

II.8.1. Parasite inoculation and grouping of animals

Three albino mice were infected with *P. berghei* (BEI-Resources, Manassas, VA, USA) and monitored until a parasitemia of 6-8% was attained and used as donor mice. The donor mice were sacrificed, and blood was collected by retro-orbital bleeding into an EDTA tube and diluted with 7.4mL of normal saline (0.9%) based on the parasitemia level. Each mouse was then inoculated with 0.2ml of blood suspension containing 1×10^7 *P. berghei* parasitized erythrocytes intraperitoneally. Forty-five infected mice were randomly grouped into nine groups (six treatment groups, two controls, and one reference group) of five mice each. Group I mice were not infected but treated with distilled water (served as negative control), group II mice were infected and treated with distilled water, and group III mice were infected and treated with standard drug (chloroquine, 20mg/kg/day, served as positive control), group IV, V and VI were infected and treated with 400, 200 and 100 mg/kg/day of *A. afra* respectively, group VII, VIII and IX, were infected and treated with 400, 200 and 100 mg/kg/day of *A. afra*/*H. Sabariffa* combination respectively. Based on the acute toxicity test, three doses (100, 200, and 400mg/kg/day) were selected for the *in vivo* antimalarial study of the extracts.

II.8.2. Curative test

After parasite inoculation, the mice were grouped as described. On the first day (D0), the mice were inoculated intraperitoneally with 0.2 mL standard inoculum of 1×10^7 PRBCs and left untreated for 72hours (D0-D3) for infection to establish. On day three post parasite inoculation, each mouse was tail-bled, and parasitemia was determined by Giemsa thin blood smears. After the baseline blood sampling, all inoculated mice were randomly divided into

their various groups and treated orally for five consecutive days with extracts and standard drugs as described above. Post-treatment parasitemia levels were determined, and the animals were anesthetized with ketamine. Blood was collected by retro-orbital bleeding, and packed cell volume (PCV = volume of erythrocyte in each volume of blood divided by the total blood volume) was measured. Body weight and temperature of each mouse were measured daily.

II.9. Acute and sub-chronic oral toxicity assessment of ethanol extracts

This was performed as per the Organization for Economic Cooperation and Development guidelines 407 ([OECD, 2008](#)). For the acute test, adult female mice were fasted for 4 hours, weighed, and separated into four distinct groups (5 animals each): a control group and three test groups. Distilled water was administered to the control group at a dose of 1 mL per 100 g of body weight, while the test groups received a single dose of AA, HS, and AA/HS combination at 2000 mg/kg. Following extract administration, each animal of the different groups was observed for 2 hours, after which they were allowed access to food and water. The animals were then observed at least once daily for the next 14 days. During this time, animals were observed for any mortality and other signs of toxicity, including changes in the skin and fur, eye color, convulsions, salivation, diarrhea, or coma. Body weight was measured daily throughout the experiment.

For the sub-chronic test, mice were divided randomly into 10 different groups of 6 mice each. The groups were assigned different treatments; Group I, negative control (distilled water), Group II, III and IV, received (500, 1000 and 2,000 mg/kg BW of AA), Group V, VI and VII, received (500, 1000 and 2,000 mg/kg BW of HS) while group VIII, IX and X, received combination of AA/HS (500, 1000 and 2,000 mg/kg BW). All treatments were administered via gavage at the same time each day for 28 consecutive days. All the experimental animals were observed daily for any abnormal clinical signs, body weight change, and mortality for 28 days. On day 28, the animals were subjected to overnight fasting and then anesthetized with ketamine/xylazine (80/5 mg/kg body weight). Blood was collected by retro-orbital bleeding using capillary tubes and through cardiac puncture into non-heparinized tubes for biochemical analysis and EDTA tubes for hematological analysis. The liver, heart, and kidneys were harvested and weighed. The non-heparinized tubes were then allowed for 30 minutes to coagulate and

centrifuged at 2000 rpm for 10 min to obtain serum for the determination of lipid profile (high-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol and triglycerides), markers of liver (ALT, AST, ALP and TB) and kidney (creatinine, urea levels) functions.

III. Results

III.1. HPTLC fingerprints of extracts

Two set of mobile phases were used to acheive separation of polyphenols in all extracts. The first solvent system separated fewer polar components (**Figure 53a**), while a second solvent system achieved separation of metabolites with high polarity (**Figure 53b**). High Performance Thin Layer Chromatography (HPTLC) fingerprint shows similarities and variation in chemical composition of metabolites present in AA, HS, AA/HS infusions. For instance, rutin, hyperoside, cafeic acid, chlorogenic acid and scopoletin were both common in the AA, HS, HS infusions and in the ethanol extract of AA (**Figure 53a**). However, luteolin was not found in the infusion of HS but was present in the AA/HS combinations (A18 and A19) (**Figure 53b**).

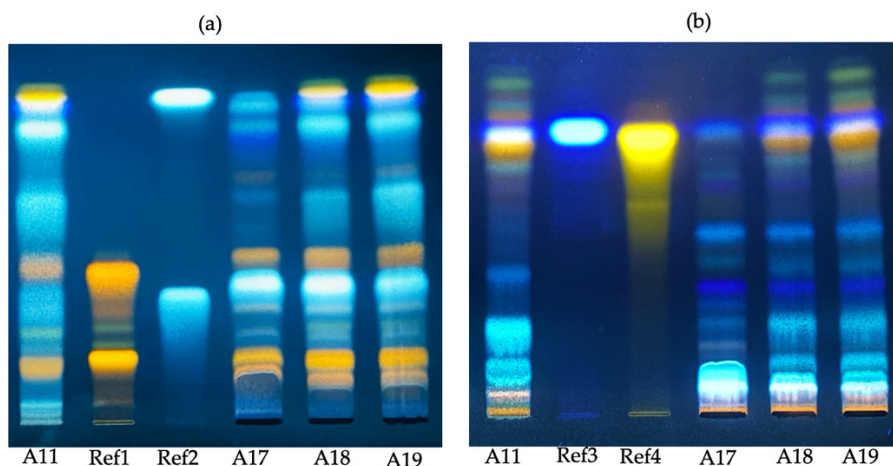
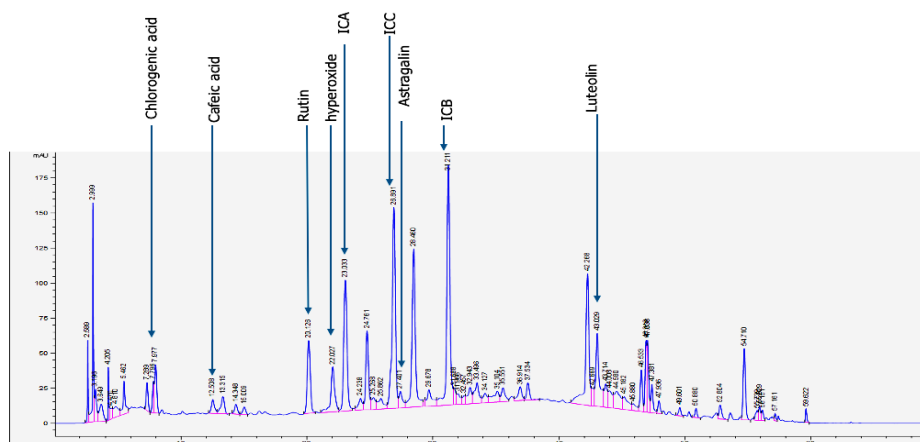


Figure 53: HPTLC analysis of infusions of A11 and A17, A18 and A19 (a) fingerprint of polyphenol composition using MP1, (b) fingerprint of polyphenol composition using MP2. A11 = 5g of *A. afra* /1L of tap H₂O, A17= 5g of *H. sabdariffa* /1L of tap H₂O, A18= 5g of *A. afra* + 5g of *H. sabdariffa* in 1L of tap H₂O, A19= 50:50 combination of infusions of A11 and A17, Ref1 = rutin and hyperoxide in order of increasing retention factor, Ref2= Chlorogenic acid and caffeic acid in order of increasing retention factor, Re3= Scopoletin, Ref4= luteolin.

III.2. UPLC analysis of infusions

UPLC analysis of all samples shows similarities and variations in the metabolite chemical pattern which was affected by method of extract preparation. By comparing the UV-spectrum and retention time (RT) of standard and those of analytes, several polyphenols were identified, including chlorogenic acid, caffeic acid, iso-chlorogenic acid A, B, and C, rutin, luteolin, hyperoside, and astragalín in the infusions of *A. afra* (**Figure 54**). These metabolites were present in both plants except for luteolin and astragalín that were absent in *H. sabdariffa*. Additionally, the ethanol extract of *A. afra* presented a UPLC fingerprint with more metabolites and higher intensities compared to the infusions (**Figure 55**). In the same sequence, the combination AA/HS (A19; 50:50 combination of infusions of A11 and A17) presented a fingerprint dominated by Chlorogenic and its derivatives with much lower intensities compared to infusions and ethanol extracts of AA (**Figure 56**).



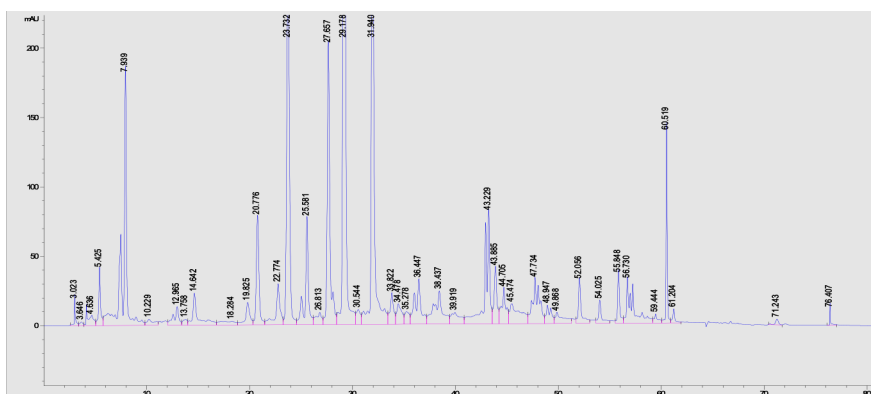


Figure 55: Chromatogram of ethanol of *A. afra*.

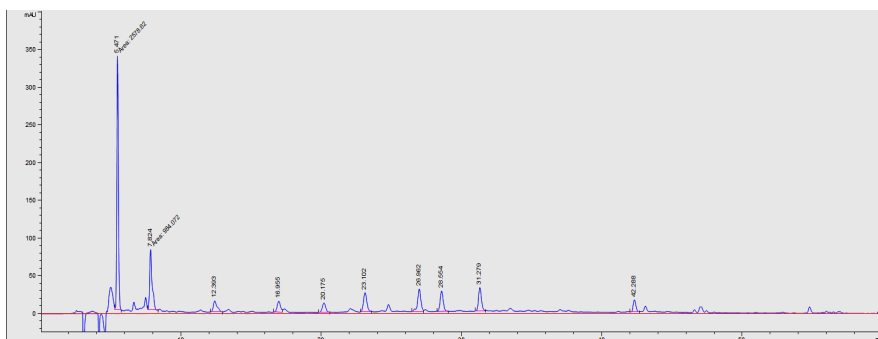


Figure 56: Chromatogram of infusion combination of *A. afra* /*H. sabdariffa* (A19).

III.3. Sensory analysis

Sensory analysis of infusions of *A. afra*, *H. sabdariffa*, extract combinations and Lipton (a locally consumed tea by the Cameroonian population) was done as described above. The degree of acceptability was assessed based on 5 parameters (color, taste, flavor, consistency/texture and bitterness) (**Figure 57**). A 50:50 combination of infusions of *A. afra* and *H. sabdariffa* was the second most accepted tea after Lipton tea. (**Table 9**).

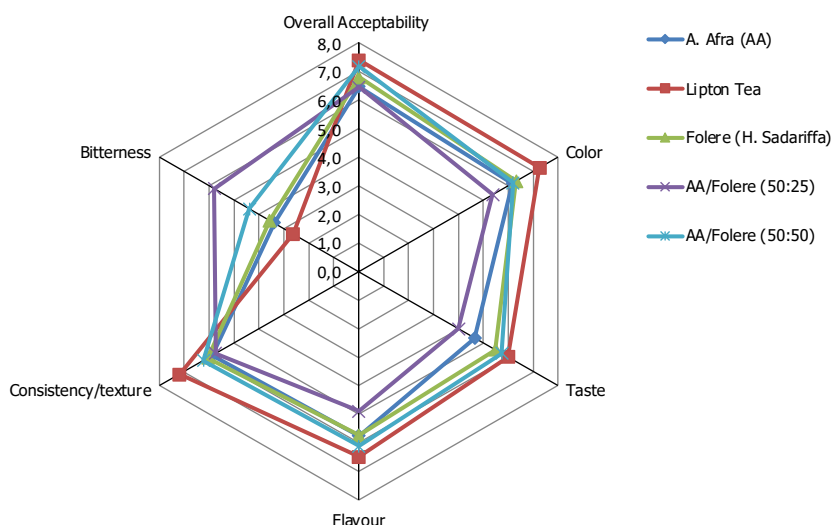


Figure 57: A 9-scale hedonic score of infusions

Table 9: Hedonic score and acceptability of different tea infusions by the study participants.

9 Scale hedonic scores		
Sample	Mean $\pm$ SD	Acceptance classification
Lipton tea	7.4 $\pm$ 0.22	First
ARTEFOL (50/50)	7.2 $\pm$ 0.14	Second
HS	6.8 $\pm$ 0.16	Third
AA	6.5 $\pm$ 0.25	Fourth
ARTEFOL (50/25)	6.4 $\pm$ 0.32	Fifth

III.4. *In vitro* antiplasmodial properties of infusions

Antiplasmodial test was performed on a chloroquine-sensitive *Pf3D7* strain as described above. Ethanol extracts of *A. afra* (EAA) and equal combination of ethanol extract of *A. afra* and *H. sabdariffa* (EC) demonstrated good antiplasmodial activity with IC₅₀ values < 20 μ g/mL, whereas ethanol extract of *H. sabdariffa* (EHS) was inactive at the tested concentration. For the

infusion extracts, A11 (5g of *A. afra* /1L of tap H₂O) and (50:50 combination of infusions of A11 and A17) demonstrated weak antiplasmodial properties while A18 was not active (**Table 10**). In general, the ethanol extracts were more active than the infusions.

Table 10: Inhibitory concentration (IC₅₀) of extracts

Infusion/standard drug	IC ₅₀ (µg/ml) ± SD
	<i>Pf3D7</i> strain
A11	46.81 ± 2.12
A17	>100
A18	53.28 ± 4.10
A19	45.81 ± 5.92
EAA	16.14 ± 4.21
EC	15.34 ± 3.74
EHS	>100
Artemisinin	0.006 ± 0.001

AA= *A. afra*, HS= *H. sabdariffa*, A11 = 5g of *A. afra* /1L of tap H₂O, A17= 5g of *H. sabdariffa* /1L of tap H₂O, A18= 5g of *A. afra* + 5g of *H. sabdariffa* in 1L of tap H₂O, A19= 50:50 combination of infusions of A11 and A17, EAA= Ethanol extract of *A. afra*, EHS= Ethanol extract of *H. sabdariffa*, and EC= Equal combination of EAA and EHS extracts.

III.5. *In vivo* antimalarial properties

III.5.1. Percentage parasitaemia suppression

The parasitemia suppression was dose-dependent. Among the test groups, maximum suppression was attained with a 400 mg/kg dose of AA (83.0%), followed by a 400 mg/kg dose of AA/HS combination (81.2%) (**Figure 58**). All groups showed a statistically significant difference when compared to the control (Pb + DW). The 400 mg/kg dose of the AA/HS showed no significant difference compared with the 400 mg/kg dose of the AA, as well as to the standard drug (CQ 20mg/kg).

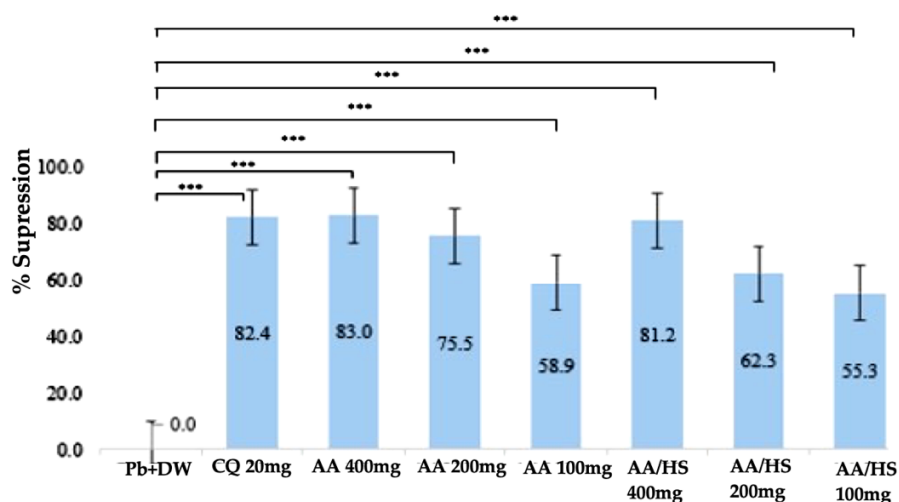


Figure 58: Percentage suppression of *P. berghei*-infected mice treated with *A. afra* and *A. afra/H. Sabdariffa* extracts in a 5-day curative test (*= significant, ** = highly significant, *** = very highly significant).

III.5.2. Pack cell volume

A dose-dependent protection against the reduction of pack cell volume (PCV) was observed in the test groups. The rank order of protection from infection-induced reduction in PCV was 20 mg/kg dose of CQ > 400 mg/kg of AA > 400 mg/kg of AA/HS > 200 mg/kg AA/HS > 200 mg/kg AA > 100 mg/kg of AA/HS > 100 mg/kg of AA (**Figure 59**). No statistical difference was observed between the PCV in the group infected and treated with distilled water and the other treatment groups. However, all groups showed a statistical difference in PCV compared to the group not infected but treated with distilled water.

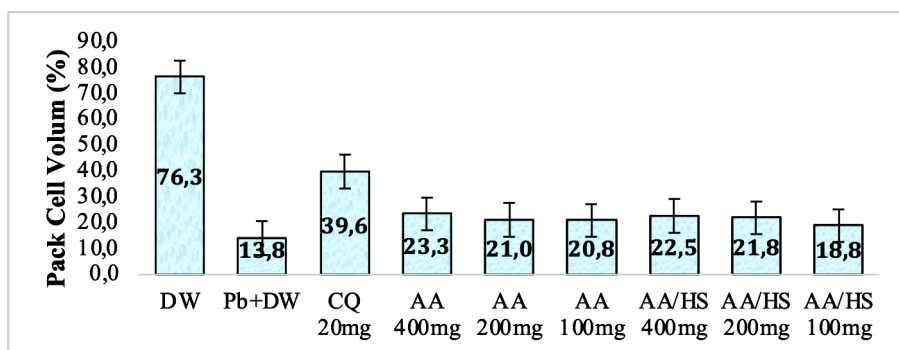


Figure 59: Changes in PCV of mice infected with *P. berghei* and treated with ethanolic extract AA, and AA/HS at the different doses.

III.5.3. Change in food consumption, body Weight, and temperature

The change in food consumption by the mice from day 1 to day 7 was measured with day 1 considered as baseline. A steady increase in food consumption was observed in the group not infected but treated with distilled water (**Figure 60a**). Generally, inconsistency in food intake was observed in all other groups, with no statistical difference between the extract or extract combination to the group infected and treated with CQ. The highest increase in body weight was observed in the group that was not infected but treated with distilled water (21%), followed by the group infected and treated with standard drug, CQ 20 mg/kg (12.5%) (**Figure 60b**). Among the groups treated with extract and extract combination, the 400 mg/kg of AA had the highest increase in body weight (11.2%) (**Figure 60b**). In the negative control group (DW), rectal temperature remained relatively constant (+0.1 °C), whereas malaria infection in the group infected and treated with distilled water caused a marked decrease in rectal temperature (−5.3 °C) (**Figure 60c**). However, this was most significantly ameliorated by standard drug; CQ 20 mg/kg (+0.4 °C, $p = 0.04$) followed by 200 mg/kg of AA (+0.8 °C, $p = 0.001$) and 100 mg/kg of AA/HS (+0.6 °C, $p = 0.001$). No significant difference in temperature change was observed when comparing the different doses of AA and AA/HS.

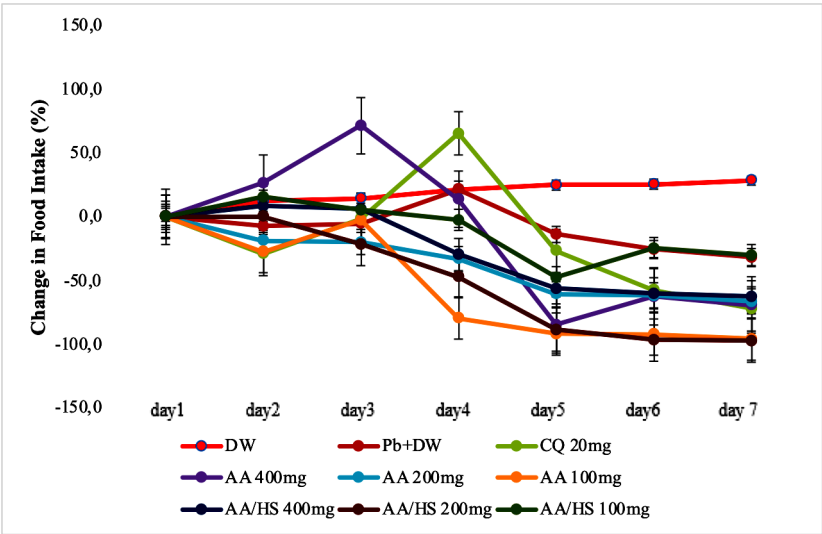


Figure 60a: Effect of ethanolic extract of AA, AA/HS on food consumption of *P. berghei* infected mice in a 5-day curative test. (*= significant, ** = highly significant, *** = very highly significant).

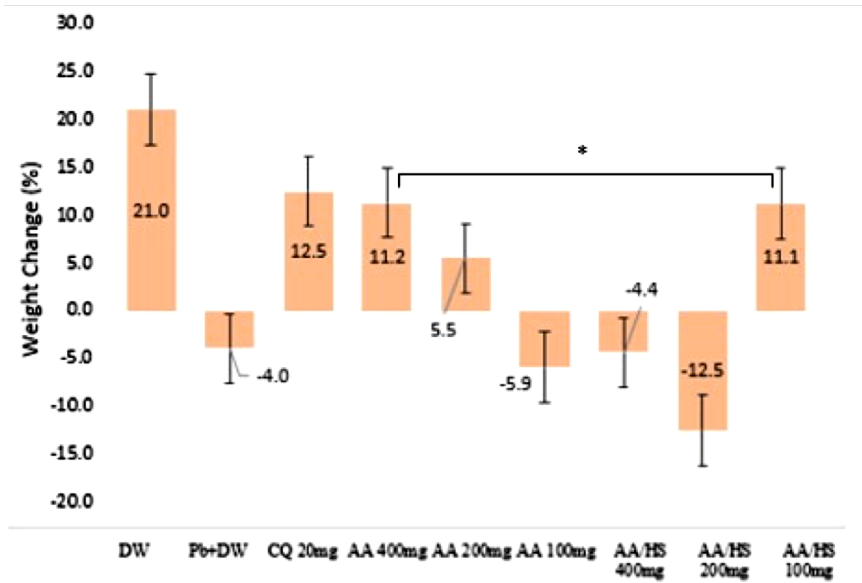


Figure 60b: Effect of ethanolic extract of AA, AA/HS on body weight of *P. berghei* infected mice in a 5-day curative test. (*= significant, ** = highly significant, *** = very highly significant).

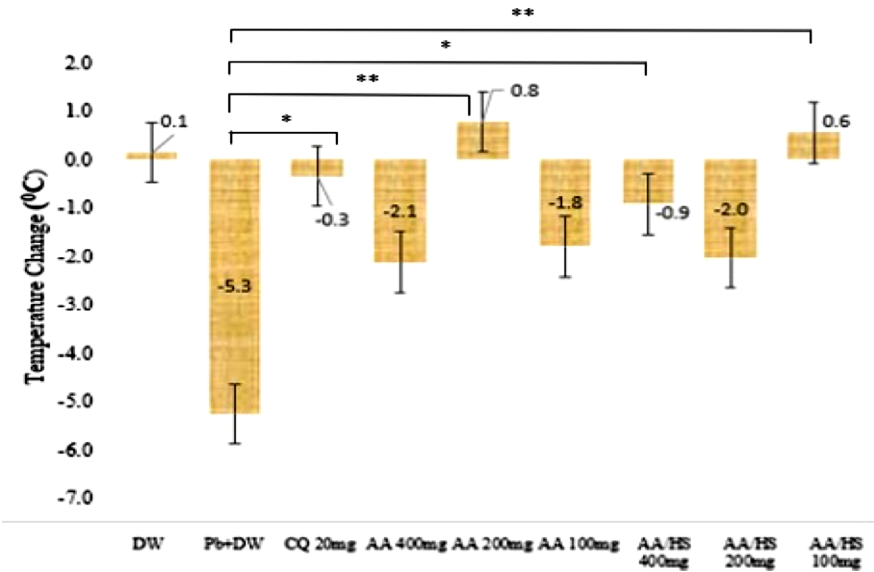


Figure 60c: Effect of ethanolic extract of AA, AA/HS on rectal temperature of *P. berghei* infected mice in a 5-day curative test. (*= significant, ** = highly significant, *** = very highly significant).

III.6. Acute oral toxicity test

There were no gross behavioral and physical changes such as rigidity, sleep, diarrhea, salivation, lacrimation, hair erection, tremors, convulsions, and reduction in feeding for the treatment groups compared to the control after acute exposure to 5000 mg/kg. The median lethal dose (LD₅₀) was found to be greater than 5000 mg/kg body weight (**Table 11**).

Table 11: Effect of ethanol extract of AF HS, and ARTEFOL combination in mice after acute oral exposure.

Ethanol extracts	Mice on day 14	Dose (mg/kg)	Toxic signs	LD ₅₀
AA	5	5 000	No behavioral changes and mortality	> 5000 mg/kg
HS	5	5 000		
ARTEFOL	5	5 000		

There was a proportional increase in the body weight of all the mice in the test groups compared to the distilled water-treated group after acute exposure (**Figure 61a**).

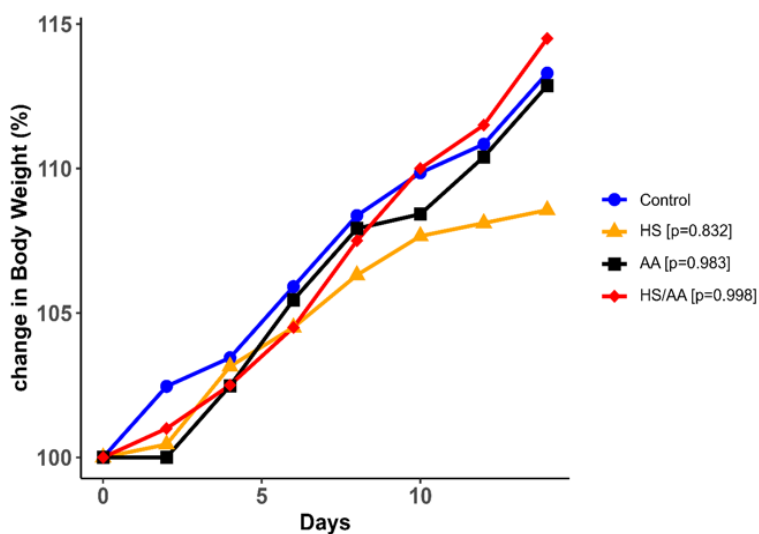


Figure 61a: Percentage change in body weight of mice treated with 5000 mg/kg of extract of in the 14-day acute toxicity study. AA = *A. afra*, HS = *H. sabdariffa*, HS/AA = extract combination (ARTEFOL).

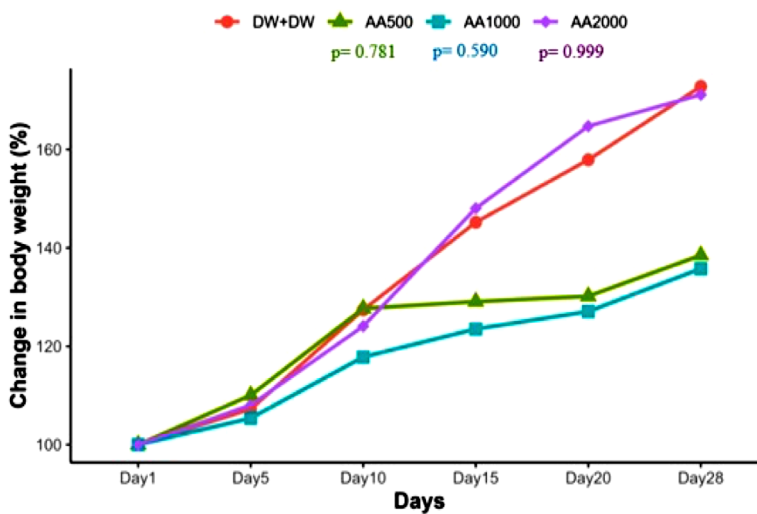


Figure 61b: Percentage change in body weight of mice treated AA after sub-chronic exposure. AA = *A. afra*, DW = Distilled water

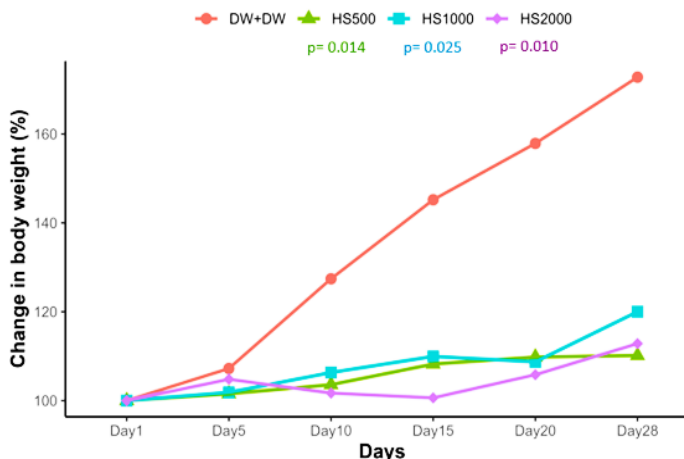


Figure 61c: Percentage change in body weight of mice treated with HS after sub-chronic oral exposure. DW = Distilled water. HS = *H. sabdariffa*, DW = Distilled water .

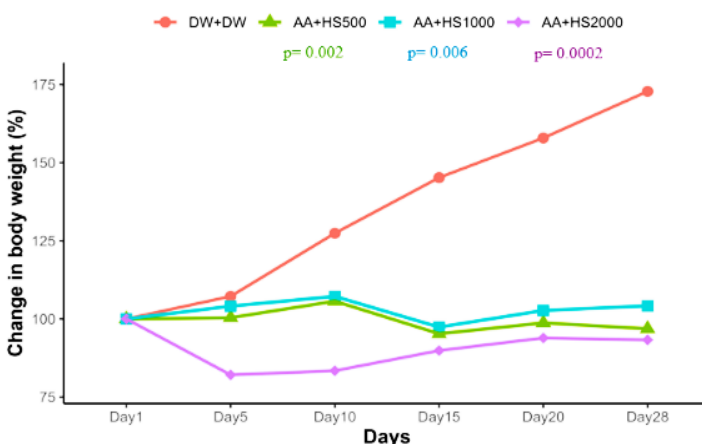


Figure 61d: Percentage change in body weight of mice treated ARTEFOL after sub-chronic oral exposure. AA = *A. afra*, HS = *H. sabdariffa*, HS/AA = extract combination (ARTEFOL), DW = Distilled water

III.7. Sub-acute toxicity

No significant changes occurred in the weight of treated mice with AA extracts at the tested doses compared to the distilled water-treated group (500, 1000, and 2,000 mg/kg with p-values of 0.781, 0.590, and 0.999, respectively) (**Figure 61b**). However, there was a statistically significant difference in body weight in all doses of HS and AA/HS compared to the distilled water-treated group (**Figures 61c and 61d**).

III.8. Effect of extracts on biochemical, hematological parameters, and organ weight

Biochemical analysis of serum samples was performed using a semi-automatic chemistry analyzer (Thermo Scientific Multiskan FC). There was no significant difference in biochemical parameters in groups treated with AA, HS, and ARTEFOL at 500 and 1000 mg/kg compared to the control group (**Table 12**). However, the daily oral administration of the ARTEFOL provoked a significant difference in AST, ALT, and ALP at a dose of 2000 mg/kg ($P < 0.05$). Regarding the organ weight, there was no statistically significant variations in the mean organ index of the liver, heart and kidney of the test groups compared to the distilled water treated group (**Table 13**). Moreover, there was no statistically significant difference in the RBCs and Hb for all treated groups compared to the distilled water-treated group (**Table 14**). However, 2000 mg/kg of ARTEFOL resulted in statistical significant increases in WBCs, HCT, MCHC, MCH and neutrophils.

Table 12: Effect of sub-acute treatment of mice with ethanol extract of AA, HS, and ARTEFOL on biochemical parameters.

	NoGP	<i>Artemisia annua</i> (AA)			<i>Hibiscus sabdariffa</i> (HS)			AA + HS		
Dose (mg/kg)		500	1000	2000	500	1000	2000	500	1000	2000
AST (IU/L)	216.80 ± 0.99	221.10 ± 3.16	222.50 ± 1.43	232.90 ± 2.74 ^c	218.30 ± 1.93	244.40 ± 5.36 ^c	247.10 ± 1.34 ^b	205.20 ± 1.10 ^b	206.60 ± 0.96 ^a	211.40 ± 0.44
ALT (IU/L)	56.28 ± 1.64	57.27 ± 1.75	56.74 ± 1.39	56.55 ± 0.84	54.89 ± 1.25	55.86 ± 1.10	52.90 ± 1.04	52.25 ± 0.26	55.08 ± 0.98	53.99 ± 0.63
ALP (IU/L)	334.90 ± 2.36.54	315.20 ± 4.03 ^b	331.80 ± 2.39	321.70 ± 2.17	336.90 ± 0.68	305.30 ± 6.89 ^c	327.60 ± 4.06	315.80 ± 3.19 ^b	315.50 ± 4.01 ^b	322.00 ± 4.44
TB (mg/L)	5.86 ± 0.15	7.01 ± 0.38 ^b	6.30 ± 0.04	7.19 ± 0.40 ^c	5.93 ± 0.12	5.58 ± 0.07	6.05 ± 0.22	6.36 ± 0.06	6.11 ± 0.09	6.29 ± 0.01
Creatinin (mg/L)	7.74 ± 0.23	7.62 ± 0.32	6.42 ± 0.13 ^c	7.41 ± 0.08	7.44 ± 0.29	7.89 ± 0.24	7.89 ± 0.07	8.64 ± 0.31 ^a	9.07 ± 0.12 ^c	7.22 ± 0.01
Urea (mg/L)	0.34 ± 0.02	0.32 ± 0.02	0.25 ± 0.02	0.42 ± 0.02	0.30 ± 0.03	0.40 ± 0.05	0.30 ± 0.00	0.35 ± 0.02	0.39 ± 0.02	0.39 ± 0.00
TC (g/L)	0.56 ± 0.01	0.63 ± 0.01 ^c	0.55 ± 0.01	0.58 ± 0.01	0.61 ± 0.01 ^b	0.58 ± 0.00	0.60 ± 0.01	0.55 ± 0.01	0.57 ± 0.00	0.51 ± 0.01 ^b
LDL (g/L)	0.10 ± 0.00	0.11 ± 0.00	0.12 ± 0.01 ^c	0.13 ± 0.00 ^c	0.10 ± 0.00	0.10 ± 0.00	0.12 ± 0.01 ^c	0.11 ± 0.00 ^a	0.11 ± 0.00	0.11 ± 0.00
HDL (g/L)	0.52 ± 0.01	0.51 ± 0.02	0.49 ± 0.01	0.50 ± 0.01	0.49 ± 0.02	0.48 ± 0.01	0.51 ± 0.02	0.53 ± 0.01	0.49 ± 0.01	0.53 ± 0.01
TG (g/L)	1.45 ± 0.06	1.56 ± 0.02	1.75 ± 0.02	1.47 ± 0.02	1.52 ± 0.02	1.48 ± 0.02	1.56 ± 0.02	1.33 ± 0.02	1.42 ± 0.02	1.54 ± 0.02

		0.07	0.03 ^b	0.08	0.05	0.05	0.07	0.04	0.04	0.06
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P < 0.05 Statistically significant (one-way ANOVA, followed by Tukey's multiple comparison test). ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, TB: total bilirubin, TG: triglyceride, TC: total cholesterol, LDL: low density lipoprotein, HDL: high density lipoprotein. a P < 0.05, b P < 0.01, c P < 0.001. Biochemical values in all the treated groups remained within the physiological range after the experimental period.

Table 13: Mean organ index of treated mice orally with AA, HS, and ARTEFOL

Organ	NoGp	<i>Artemisia afra</i> (AA)			<i>Hibiscus sabdariffa</i> (HS)			AA + HS		
		500	1000	2000	500	1000	2000	500	1000	2000
Liver	5.26 ± 0.27	4.96 ± 0.19	5.16 ± 0.21	5.08 ± 0.26	5.57 ± 0.33	5.22 ± 0.42	4.74 ± 0.24	4.97 ± 0.13	5.11 ± 0.27	5.10 ± 0.33
Heart	0.46 ± 0.02	0.49 ± 0.01	0.53 ± 0.02	0.47 ± 0.01	0.51 ± 0.02	0.47 ± 0.01	0.47 ± 0.01	0.47 ± 0.01	0.47 ± 0.03	0.66 ± 0.17
Kidney	1.52 ± 0.12	1.45 ± 0.14	1.61 ± 0.08	1.57 ± 0.11	1.53 ± 0.20	1.48 ± 0.08	1.36 ± 0.08	1.39 ± 0.10	1.38 ± 0.09	1.24 ± 0.08

Table 14: Effect of sub-acute oral administration of AA, HS, and ARTEFOL on hematological parameter

	NoGP	<i>Artemisia afra</i> (AA)			<i>Hibiscus sabdariffa</i> (HS)			AA + HS		
Dose (mg/kg)		500	1000	2000	500	1000	2000	500	1000	2000
WBC (×10 ³ µL ⁻¹)	11.40 ± 0.08	13.02 ± 0.65 ^c	11.32 ± 0.11	12.02 ± 0.01	11.47 ± 0.10	12.97 ± 0.71 ^c	10.86 ± 0.58	11.03 ± 0.01	11.98 ± 0.15	13.02 ± 0.68 ^c
RBC (×10 ⁶ µL ⁻¹)	5.97 ± 0.27	6.57 ± 0.10 ^b	5.89 ± 0.22	6.18 ± 0.15	6.28 ± 0.08	6.19 ± 0.35	6.14 ± 0.19	6.11 ± 0.02	6.31 ± 0.08	6.21 ± 0.08
Haemoglobin (dL)	11.88 ± 0.13	12.28 ± 0.03	11.63 ± 0.22	11.96 ± 0.09	11.88 ± 0.27	12.26 ± 0.46	11.79 ± 0.28	11.80 ± 0.26	12.18 ± 0.23	12.43 ± 0.39
Haematocrit (%)	32.62 ± 0.50	31.75 ± 0.43	33.31 ± 0.64	32.38 ± 0.44	35.64 ± 0.62 ^c	35.32 ± 0.69 ^c	34.11 ± 0.48 ^a	34.54 ± 0.90 ^b	35.15 ± 0.37 ^c	36.26 ± 0.52 ^c
Platelets (×10 ³ µL ⁻¹)	539.33 ± 5.42	550.33 ± 5.14	468.33 ± 29.52 ^b	466.83 ± 2.19 ^b	465.50 ± 25.14 ^b	455.66 ± 17.14 ^c	476.83 ± 22.09 ^b	461.00 ± 22.85 ^c	463.66 ± 26.85 ^c	491.33 ± 18.66
Lymphocytes (%)	60.13 ± 0.30	65.65 ± 1.68	68.57 ± 4.58	71.13 ± 1.68 ^a	67.82 ± 6.87	73.29 ± 4.94 ^b	72.82 ± 5.88 ^b	61.98 ± 0.74	69.07 ± 0.68	63.68 ± 2.85
Monocytes (%)	12.50 ± 0.27	13.62 ± 0.12 ^a	6.88 ± 0.29 ^c	6.39 ± 0.37 ^c	6.94 ± 0.19 ^c	6.87 ± 0.22 ^c	6.93 ± 0.51 ^c	12.87 ± 0.66	13.02 ± 0.29	12.60 ± 0.45
Granulocytes (%)	20.48 ± 0.23	22.05 ± 0.65 ^a	20.78 ± 0.53	22.70 ± 0.52 ^b	23.57 ± 1.10 ^c	19.63 ± 0.47	20.57 ± 0.78	21.94 ± 0.16	21.26 ± 0.61	21.30 ± 0.57
Neutrophils (%)	22.43 ± 0.23	23.00 ± 0.04	21.52 ± 1.18	20.72 ± 0.27 ^c	21.99 ± 0.54	21.18 ± 0.29 ^a	21.00 ± 0.27 ^b	21.67 ± 0.07	21.79 ± 0.04	21.03 ± 0.30 ^b
Eosinophils	2.40 ±	2.60 ±	2.92 ±	2.46 ±	2.86 ±	2.92	2.89 ±	2.80 ±	2.70 ±	2.67 ±

(%)	0.18	0.24	0.17 ^a	0.09	0.15	± 0.30 ^a	0.21	0.25	0.07	0.22
MCV (fL)	52.89 ± 0.34	53.13 ± 0.28	55.79 ± 0.43 ^c	55.20 ± 0.15 ^c	55.91 ± 0.35 ^c	56.30 ± 0.31 ^c	55.95 ± 0.44 ^c	53.46 ± 0.10	55.52 ± 0.30 ^c	52.69 ± 0.15
MCh (pg)	15.50 ± 0.12	16.08 ± 0.31 ^b	16.73 ± 0.11 ^c	15.68 ± 0.08	16.84 ± 0.11 ^c	16.91 ± 0.12 ^c	16.86 ± 0.12 ^c	16.25 ± 0.26 ^c	15.73 ± 0.23	16.95 ± 0.11 ^c
MCHC (g/dL)	29.10 ± 0.34	29.36 ± 0.35	30.28 ± 0.05 ^c	30.40 ± 0.08 ^c	31.03 ± 0.33 ^c	31.10 ± 0.17 ^c	30.44 ± 0.33 ^c	29.97 ± 0.03 ^a	30.22 ± 0.22 ^c	31.01 ± 0.55 ^c

RBC: red blood cell, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, WBC: white blood cell. P < 0.05 statically significant (one-way ANOVA, followed by Tukey's multiple comparison test), ^a P < 0.05, ^b

IV. Discussion

More than 60 percent of the African population relies on traditional herbal medicine to meet their primary healthcare demand. However, the lack of validated information on antimalarial efficacy and safety has been a major concern regarding the use of these medicinal plants in the fight against malaria (Assefa et al., 2020). Regardless of the efficacy of *A. afra* against sexual and asexual *Plasmodium* parasites, its characteristic bitter taste when taken in the form of an infusion could impact the overall acceptance or compliance with the herbal product, especially by asymptomatic patients. These asymptomatic malaria patients constitute a vast majority of people in endemic areas and perpetuate the disease transmission. To address this, the present study explores the formulation of ARTEFOL, a combination of *A. afra* and *H. sabdariffa*, the latter known for its acidic-sweet flavor and antioxidant properties. The goal was to enhance palatability while maintaining or improving therapeutic efficacy. Detailed knowledge about the chemical composition, toxicity, and efficacy of this extract combination in malaria therapy is provided in these findings.

High-Performance Thin-Layer Chromatography (HPTLC) analysis revealed distinct chemical profiles for AA, HS, and ARTEFOL (Figure 51). The presence of bioactive compounds such as scopoletin, scopolin, chlorogenic acid and its derivatives, rutin, hyperoside, astragalin and luteolin in AA, HS and ARTEFOL in the HPTLC analysis was further confirmed in the UPLC analysis. However, luteolin and astragalin were absent in HS.

Sensory analysis of infusions was done through the assessment of the hedonic rating of the food product. Four teas were prepared as described above and administered to 100 participants who tested the infusions, A11, (5g of *A. afra* /1L of tap H₂O), A17 (5g of *H. sabdariffa* /1L of tap H₂O), A19 (50:50 combination of infusions of A11 and A17) and a positive control (lipton; a tea locally consume by Cameroonian population) and rated them on a scale of 9 based on the following parameters; bitterness, color, taste, consistency, flavour and overall acceptability. In this study, an equal combination of AA and HS infusions masked the bitterness of the former, leading to an improved acceptance level with a hedonic score of 7.2 ± 0.14 on a scale of 9 compared to AA alone (6.5 ± 0.25) (Table 9). This combination was thus the second most accepted tea in the study after Lipton, a local tea consumed by the Cameroonian population (Table 9). The presence of anthocyanins in HS

characterised its red color, while the acidic sweet taste is attributed to the organic acids such as citric, malic, and tartaric acid (Izquierdo-Vega et al., 2020), which may have played a significant role in masking the bitterness of AA, leading to an improved acceptance level.

To validate the therapeutic effect of the combination on malaria parasites, we investigated the *in vitro* antiplasmodial properties of all extracts and the combination on a chloroquine-sensitive *Pf3D7* strain and *in vivo* studies on *P. berghei*-infected albino mice. The results showed good antiplasmodial properties for all ethanol extracts and combinations, while the tea infusions demonstrated weak antiplasmodial activity (Table 10). The observed activity could be ascribed to the presence of phenolic compounds such as chlorogenic acid, rutin and coumarins, scopoletin, which have been shown to demonstrate weak antiplasmodial activity (Shinyuy et al., 2023). However, a combination of these metabolites could work in synergy, leading to the observed antimalarial effects.

In order to better confirm that HS does not influence the antiplasmodial properties of AA, ethanol extracts that demonstrated good activity *in vitro* were further evaluated *in vivo*. The *in vivo* component of the study corroborated the *in vitro* findings, demonstrating the effectiveness of the combined herbal treatments against *Plasmodium* parasites in an animal model. Both the AA and extract combination showed a significant ($P > 0.001$) curative effect at all doses compared to the negative control, with maximum suppression at the highest dose. Like the standard drug, all the treated groups reduced parasitemia levels. This might be an indication of the comparative onset of the defensive action of the extract and standard drug. Both the AA and extract combination showed a dose-dependent curative antimalarial effect (Figure 56).

Pack cell Volume (PCV) reduction in the test groups compared to the negative control is indicative of the effectiveness of the crude extract in preventing hemolysis due to the rising parasitemia level (Figure 57). Escalating parasitemia causes the clearance and/or destruction of infected RBCs, clearance of uninfected RBCs, and erythropoietic suppression. The prevention of PCV reduction may be attributed to the antimalarial activity of the extracts against parasitized red blood cells (RBCs) and the causative parasite, thereby maintaining the availability of new RBCs produced in the bone marrow. The antioxidant properties of HS can stimulate the production of ROS that are

involved in the fight against oxidative stress, hence causing less damage to the host. (Nadia et al., 2020 and Girmaw et al., 2022). Additionally, rectal temperature reduction was prevented in the test groups compared with the negative control, indicating the antimalarial effect of the extracts, as hypothermia is a symptom associated with escalating parasitemia (**Figure 58**). Weight loss observed during treatment may be attributed to catabolic activity, appetite suppression, and metabolic dysfunction associated with malaria.

After detailing the therapeutic effect of the AA and AA/HS combination, the focus was to ascertain the safety profile through evaluation of acute and subchronic oral toxicity assessment. In the acute toxicity study which is aim to check the harmful effects of an agent on the organism given as a single or short-term exposure (Raji et al., 2021), there were no signs of toxicity or mortality at a dose of 5000 mg/kg for AA and AA/HS combination, indicating an LD₅₀ > 5000 mg/kg (OECD, 2008). Treated mice exhibited normal activity and progressive weight gain, supporting the safety of the formulation (Raji et al., 2021; Mokenen et al., 2020; Njinga et al., 2020).

To further confirm the safety of AA and AA/HS combination, a subchronic oral toxicity test was employed over 28 days by administering repeated doses (500, 1000, and 2000 mg/kg body weight) of AA and AA/HS to mice in the different treatment groups. In this study, all animals were active and responded positively to stimuli. No deaths and no signs of toxic effects were observed for all treatment groups. Additionally, the mean organ weights of all treated mice did not differ significantly ($P > 0.005$) from those of the control groups (**Table 13**). Liver function and renal function tests were performed, and metabolic biomarkers were measured. In this study, AST levels decreased at 500/500 mg/kg but increased at 2,000/2,000 mg/kg for the extract combination. In contrast, AST levels in mice treated with HS were slightly elevated at doses of 1,000 and 2,000 mg/kg. While ALP levels at doses of 1000 and 2,000mg/kg in mice treated with AA, HS, and AA/HS combination were decreased compared with the distilled water-treated group (**Table 12**). Additionally, there was no significant alteration in the serum levels of urea and creatinine concentrations in mice in the test groups as compared to the distilled water-treated group. The absence of change in the levels of the above renal function markers suggests that the extract combination does not

cause deterioration in renal function (Eshetu et al., 2026, and Njinga et al., 2020).

Previous studies have shown that hematological parameters are very sensitive and could be used as reliable indicators for determining the intrusion of toxic substances (Arika et al, 2016). In this study, mice treated with HS and AA/HS combination at doses of 500, 1000, and 2,000 mg/kg developed a significant increase in HCT and MCHC ($p < 0.05$). However, these values are within normal range (Table 14). Nonetheless, the significant decline of platelet counts in mice at a dose of 500 and 1000 mg/kg of HS, as well as extract combination, and 2,000 mg/kg of AA and HS, compared to the distilled water-treated group, suggested that the extract can induce thrombocytopenia. The fact that no significant change was observed in TC and TG levels in this study suggests that the extract combination may not have side effects on cholesterol metabolism in mice. The fact that HS did not exacerbate toxicity concerns associated with AA is encouraging. The absence of significant toxicity implies that these herbal combinations may be well-tolerated, a critical factor for their eventual application in clinical settings.

V. Conclusion

This study underscores the potential of combining HS with AA as a novel strategy to increase compliance with the herbal remedy while maintaining the therapeutic benefits of AA and a favorable toxicity profile. The *in vivo* results corroborated *in vitro* findings, demonstrating significant anti-plasmodial activity of both AA and ARTEFOL. The dose-dependent suppression of parasitemia and the curative effects observed across all tested concentrations underscore the efficacy of the extracts. Moreover, the study provides a foundation for future investigations, potentially leading to the development of standardized herbal formulations for antimalarial interventions. The positive *in vitro* and *in vivo* outcomes, coupled with the absence of significant toxicity, pave the way for further research and development of these herbal combinations as alternative and complementary approaches to combat malaria.

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Chapter Seven

**Toxicity Profile, Antiplasmodial and
Gametocytocidal Properties of *Erigeron
bonariensis* L. (anc. *Conyza bonariensis* (L.)
Cronquist).**

7.1 Foreword

In Cameroon, *Artemisia annua* and *Artemisia afra* are being cultivated, processed and sold in local markets for the management of ailments, particularly malaria in several regions. This activity generates income and thus improves the livelihood of local farmers. However, in the Adamawa region of Cameroon, the commercialisation of *Artemisia annua* and *Artemisia afra* for the treatment of malaria is not as effective as in other regions of Cameroon. Ethnopharmacological investigation revealed that the local population in this region used *Erigeron bonariensis* (locally called bitter-bitter in the Adamawa region) as a traditional remedy for the treatment of malaria. This is mostly taken in the form of an infusion or a decoction. Like *A. annua* and *A. afra*, *Erigeron bonariensis* is a member of the Asteraceae family and as has been shown to be of ethnopharmacological relevance in several African countries for the management of several ailments including malaria. *Erigeron bonariensis* has a rich repertoire of bioactive molecules including polyphenols, coumarins, flavonoids, terpenoids essential oils among others. Due to this observation, it was important to provide and document scientific evidence on the antimalarial property and safety profiles of *Erigeron bonariensis*. In this chapter, we focused to investigate the antiplasmodial and gametocytocidal properties of *E. bonariensis*. Additionally, we detailed the safety profiles of *E. bonariensis* by assessing cytotoxicity, acute and subchronic oral toxicity in an albino mice. The data presented herein provide scientific justification and supports the use of *E. bonariensis* as a traditional remedy for the fight against malaria especially in resource limited settings where access to modern therapy is problematic.

7.2 Toxicity profile, antiplasmodial and gametocytocida properties of *Erigeron bonariensis* L. (anc. *Conyza bonariensis* (L.) Cronquist) (Article published)

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Toxicity profile, antiplasmodial and gametocytocidal properties of *Erigeron bonariensis* L. (anc. *Conyza bonariensis* (L.) Cronquist)

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ABSTRACT

Ethnopharmacological relevance: *Erigeron bonariensis* L. (anc. *Conyza bonariensis* (L.) Cronquist) is a medicinal plant with traditional use in Cameroonian folk medicine and other parts of Africa for the management of ailments, including malaria.

Aim: This study focused on investigating the antiplasmodial, gametocytocidal properties and toxicity profile of *E. bonariensis*.

Materials and methods: Extracts of *E. bonariensis* were prepared by sequential maceration using n-hexane, ethyl acetate, and methanol, and their antiplasmodial properties were assessed on both sexual and asexual *Plasmodium* parasite stages. Additionally, cytotoxicity was assessed on the MDA-MB-231 breast cancer cell line, while acute and sub-chronic toxicity were evaluated using albino mice.

Results: *Conyza* spp. all extracts were active against both stages of the parasite at different levels. Hexane extract

Figure 62: Screenshot of the cover page of the published article.

Abstract

Ethnopharmacological relevance: *Erigeron bonariensis* L. (anc. *Conyza bonariensis* (L.) Cronquist) is a medicinal plant with traditional use in Cameroonian folk medicine and other parts of Africa for the management of ailments, including malaria.

Aim: This study focused on investigating the antiplasmodial, gametocytocidal properties and toxicity profile of *E. bonariensis*.

Materials and methods: Extracts of *E. bonariensis* were prepared by sequential maceration using n-hexane, ethylacetate, and methanol, and their antiplasmodial properties were assessed on both sexual and asexual *Plasmodium* parasite stages. Additionally, cytotoxicity was assessed on the MDA-MB-231 breast cancer cell line, while acute and sub-chronic toxicity were evaluated using albino mice.

Results: Generally, all extracts were active against both stages of the parasites at different levels. Hexane extract exhibited good antiplasmodial activity (IC₅₀ 6.02 ±1.13 µg/mL) against asexual parasites while also demonstrating the highest gametocytocidal activity (IC₅₀ 1.863 ±0.717 µg/mL). All extracts were relatively non-toxic on the MDA-MB-231 breast cancer cell line. No significant adverse effects at the tested dose were observed following an acute exposure with an LD₅₀ > 5000 mg/kg BW. In the sub-chronic toxicity evaluation, no significant changes in body weight, or hematological and biochemical parameters were observed across the dosed groups compared to the control, with a p-value <0.05. Additionally, histopathological examination of the liver indicated no signs of toxicity or abnormalities.

Conclusion: The findings show that *E. bonariensis* has effective antiplasmodial and gametocytocidal properties *in vitro* and has relatively low toxicity. This study supports the use of *E. bonariensis* as a culturally appropriate alternative for malaria management. However, further studies are needed on the safety profile and in harnessing the therapeutic potential of *E. bonariensis* for the development of novel antimalarials.

Keywords: Malaria, Medicinal plants, *Erigeron bonariensis*, Antiplasmodial, Toxicity

I. Introduction

Malaria continues to pose a significant global health challenge, particularly in resource-limited regions. It is responsible for over 251 million cases and has caused over 610,000 deaths across 85 malaria-endemic countries in 2023 globally (WHO, 2024). Cameroon is among the 11 countries with the highest malaria burden, contributing to 70% of global malaria cases and reporting over 2.7 million cases, according to the World Health Organization (WHO, 2024). The primary chemotherapeutic medication used to treat malaria is Artemisinin-based Combination Therapies (ACTs). However, the development and spread of drug-resistant strains of *Plasmodium falciparum* to ACTs (WHO, 2024) and to other antimalarials have impeded disease control efforts, emphasizing the critical need for alternative therapeutic agents. In response, there has been a growing interest in natural products research, particularly plant-derived compounds, which have become increasingly important in modern medicine for their potential to combat malaria and other diseases (Kamaraj et al., 2023; Nchang et al., 2023; Shinyuy et al., 2025).

Among these natural sources, *Erigeron bonariensis* L. (anc. *Conyza bonariensis* (L.) Cronquist), belongs to the Asteraceae family, and commonly known as fleabane has attracted attention due to its traditional use in folk medicine (Hanwen, 2007). Indigenous to South America, *Erigeron bonariensis* (EB) is a species of flowering plant with alternate lance-shaped leaves covered with fine hairs. EB has upright stems covered in thick hairs. At the tips of the branches are clusters of tiny, daisy-like blooms (Figure 63). EB flourishes throughout roadsides, meadows, pastures, and waste places thanks to its fibrous root system (Hanwen, 2007). EB is an invasive species in some parts of North America, Europe, Africa, and Asia (Hanwen, 2007). It is widely distributed across the temperate and tropical regions and has a long history of use as a traditional remedy for treating fevers related to malaria (Araujo et al., 2013). EB has a rich phytochemical composition, including flavonoids, glucosids, terpenoids, essential oils, phenolic compounds, and sesquiterpene lactones, among others (Ferreira et al., 2023; Opiyo, 2023). Due to its rich phytochemical profile, EB has demonstrated activity against several ailments. It is known for its antifungal, antibacterial, antioxidant, analgesic, anti-inflammatory, and antimicrobial properties (Boniface and Pal, 2013). EB is used traditionally for a variety of illnesses, including asthma, bronchitis, hypertension, anti-inflammatory, antimicrobial, malaria, and diarrhea

(Espinoza et al., 2020; Kamaraj et al., 2023; Opiyo, 2023). Hence, a product of high demand, especially in malaria-endemic regions and therefore, a plant of high ethnomedicinal and ethnopharmacological relevance. However, the daily massive consumption of natural plant products to manage diseases such as malaria and the integration of traditional medicine into conventional modern medicine make it imperative to determine the efficacy and document the safety profile of this natural plant product (Bandaranayake et al., 2006). Although EB has a long history of use and is of ethnopharmacological importance in alleviating fevers related to malaria (Opiyo et al., 2023), there is little or no data on both the safety profile and efficacy on *Plasmodium* gametocytes (the form of the parasite responsible for the disease transmission) and the asexual parasites (causes symptoms and pathology of malaria). It is therefore important to elucidate and document the toxicity profile as well as the efficacy of EB on malaria parasites. This study is focused on investigating the antiplasmodial potentials of leaf extracts of EB against the gametocytes and asexual forms of *P. falciparum* and evaluating its toxicity profile by assessing cytotoxicity, acute and sub-chronic oral toxicity. Moreover, targeting the *Plasmodium* gametocyte is crucial as it will serve as a vital tool to block transmission of malaria while also alleviating the disease pathologies. This would provide valuable insights into the potential therapeutic application of EB and substantiate its traditional use, particularly in resource-limited settings where malaria burden remains high. Leveraging EB's pharmacological properties might offer new insights into the search and development of biologically active compounds with broader application in the management of infectious diseases.



Figure 63: Field picture of EB in the Adamawa region of Cameroon

II. Materials and methods

II.1. Collection and processing of plant material

Fresh leaves of EB were collected from the Adamawa region, Cameroon, and voucher specimens were deposited at the Limbe Botanical Gardens, authenticated, and a voucher number assigned (SCA 1678). The leaves were sorted, oven-dried at 30°C, powdered, and kept in appropriate condition for the preparation of crude extracts.

II.2. Preparation of crude extracts

Crude extracts were prepared by sequential maceration as described by [Mbah et al. \(2012\)](#). In brief, the plant powder was successively macerated at room temperature for three days, starting with n-hexane, followed by ethyl acetate, and finally methanol. Whatman No. 1 filter paper was used to filter the mixture, and Büchi brand Rota vapor was used for rotary evaporation under pressure to concentrate the filtrate at 30°C. After being recovered using the appropriate solvents, each crude extract was dried, weighed, and

kept for further use at 4°C. Additionally, ethanol extract was prepared as described above and used to evaluate the safety profile of EB.

II.3. *Plasmodium falciparum* 3D7 *in vitro* culture and viability test

As described by [Trager and Jensen \(1976\)](#), a chloroquine-sensitive 3D7 *P. falciparum* strain was maintained *in vitro* in 3 % hematocrit (human type A or O-positive red blood cells). Complete Culture Medium (CCM) used in the assay was prepared by supplementing RPMI 1640 (Gibco, Fisher Scientific, Merelbeke, Belgium) medium containing NaHCO₃ (32 mM), HEPES (25 mM), and L-glutamine with 10 % heat-inactivated human plasma, 1.76 g/L of glucose (Sigma-Aldrich, Overijse, Belgium), 44 mg/mL of hypoxanthine (Sigma-Aldrich, Overijse, Belgium), and 100 mg/L of gentamycin (Gibco, Fisher Scientific, Merelbeke, Belgium). Parasitemia was verified by microscopy using Giemsa-stained thin films. All cultures were placed in a humidified incubator at 37 °C with a standard gas mixture of 5 % O₂, 5 % CO₂, and 90 % N₂. A stock solution of crude extracts was prepared by dissolving 10 mg of extract in 1 mL DMSO, and an antiplasmodial assay was conducted using an initial extract concentration of 100 µg/mL, which was subsequently serially diluted to eight concentrations. Each extract was tested in duplicate wells with the parasites at a final volume of 250 µL, making a final DMSO concentration of < 1%. The positive control consisted of wells with parasites only, while the negative control consisted of wells with only culture medium. As a standard, a stock solution of artemisinin (1µg/mL) (Gibco, Fisher Scientific, Merelbeke, Belgium) was made and serially diluted as described above. During a 48-hour period of incubation, plasmodial lactate dehydrogenase activity was estimated to assess parasite growth, as previously reported ([Kenmogne et al., 2006](#)), and linear regression was used to determine the IC₅₀ (concentration that kills 50% of parasites). All tests were repeated at least twice.

II.4. Induction of gametocytogenesis, culture, and viability assay

Gametocyte induction and culturing were done as described elsewhere ([Gebru et al., 2017](#)). Before the start of initiation of gametocyte culture, synchronization of asexual chloroquine-sensitive NF-54 *P. falciparum* strain was performed by D-sorbitol (5% w/v) and magnetically aided magnetic cell separation with LD-MACS. Upon synchronisation, gametocyte culture was started with a 6% haematocrit, 0.3% ring-stage parasitaemia, and a daily change of medium for 14 days. Haematocrit was dropped to 3% when a

parasitaemia of 3% was reached. N-acetyl-D-glucosamine was added to a final concentration of 50 mM between days 10 and 14 to eliminate all parasite forms other than late-stage gametocytes (stage IV and V), which were then subjected to *in vitro* viability assays. Working concentrations were prepared by serially diluting the stock solution of extracts (prepared as previously described) in at least 1:500 (DMSO < 0.4 %). Methylene blue (50µM) prepared in sterile distilled H₂O was used as a gametocytocidal drug control. Medium (80µL) was introduced in each well, and 40µL of the extract/drug was added in well A and serially diluted (1:3 dilution) to well G. The last wells were used as growth control (well H). A column for the gametocyte dilution control was also prepared. Gametocyte cultures were purified with NycoPrep and MACS separation ([Held et al., 2014](#)), and an improved Neubauer counting chamber was used to quantify the parasites. 20µL of parasite suspension containing about 50000 gametocytes was added to each well. Plates were incubated for 48 h at 37°C, 5% CO₂, and 5% O₂. A luminescent assay based on the quantitation of ATP produced by the parasites (BacTiter-Glo) was used following the protocol described by [Lelièvre et al. \(2012\)](#) to assess the viability of gametocytes, and data were analyzed using R and the dcr-package to obtain IC₅₀ values. The assay was repeated at least twice in technical duplicate.

II.5. Cytotoxicity assays

Using a resazurin-based assay, cytotoxicity of EB extracts was assessed on MDA-MB-231 cells (triple-negative breast cancer) that were seeded in 96-well cell culture plates at a density of 2,500 cells per well (2.5×10^4 cells/mL). Cells were allowed to grow and adhere for 24 hours ([Bowling et al., 2012](#)) and subsequently exposed to EB extracts for 72 hours in complete medium (DMEM + 10% FBS), at a final DMSO concentration of < 0.5%. Positive control wells received DMEM containing 0.5% DMSO, whereas negative control wells were treated with doxorubicin (5 µM). The plates were kept in an incubator set at 37°C with 5% CO₂ in a humidified environment for the whole 72-hour incubation period.

Cell viability assay was performed after 72 h incubation (old media removed) by adding 75µL of resazurin reagent in each well (PrestoBlue, Thermo Fisher Scientific, ref. A13261) and fluorescence was measured using FlexStation microplate reader at an excitation/emission wavelength of 560/590 nm after 2 hours of incubation. The fluorescence values obtained were used to

compute the IC_{50} values (concentration needed to inhibit 50% of the cell population relative to the controls).

II.6. Ethical clearance

Ethical clearance for work with animals was granted by the University of Buea Institutional Animal and Use Committee (UB-IACUC), Cameroon, with a permission number: UB-IACUC N°23/2024.

II.7. Animals

Three-month-old female albino mice weighing between 17-25 g obtained from the Faculty of Medicine and Pharmaceutical Sciences, University of Douala, Cameroon, were used for acute toxicity study, while 8-week-old female albino mice weighing between 18-24g were used to evaluate sub-chronic toxicity. Animals were divided into several test and control groups, maintained in standard environmental conditions ($30 \pm 3^{\circ}\text{C}$, 12 h light/ 12 h dark cycle, free access to water and food. All methods used in this study complied with the Organization for Economic Co-operation and Development (OECD) guidelines and regulations for the testing of chemicals ([OECD, 2001](#)).

II.8. Acute Toxicity

Female adult mice were starved overnight, weighed, and divided into three distinct groups of 5 animals each. These groups were as follows: a control group and two test groups. Through an oral route using a gavage, the test groups received a single dose of 2000 and 5000 mg/kg BW of ethanol extract of EB, whereas the control group received 1 mL of distilled water per 100 g of body weight. Animals in all groups were observed for 2 hours and then allowed access to food and water. Animals were then observed once daily for the next 14 days for any mortality and other signs of toxicity, including changes in the skin and fur, eye color, convulsions, salivation, diarrhea, or coma. Body weight was measured daily throughout the experiment.

II.9. Sub-chronic toxicity

For the sub-chronic toxicity evaluation, 24 non-pregnant and nulliparous female mice were employed and randomly divided into 4 groups of 6 mice each. The test groups (group I, II, and III) received daily doses of 400, 800, and 1600mg/kg BW of ethanol extract of EB, respectively ([El Kabbaoui et al., 2017](#)), whereas the fourth group (control group) received distilled water. All

treatments were administered via gavage at the same time each day over 28 days. Animals were concurrently observed for signs of abnormalities, and body weights were recorded. After this treatment period, all animals were allowed free access to water but not food overnight, then put under ether anesthesia. Blood was collected with and without anticoagulant, Ethylenediaminetetraacetic acid (EDTA) by retro-orbital puncture for hematological and biochemical studies, respectively. After blood collection, mice were sacrificed by decapitation and dissected. The liver was collected and fixed in 10% formaldehyde for histological analysis.

II.10. Hematological and biochemical parameters assessment

An automatic hematological analyzer (SysmexKX-21N) was used for hematological analysis including red blood cell (RBC) count, leukocyte (WBC) count, hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, lymphocyte, monocyte, neutrophil, basophil and eosinophil counts (Costa et al., 2020).

Blood was centrifuged at 3000 rpm for 10min using a centrifuge (ROTOFIX 32A®, Mettich Zentrifugen, Germany) for the analysis of biochemical parameters in sera. This included the determination of glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglyceride (TG), alkaline phosphatase (ALP), total bilirubin (TB), conjugated bilirubin (EB), total protein (TP) and albumin (ALB). Additionally, concentrations of urea and creatinine were measured to assess renal function as well as other metabolites and minerals; uric acid, Na⁺, Cl⁻, K⁺, Ca²⁺, Mg²⁺, and inorganic phosphorus. The assays were performed using an automatic biochemical analyzer ([Hitachi902](#), [Roche](#)) with a spin react biochemical kit.

II.11. Liver's histopathological evaluation

This was achieved by microscopy, wherein tissues of the liver were processed with a microtome and an automated tissue processor. Each sample was afterward embedded, placed on a glass slide, and stained with Hematoxylin and Eosin (HE). Tissues were visualized by microscopy to note changes in their structure, and pictures were taken.

II.12. Data analysis

A combination of statistical packages, including R programming, SPSS (version 25.0), and GraphPad Prism (version 10.2.0), was used for statistical analysis. RM-ANOVA was used to analyze the body weight of the mice, and one-way ANOVA, followed by Tukey's multiple comparisons, was used to analyze liver function indices and lipid parameters, hematological, and kidney parameters. A p-value of < 0.05 was considered statistically significant by comparing the test results with the control group.

III. Results

III.1. Inhibition of *P. falciparum* asexual and sexual blood stages by EB extracts

The antiplasmodial property of EB was evaluated by exposing asexual chloroquine-sensitive (*Pf3D7*) and sexual NF-54 strains of *P. falciparum* to n-hexane, ethyl acetate, and methanol extracts of EB. All three extracts demonstrated strong gametocytocidal activity with IC_{50} values $< 5 \mu\text{g/mL}$ (**Table 15**). The n-hexane extract was the most active against both the gametocytes and asexual parasites with IC_{50} values $< 7 \mu\text{g/mL}$ (**Table 15**). The three extracts recorded CC_{50} values $> 100 \mu\text{g/mL}$ against MDA-MB-231 cells (**Table 15**).

Table 15: Dual-stage drug sensitivity assays (n=3).

Extract/ standard drug	IC₅₀ (µg/mL) ± SD		CC₅₀ (µg/mL)
	Gametocytes	Asexual parasites	MDA-cells
n-Hexane	1.86 ± 0.72	6.02 ± 1.13	>100
Ethyl acetate	3.53 ± 1.35	30.61 ± 4.80	>100
Methanol	2.47 ± 1.06	10.53 ± 2.82	>100
Methylene blue	0.12 ± 0.46	–	–
Artemisinin	–	0.004 ± 0.01	–

III.2. Acute toxicity

Following an acute study, no mortality was recorded in either the control group (vehicle) or the group that was treated with a single dose of 2000 mg/kg BW of EB during the 14-day follow-up period. However, at a higher dose of 5000 mg/kg BW of the extract, a 1/5 mortality rate was recorded on day 3 of the follow-up period. Loss of appetite, hypoactivity, and dizziness were recorded in the treatment groups (2000 and 5000 mg/kg BW) during the follow-up period. Lack of sensitivity to noise was observed in the 5000 mg/kg BW treatment group, but not in a lower dose group (**Table 16**). Following OECD guidelines, the median lethal dose (LD₅₀) can therefore be estimated to be greater than 5000 mg/kg BW.

Table 16: An overview of toxicity signs after an acute oral administration of ethanol extract of EB.

	Dose (mg/kg BW)	D/T	Number of mice	Symptoms of toxicity
1	Vehicle	0/5	5	None
2	2000	0/5	5	Loss of appetite Hypo-activity Dizziness
3	5000	1/5	5	Loss of appetite Hypo-activity Dizziness Lack of sensitivity to noise

III.3. Sub-chronic toxicity of ethanol extract of EB

III.3.1. Visible indicators of toxicity and body weight

All the animals were observed for two hours post administration of ethanol extracts of EB at the different doses daily to identify signs and symptoms of toxicity and behavioral changes. This study reveals no alteration in behavior or indication of toxicity with no mortality during the study, which attests to the safety of EB following a sub-chronic oral dosing. Generally, there was an increase in the relative body weights of the animals both in the control and test groups during the 28-day study period (**Figure 64**). The highest growth rate was observed in the control group, with a relative body weight increase

of up to 17%. At increasing doses, the rate of increase in the percentage body weight in the test groups was significantly lower than the control (400 mg/kg BW ($p < 0.05$) < 800 mg/kg BW ($p < 0.001$) < and 1600 mg/kg BW ($p < 0.001$))

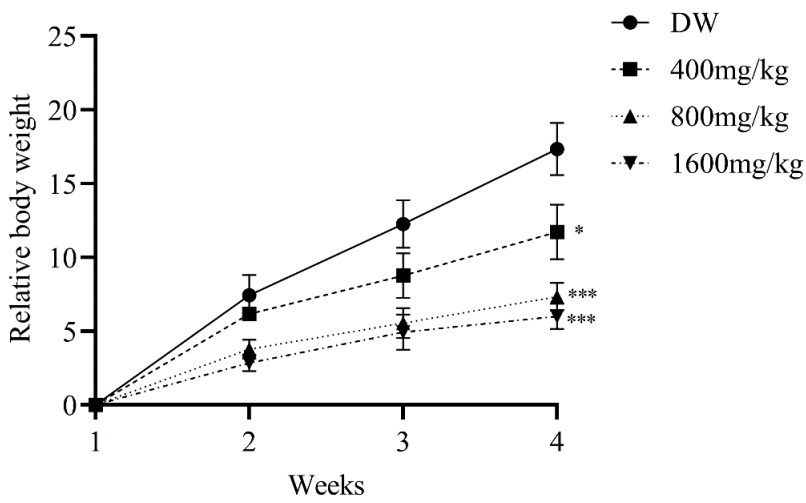


Figure 64: Effect of EB extracts on body weight of mice following sub-chronic treatment.

III.3.2. Effect of ethanol extract of EB on Liver function indices and lipid parameters

In the sub-chronic toxicity study of ethanol extract of EB, biochemical analysis of serum samples showed that the serum levels of marker enzymes did not change significantly in the test groups compared to the group treated with distilled water, except serum AST, ALT, ALP, and total cholesterol (TC) levels. Generally, there was a significant decrease ($p < 0.05$) in the serum levels of AST, ALT, ALP, and TC in the test groups (400, 800, and 1600 mg/kg BW) compared to the distilled water-treated group (**Table 17**)

Table 17: Liver function indices of mice treated with ethanol extract of EB for 28 consecutive days.

	Normal group	Ethanol extract of EB		
		400mg/kg BW	800mg/kg BW	1600mg/kg BW
Glu (g/L)	0.57 ± 0.02	0.57 ± 0.05	0.58 ± 0.03	0.55 ± 0.02
AST (IU/L)	227.31 ± 3.93	216.58 ± 2.62**	210.65 ± 4.25***	206.09 ± 4.01***
ALT (IU/L)	52.78 ± 1.05	50.08 ± 1.54	44.04 ± 1.02***	40.42 ± 0.88***
ALP (IU/L)	337.92 ± 1.25	318.66 ± 11.69*	315.38 ± 8.18***	301.86 ± 5.10***
TP (g/L)	72.14 ± 0.69	70.62 ± 0.62	71.94 ± 0.50	71.14 ± 0.50
ALB (g/L)	20.22 ± 0.11	20.77 ± 0.68	20.46 ± 0.18	20.49 ± 0.10
TB (mg/L)	6.03 ± 0.13	6.43 ± 0.70	6.85 ± 0.08	6.47 ± 0.73
EB (mg/L)	3.67 ± 0.08	3.22 ± 0.40	3.16 ± 0.53	3.10 ± 0.30
TC (g/L)	0.63 ± 0.01	0.63 ± 0.02	0.57 ± 0.03***	0.59 ± 0.03***
LDL (g/L)	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
HDL (g/L)	0.53 ± 0.01	0.51 ± 0.03	0.51 ± 0.02	0.51 ± 0.02
TG (g/L)	1.42 ± 0.14	1.49 ± 0.20	1.44 ± 0.11	1.49 ± 0.11

Data was analyzed by one-way ANOVA, followed by Tukey's multiple comparison test, and presented as mean ± SEM with *p < 0.05, ***p < 0.001, significantly different from the control group. Glu: glucose, ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, ALB: albumin, TP: total protein, TB: total bilirubin, EB: conjugated bilirubin, TG: triglyceride, TC: total cholesterol, LDL: low density lipoprotein, HDL: high density lipoprotein

III.3.3. Effect of ethanol extract of EB on hematological parameters

There were no significant modifications in hematological parameters of test groups compared to the distilled water-treated group after a twenty-eight-day treatment of animals with different doses of EB, except for WBC count, which increased significantly ($p < 0.05$) in the group treated with the dose 600 mg/kg BW relative to the distilled water-treated group. RBC count was also seen to increase significantly ($p < 0.001$) in all the treatment doses (400, 800, and 1600 mg/kg BW). There was a general increase in all the other hematological parameters of EB-treated mice as compared to the distilled water-treated group (**Table 18**). However, these increases were not significant.

Table 18: Effect of EB ethanol extract on hematological parameters in mice

parameter	Ethanol extract of EB			
	Normal group	400 mg/kg BW	800 mg/kg BW	1600 mg/kg BW
WBC (X103 U L ⁻¹)	11.18 ± 0.13	10.78 ± 0.15	11.56 ± 0.03	12.07 ± 0.89 *
RBC (X106 UI ⁻¹)	6.02 ± 0.15	6.72 ± 0.13***	6.72 ± 0.11***	6.87 ± 0.23 ***
Hemoglobin (G/Dl)	11.66 ± 0.27	11.90 ± 0.31	12.05 ± 0.127	11.57 ± 0.47
Hematocrit (%)	34.14 ± 0.40	34.72 ± 0.49	34.31 ± 0.24	33.79 ± 0.73
Platelets (x103 µl ⁻¹)	543.66 ± 7.44	530.66 ± 8.33	535.83 ± 8.22	542.83 ± 24.88
Lymphocytes (%)	59.26 ± 0.32	59.46 ± 4.44	61.16 ± 3.57	64.79 ± 3.81
Monocytes (%)	12.58 ± 0.65	12.68 ± 1.10	12.99 ± 0.48	12.94 ± 0.55
Granulocytes (%)	20.82 ± 0.36	20.98 ± 0.73	20.91 ± 0.55	21.00 ± 0.43
Neutrophils (%)	21.53 ± 0.31	21.29 ± 0.22	21.66 ± 0.38	21.44 ± 0.43
Eosinophils (%)	2.30 ± 0.20	2.29 ± 0.13	2.515 ± 0.07	2.48 ± 0.10
Basophils (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.01
MCV (fl)	55.54 ± 0.38	55.55 ± 0.39	55.55 ± 0.32	56.04 ± 0.41
MCH (Pg)	16.73 ± 0.13	16.65 ± 0.19	16.66 ± 0.20	16.60 ± 0.22
MCHC (G/Dl)	28.92 ± 0.14	28.89 ± 1.03	29.40 ± 0.63	28.79 ± 0.74

Data were presented as mean ± SEM (n=6 (3 females+3males)/group). *p < 0.05, **P < 0.01, *** < 0.001, significantly different from the distilled water treated group using one-way ANOVA, followed by Tukey's multiple comparison test.

III.3.4. Histopathological assessment of the liver

No pathological abnormalities were observed in the liver profile of the test groups compared to the distilled water-treated group. For example, after 28 days of treatment, there was no significant distortion of the liver cytoarchitecture at all doses of EB relative to the distilled water-treated group. Additionally, analysis of the histological profiles of the liver showed that the control or distilled water-treated group had normal liver profiles comprising hepatocytes well arranged in cords radiating from a central vein. (Figure 63).

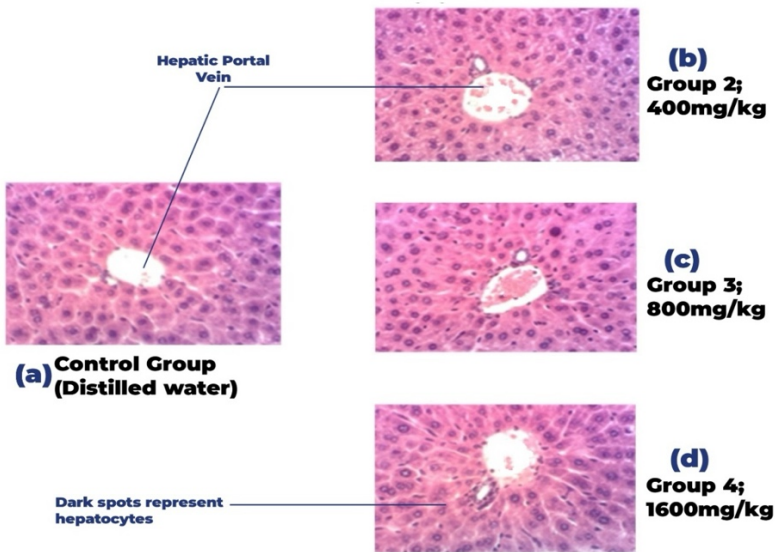


Figure 65: Histological section of the liver of mice subjected to various treatments with the ethanol extract of EB.

IV. Discussion

E. bonariensis (EB) is a medicinal plant with traditional use in Cameroonian folk medicine and other malaria-endemic countries. However, there is limited information on the safety profile and efficacy of EB against *P. falciparum* gametocytes. This study focused on investigating the safety profile of EB by assessing the acute and sub-chronic exposure of ethanol extract of EB in an appropriate animal model and investigating the *in vitro* antiplasmodial activity and gametocytocidal activity. As mentioned earlier, n-hexane, ethyl acetate, and methanol extracts of EB were prepared by

sequential maceration and subjected to *in vitro* assay to assess their antiplasmodial properties on both sexual and asexual chloroquine-sensitive NF-54 and 3D7 *P. falciparum* strains, respectively. Additionally, ethanol extract was prepared and used for both acute and sub-chronic oral toxicity assessment.

According to the classification describe by [Jansen et al. \(2017\)](#), antiplasmodial activity levels of plant crude extract are conventionally categorized as follow; highly active ($IC_{50} \leq 5 \mu\text{g/mL}$), good activity (IC_{50} 5 - 15 $\mu\text{g/mL}$), moderate activity (IC_{50} 15 - 30 $\mu\text{g/mL}$), weak activity (IC_{50} 30 - 50 $\mu\text{g/mL}$) and lack of activity with $IC_{50} \geq 50 \mu\text{g/mL}$. In this context, the n-hexane and methanol extracts demonstrated good activity against the asexual *Pf3D7* strain, whereas the ethyl acetate extract showed weak activity. Interestingly, all extracts demonstrated high gametocytocidal activity against the *P. falciparum* NF-54 strain. The rich phytochemical composition of EB, especially flavonoids and phenolic acids, could account for the observed antiplasmodial properties. For example, phenolic compounds such as caffeic acid and chlorogenic acid are present in EB ([Barhoumi et al., 2013](#)) and hence, could contribute to the plant's antimalarial effects. By inducing oxidative stress, phenolic compounds are known to disrupt the life cycle of the malaria parasite and diminish its virulence ([Mamede et al., 2020](#)). Additionally, quercetin and kaempferol, which are naturally occurring compounds in EB ([Barhoumi et al., 2013](#)), have been shown to interfere with the growth of *P. falciparum* ([Shinyuy et al, 2023](#)). The reported antimalarial effect may also be possibly explained by the presence of conyzine, an alkaloid isolated from EB ([Opiyo, 2023](#)) that could interfere with parasite metabolism and protein production, leading to death.

In the acute toxicity study, the aim was to classify acute toxicity of EB extract after a single exposure according to the Globally harmonized system of classification and labelling of chemicals (GHS). The acute toxicity of the ethanolic extract of EB was evaluated by administering a single dose of 2000 mg/kg BW and 5000 mg/kg BW to mice. During the 14 days post administration, there were no overt signs of toxicity in both the control and the test groups. No mortality was observed in the test group (2000 mg/kg BW of extract), while one death was recorded in the test group (5000 mg/kg BW) on day 3. Following the 14-day post administration, both the test group and the distilled water-treated groups showed no significant signs of toxicity.

Behavioral and physical parameters such as grooming, locomotion, and physical appearance remained normal, indicating that EB is well tolerated in an acute exposure, with an LD₅₀ > 5000 mg/kg BW. This agrees with the findings of [Zalabani et al. \(2012\)](#), who reported an LD₅₀ > 5000 mg/kg BW after an acute oral administration of methanol extracts of EB in mice.

A repeated daily oral administration of the ethanolic extract of EB in mice for 28 consecutive days was performed to determine the subchronic effect of EB. In this study, the ethanolic extract of EB demonstrated a safety profile as evidenced by the absence of significant changes in the general behavior or body weight of mice after a subchronic oral exposure. The fact that there were no significant differences or appreciable variations in the percentage of body weight between the test and the distilled water-treated or control groups indicates that the ethanolic extract had no direct impact on the animals' overall health. There was a significant increase in the relative body weight of mice in the control group during the experiment. Treatment with different doses of the extract resulted in a significant reduction in the growth rate of mice compared to the control group. This may likely be due to a decrease in appetite and or slowdown in metabolism, evidenced by the significant decrease in liver parameters, including total cholesterol (TC, synthesized mainly in the liver).

Biochemical assessments focused on markers of liver and kidney function. A significant reduction in the activities of AST, ALT, ALP, and the level of total cholesterol (TC) in the test groups, with respect to the control group, indicates the hepatoprotective and lipid-lowering properties of the extract. Reduction in AST and ALT activities indicates a decrease in liver metabolic activity, leading to decreased synthesis of enzymes; this may occur at the level of nucleic acid metabolism or protein synthesis. Additionally, a decrease in total cholesterol (TC) suggests that the plant extract may have cholesterol-lowering properties by modulating lipid metabolism or reducing the synthesis of cholesterol.

In our present study, there were no appreciable variations in the serum creatinine levels between the treatment and control groups, whereas only urea was significantly decreased. During deamination, ammonia (NH₃) is removed from the blood by conversion into urea, and an increase may be because of high glomerular filtration ([Olaniyan et al., 2016](#)). A decrease in urea concentration with sub-chronic toxicity administration of ethanol

extract of EB suggests efficient kidney function produced by the extract for the removal of NH_3 . Also, we observed a progressive decrease in the level of uric acid. Excess uric acid is caused by defects in the metabolism of purine nucleotides. The observed reduced production of uric acid in the treated mice points to either a decline in nucleic acid catabolism or increased excretion; however, decreased synthesis (mainly in the liver) agrees with the decrease in hepatic enzymes and TC. Estimating the levels of electrolytes (K^+ , Na^+ , Cl^- , Ca^{2+} , Mg^{2+} , and inorganic phosphorus) in serum may be important in the assessment of renal function since the outcome of the regulatory mechanism of osmotic balance and ionic charges can be determined by the levels of electrolytes in the blood ([Opoku-Okrah et al., 2015](#)). Sodium is the major cation of the extracellular fluid, where it regulates water-electrolyte balance and protects the body against excessive fluid loss. Potassium is the major intracellular cation with a similar role to that of Sodium. In the extracellular and intracellular fluid, sodium and potassium, respectively, are the main cations that control the water-electrolyte balance and shield the organism from excessive fluid loss. Sodium and potassium levels were not significantly different in the test groups relative to the control group, confirming that the plant extract had little or no impact on the water-electrolyte balance in mice. However, the extract could specifically impact divalent cation homeostasis, possibly through hormonal and/or renal function, evidenced by the depletion in Ca^{2+} and Mg^{2+} ions.

From the findings of [Mukinda and Syce \(2007\)](#), the hematopoietic system is more susceptible to harmful substances, and therefore, assessing the effect of the ethanol extract of EB on the hematopoietic system is important to further validate its safety profile. This is possible through the evaluation of hematological parameters after a sub-chronic oral administration in mice ([Adebayo et al., 2017](#)). Results from this study indicate that, except for RBCs and neutrophils (decreased concentration), WBCs, lymphocytes, and hemoglobin (increased concentration) that differ sufficiently in the test groups compared to the distilled water treated group, most of the investigated hematological parameters did not show significant variation. The mean increase in WBCs level in all test groups is most likely induced by inflammation which could be partly confirmed by the mean increase in Lymphocyte levels in the test groups. The mean rise in WBCs levels in the test groups may partially supports the hypothesis that inflammation is the cause

of the mean increase in lymphocyte levels in all test groups (Yu et al., 2020). The mean increase in WBC counts in this study indicates that EB may possess immunomodulatory properties (Berinyuy et al., 2015). In our investigation, the mice treated with the plant extract showed a progressive increase in the RBC count from group 2 (400 mg/kg BW) to group 4 (1600 mg/kg BW) and a mean increase in the hemoglobin levels ($p < 0.05$). Therefore, these increases are not attributed to any sign of toxicity; the mean increase in hemoglobin in all 3 treatment groups suggests that the ethanol extract of EB likely stimulated erythropoiesis.

It was found that mice in the test and distilled water-treated groups had normal hepatocytes and portal tracts in their livers after a macroscopic and histological assessment. No lesions or abnormal pathology in the profile of the liver susceptible to the graded doses of plant extract were seen. These are good indicators for the liver functions, further suggesting that oral treatment with EB does not alter hepatocytes. Additionally, the absence of histopathological changes is a key indicator that the biochemical and hematological alterations were probably mild and likely reversible.

V. Conclusion

These findings demonstrate that EB has strong antiparasmodial and gametocytocidal properties *in vitro*, which suppress or inhibit parasite growth and could interrupt transmission. With an oral LD₅₀ value > 5000 mg/kg BW, the ethanol extract of EB can be considered practically nontoxic for a single dose or short-duration administration. Additionally, the findings from the 28-day subchronic toxicity experiment further confirm the safety of this plant extract, as the plant extract did not induce damage to hepatocytes, alter blood parameters, alter kidney profile parameters, or affect the body weight of animals during subchronic administration. In this context, this study supports the use of EB as a culturally sound alternative for managing malaria. However, further studies are needed to fully understand the safety profile and harness the therapeutic potential of EB for the development of novel antimalarials.

CRedit authorship contribution statement

Methodius S. Lahngong: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation,

Conceptualization. Thomas M. Schaefer: Writing – review & editing, Software, Methodology, Investigation, Formal analysis. Bertrand Y. Wanyu: Writing – review & editing, Investigation, Formal analysis. Carla Hamann: Methodology, Investigation, Data curation. Lais P. de Carvalho: Software, Methodology, Investigation, Formal analysis, Data curation. Mbua J. Vekima: Methodology, Investigation, Formal analysis. Olivia Jansen: Writing – review & editing, Validation, Supervision, Formal analysis, Conceptualization. Allison Ledoux: Visualization, Validation, Formal analysis, Data curation. Robert A. Shey: Writing – review & editing, Validation, Formal analysis. Smith B. Babiaka: Writing – review & editing, Investigation, Data curation. Jana. Held: Writing – review & editing, Supervision, Conceptualization. Moses N. Ngemenya: Writing – review & editing, Visualization, Validation, Formal analysis. Germain S. Taiwe: Validation, Supervision, Investigation. Stephen M. Ghogomu: Writing – review & editing, Validation, Supervision, Project administration, Conceptualization. Gis`ele E. Loe: Writing – review & editing, Visualization, Validation, Project administration. Michel Fr´ed´eric: Visualization, Validation, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter Eight

General Discussion, Conclusion and Perspectives

8.1 General discussion

Malaria still remains one of the most infectious and deadliest parasitic disease that ravage global population especially in Sub-Saharan African countries. Over the past two and half decades, they have been about 2.2 billion cases and approximately 12.7 million deaths due to malaria, globally ([World Health Organization, 2024](#)). Children under the age of five and pregnant women, including travelers to endemic regions constitute the most vulnerable groups and hence a disease of top priority for elimination. Although malaria-related mortality has remained close to 600,000 deaths annually over the past five years, there has been a consistent increase in the number of reported malaria cases each year. Sub-Saharan African countries are the hardest hit zones accounting for over 95% and 96% of the world's malaria burden and death rate respectively ([World Health Organization, 2024](#)). Malaria being a life-threatening disease poses long-term socio-economic problems for the affected population including (i) interruption of children's education by constant absenteeism from school, (ii) reduction of wages by constant absenteeism of workers from job sites due to severe episodes of malaria, (iii) poverty as a result of high cost of antimalaria drugs, high cost of transportation to health centers, high medical service fees and expenses on funeral. This makes the attainment of United Nation's Sustainable Development Goals (SDGs) 1, 2, 3, 4 and 13 unachievable. It is important to note that the attainment of all other SDG's hinges on the attainment of SDG3 which is focused on health and wellbeing. Therefore, any contribution to the control and elimination of malaria will be valuable not only to endemic populations, but also to the world, especially in this era of globalization and increased mobility of the people.

Several control strategies have been employed for malaria management including vector control measures that rely on long-lasting insecticide-treated bed nets (LLTNs) and indoors residual sprays (IRS) ([Orok et al., 2021](#); [Lindsay et al., 2021](#); [World Health Organization, 2022](#)). In addition, the World Health Organization recommends ACTs as treatment options, and recently the development of RTS vaccines for the management of the disease ([Laurens, 2020](#); [World Health Organization, 2021](#)). Despite all of these measures, malaria continues to exert a serious public health and socio-economic burden on the affected communities. This can be attributed to increasing antimalarial drug resistance to all chemotherapeutic measures

currently recommended including ACTs ([Dondorp et al., 2010](#); [World Health Organization, 2015](#)). The situation is further aggravated not only by Histidine-Rich Protein-2 gene (HRP2 gene) deletion, which makes diagnosis problematic, but also by the widespread mosquito resistance to insecticides as well as the rapid spread of invasive vector species (*Anopheles stephensi*) ([Eyong et al., 2022](#)). Additionally, ILTNs and IRS are indoor control measures and populations remain exposed to the disease during their professional life. Also, the lack of safer agents to target the gametocytes, the form of the parasite responsible for transmission poses a serious problem. To ensure the successful transmission of parasites via the insect vector, gametocytes represent an important mechanism for drug resistance, and increased gametocyte densities and circulation time after antimalarial therapy are predictive of resistance development. Disrupting malaria transmission therefore will require the development of new anti-gametocidal agents with safety profiles that allow for population-wide treatment.

The fight against the life-threatening disease is one of the goals of the World Health Organization's (WHO) Global Technical Strategy for malaria to reduce transmission and achieve a World free of malaria by 2030. To achieve a malaria free world, there is a need to revise the current control measures. Nevertheless, several strategies are currently being explored for development of drugs including the screening of synthetic compounds, drug repurposing, combinatorial chemistry, molecular modeling and natural product research. Among these methods, natural product has stood the test of time in the management of health-related problems either as pure molecules or as crude plant used in traditional medicine ([Newman and Cragg, 2020](#); [Barbara and Willem, 2024](#)). Over the years, there have been increased recourse to traditional medicine using *Artemisia afra* and *Artemisia annua* especially in Africa where malaria is endemic.

Artemisia afra and *Artemisia annua* are two species of the genus *Artemisia* known for their medicinal properties, particularly in treating malaria ([van Wyk, 2011](#); [Klayman, 1985](#)). Both plants contain bioactive compounds comprising of monoterpenes, diterpenes, triterpenes, sesquiterpenes, phenolic acids, flavonoids, coumarines among others ([Shinyuy et al., 2023](#)). Despite being devoid or with vestigial amount of artemisinin, *A. afra* has equally demonstrated strong antiplasmodial properties, including activity against *Plasmodium* gametocytes making it a good alternative for both

chemotherapy and for transmission blocking agents in the fight against malaria (Roesh et al., 2025; Ashraf et al., 2022; Snider and Weathers, 2020). On the other hand, *A. annua* contain significantly higher artemisinin content and demonstrate stronger antiplasmodial activity (Mamede et al., 2025, Shinyuy et al., 2025, Ashraf et al., 2022). In Cameroon like other African countries, both medicinal plants are cultivated in different regions, formulated as herbal tea, and sold for the management of malaria but without pharmacological quality control (Shinyuy et al., 2025). Morphologically, both medicinal plants look alike and this makes herbal tea authentication problematic. Additionally, there is ongoing controversy concerning the artemisinin content and its contribution to the antimalarial activity of both plants. (Mamede et al., 2025). Moreso, post-harvest handling practices have been shown to influence the composition of medicinal plants (Su et al., 2019; Zhang et al., 2024; Zhu et al., 2018). *Artemisia annua* and *Artemisia afra* are no exceptions in this context and thus, this could impact their therapeutic potential against malaria.

Our research therefore, focuses on leveraging the therapeutic potential of both plants as culturally sound alternative therapeutics and or chemoprophylaxis, to contribute to the global efforts in malaria elimination and eradication. Thus, we aimed to standardize, validate, and assess the safety of *A. afra* and or *A. annua* as potential antimalarial agent. Focus was put to explore their antiplasmodial potential, gametocidal properties, toxicity, development of galenic formulation and assessing the mode of action against *Plasmodium* parasites. Additionally, the metabolic contents of *A. afra* and *A. annua* sourced from different geographical locations and seasons from Cameroon including the impact of post-harvest handling practices on the chemical composition of both plants were assessed for optimal composition and for quality control of the herbal products. This research holds promise in contributing to global efforts to eliminate and eradicate *Plasmodium* reservoirs, offering a sustainable and culturally relevant approach to malaria control in Cameroon, across Africa, and worldwide.

The first key aspect addressed was the development of chromatography fingerprints of *A. annua* and *A. afra*, which could be used for the development of pharmaceutical monographs. This point was crucial given the close morphological similarities that exist between both plants, making

herbal tea authentication problematic. In this context, various analytical techniques, including TLC, HPLC-DAD, and GC-FID, were utilised to analyse the metabolic contents. The TLC, HPLC-DAD, and GC-FID analysis revealed differences in the chemical patterns of semi-volatile and polyphenol contents between the two species, which could potentially be used to correctly identify the medicinal products. For example, the presence of rutin, astragalin, and luteolin in *A. afra* against their absence in *A. annua*, as well as the existence of distinct TLC-bands in *A. afra* (**Supplementary Figure S2a and S2b**) could serve as the foundation for the production of specific monographs for this plant species. Furthermore, TLC fingerprints indicate the presence of artemisinin (ART), specifically in *A. annua* (**Supplementary Figure S2a**). The World Health Organisation encourages the development of herbal monographs for traditionally important medicinal plants primarily aimed to promote the harmonization in the use of herbal medicines with respect to authentication and quality control. The observed inter-species variation in the chemical pattern as seen in the TLC and HPLC fingerprints could serve as tools for the development of monograph for both plants. Thus these findings provides data that could be used to develop herbal monographs for both *A. annua* and *A. afra*. Nonetheless, a simple and cost effective TLC analysis as shown in the study could be used in resource limited settings to correctly identity and distinguish between these two medicinal plants.

After characterizing the chemical profiles of both plants, it was essential to determine whether the geographic origin and season of collection influence their secondary metabolite composition and, consequently, their antiplasmodial activity. A link between the observed metabolic fingerprint and antiplasmodial activity was thus established using both geographical and multispectral remote sensing data, and a number of analytical techniques mentioned above. The phytochemical content of *A. annua* and *A. afra* varied significantly across Cameroon's various regions and seasons. These variations included changes in artemisinin and other secondary metabolite abundance such as chlorogenic acid content and its derivatives. A higher artemisin content in *A. annua* was seen in the rainy season than in the dry season (**Figure 29**). Regardless of season of collection, artemisinin contents was highest in samples obtained from the Adamawa region (**Figure 29**). Although the various collection sites share a comparable climate, the

obtained NDVI values reveal considerable disparities in the environmental circumstances among them (**Figure 32**). The elevated artemisinin levels found in the rainy season samples were associated with enhanced antiplasmodial efficacy compared to those collected during the dry season (**Figure 31**). Furthermore, analysis utilising geographical and multispectral remote sensing data indicated that the rainy season exhibited healthier flora, which enhanced the artemisinin content responsible for the increased activity of *A. annua* compared to the dry season (**Figure 32**). The intra-species variation in the polyphenol contents for *A. afra* samples agrees with the findings of [Sotenjwa et al., 2020](#) and [Olivier et al., 2022](#) who demonstrated both geographical and seasonal effects on the composition of polyphenols in *A. afra*. In addition to variations in the polyphenol profiles, our study demonstrates the impact of both season and geographical origin on the semi-volatile composition, chlorogenic acid contents, total polyphenol contents and artemisinin content. Moreso, the intra and inter-species variation in the metabolite composition affected their therapeutic effects against *Plasmodium* parasites. Thus, there is the need to take into account both season and place of collection when choosing an *Artemisia* spp as traditional remedy in the management of malaria.

In order to contribute to the broader exploration of herbal medicine as a sustainable and culturally relevant approach to fight malaria, further analysis on the impact of post-harvest handling practices was performed. The goal was to contribute to the understanding of how post-harvest handling practices impacts the secondary metabolite composition and medicinal properties of *A. afra* and *A. annua*. Optimizing these practices can enhance the therapeutic potential of Artemisia-based products, particularly in combating malaria. Additionally, it underscores the importance of sustainable agriculture and responsible handling of medicinal plants to ensure consistent efficacy and quality of herbal remedies. Plant samples were thus, harvested from the same farm land and subjected to different post-harvest handling practices (**Table 4**) and infusions were prepared following the traditional preparation method. These infusions were lyophilised and metabolite content analyzed by HPTLC, HPLC and GC. Meanwhile antiplasmodial activity was assessed for all samples and correlated to the observed metabolite pattern. In our findings, the chemical composition of *A. afra* and *A. annua* is largely influenced by the way they are

handled after harvesting. We showed that non-fermentated and sun-dried samples retained the chemical profile of both plants (**Figure 36a-c**) as compared to fermented, oven-dried and shaded-dried samples. Interestingly, artemisinin content was higher in non-fermented and sun-dried samples compared to oven and shaded-dried method for *A. annua* (**Figure 43** and **44**). This indicates that sun-drying enhances the bioconversion process, leading to higher artemisinin concentrations as observed in this study. Moreso, dihydroartemisinic acid, the primary biosynthetic precursor of artemisinin, undergoes photooxidation in the presence of sunlight, converting into artemisinin and resulting in elevated levels in sun-dried *Artemisia annua* (Sy and Brown, 2002). Conversely, shade-drying is a prolonged method, and in regions with high tropical humidity, this extended drying period can promote fungal contamination of leaves and potentially lower artemisinin content (Laughlin, 2002). Concerning polyphenol profiles, various processing techniques, such as fermentation, enzymatic treatment, and both thermal and non-thermal methods, have been shown to significantly affect the yield of phenolic compounds (Chen et al., 2013). In this study, both fermentation and shade-drying were found to adversely affect the chemical profile of the plants, leading to decreased levels of chlorogenic acid and its derivatives (**Figure 37-41**).

Artemisia afra leaves have shown a positive effect on different *Plasmodium* parasite stages including killing the *Plasmodium* gametes. Due to these observations, It was interesting to broaden the knowledge of Artemisia-based preparations by investigating their total pharmacological effectiveness in relation to their chemical composition. This could strengthen the transmission blocking strategy and prevent further parasite-induced tissue damage resulting from hyperinflammation. To achieve this objective, a bioassay guided fractionation approach was used to isolate and characterised molecules from *A. afra* with dual stage antiplasmodial activities. Using a combination of open column chromatography and preparative thin layer chromatography, four compounds were isolated from *A. afra* and characterised by analysing spectra data from NMR, LC-MS, IR and UPLC-QDA. Out of the four compounds, two showed strong antiplasmodial property against the sexual (gametocytes) and asexual forms of a chloroquine sensitive NF-54 and 3D7 *P. falciparum* strains respectively (**Table 7**). Compound (**Y1**), was hypothesized to be a derivative sitosterol (**Figure 48**)

while (**82C**) was hypothesized to be derivative of ellagic acid (**Figure 49**); both demonstrated promising *in vitro* activity with IC₅₀ values < 1 µg/mL. Further drug development of these compounds could be used to alleviate the symptoms of the disease and also to decrease the interaction between humans and insect carriers, therefore lowering the transmission of malaria. This will enable to achieve a world free of malaria and hence increase the mobility of people in endemic regions and therefore increased productivity leading to economic growth and therefore, the attainment of United Nation's SDGs.

Although both plants have demonstrated significant antiplasmodial activity and effectiveness as a traditional remedy in the management of malaria, there could be a lack of compliance to the herbal product due to the bitter taste. The poor compliance to the herbal remedy is mostly common in asymptomatic patients who constitute a significant share of infected persons in endemic regions. These individuals serve as silent reservoirs that perpetuates the disease transmission. It was therefore necessary to formulate a herbal tea that lack the characteristic bitter taste to improve on the palatability and increase in compliance by asymptomatic patients. To achieve this objective, an equal-proportion formulation (ARTEFOL) consisting of *Artemisia afra* (AA) and *Hibiscus sabdariffa* (HS) was developed, with HS selected for its acidic-sweet taste and antioxidant properties. The characteristic red colour of HS is attributed to anthocyanins, while its acidic-sweet taste derives from organic acids such as citric, malic, and tartaric acids (Izquierdo-Vega et al., 2020), which likely contributed to masking AA's bitterness. The aim was to improve palatability while preserving or enhancing therapeutic efficacy of AA. Thus, sensory evaluation was conducted using hedonic scoring for the combination, AA, HS and Lipton (a tea commonly consumed in Cameroon). Four tea infusions, A11, A17, A19 (ARTEFOL), and a positive control (Lipton) were prepared using the traditional preparation method and administered to 100 participants. Ratings were based on bitterness, colour, taste, consistency, flavour, and overall acceptability on a 9-point hedonic scale. Results show that an equal combination of AA and HS significantly masked AA's bitterness, achieving a hedonic score of 7.2 ± 0.14 , compared to AA alone (6.5 ± 0.25) (**Table 8**). This blend was ranked second in acceptability after Lipton. High-Performance Thin-Layer Chromatography (HPTLC) revealed similarities as well as

differences in the chemical profiles for AA, HS, and ARTEFOL (**Figure 53**). Furthermore, bioactive compounds such as scopoletin, scopolin, chlorogenic acid and its derivatives, rutin, hyperoside, and luteolin detected in ARTEFOL through HPTLC were further confirmed by UPLC analysis. To validate the therapeutic potential of the combination, *in vitro* antiplasmodial activity was assessed against a chloroquine-sensitive Pf3D7 strain, followed by *in vivo* evaluation in *Plasmodium berghei*-infected albino mice. Ethanol extracts and their combinations exhibited strong antiplasmodial activity, whereas tea infusions showed weaker effects (**Table 9**). Synergistic interactions among these metabolites could explain the observed efficacy. Further *in vivo* studies confirmed that HS does not diminish AA's antiplasmodial effect. Both AA and the combined extract demonstrated significant ($P > 0.001$) dose-dependent curative effects compared to the negative control, with maximum suppression at the highest dose (**Figure 58**). Like the standard drug, treated groups showed reduced parasitaemia, suggesting a comparable onset of the defensive action of the extract and standard drug. These findings highlight the potential of ARTEFOL as an effective antimalarial formulation (**Figure 58**). The safety profile of AA, HS and ARTEFOL was done by evaluating cytotoxicity, acute and sub-chronic oral toxicity. Results show no signs of toxicity or mortality at a dose of 5000 mg/kg for AA and combination (ARTEFOL), indicating an $LD_{50} > 5000$ mg/kg (OECD, 2008) (**Table 10**). Treated mice exhibited normal activity and progressive weight gain, no altered kidney and biochemical parameters (**Table 11-13**), supporting the safety of the formulation (Raji et al., 2021; Mokenen et al., 2020; Njinga et al., 2020).

As mentioned above, *Artemisia spp.* have been used for millennia in traditional medicine to treat a variety of ailments, and remain widely used throughout Africa for healthcare purposes, notably to prevent and/or treat malaria. The Asteraceae plant family has a long history in this context and is well known due to the discovery of artemisinin from *Artemisia annua* (Ekiert et al., 2021; Feng et al., 2020; Maciuk et al., 2023; Moyo et al., 2019; Shinyuy et al., 2023). In Africa, *A. annua* and *A. afra* are traditionally employed for the management of malaria, among other diseases (du Toit & van der Kooy, 2019; Moyo et al., 2019; Shinyuy et al., 2023). Multiple *in vitro* growth inhibition assays with *A. afra* extracts have demonstrated significant activity against *P. falciparum* intraerythrocytic growth (Ashraf et al., 2022; Shinyuy

et al., 2023), though with lower potency than compared to *A. annua* extracts (Shinyuy et al., 2023). Both *A. annua* and *A. afra* infusions were shown to significantly diminish sporozoite *in vitro* infectivity of hepatocytes and impair hypnozoite re-activation, independently of artemisinin (Ashraf et al., 2022). However, the modes of action of these plant extracts remain unclear, with contradictory reports regarding the presence and role of artemisinin in both plants. A better understanding of these plant extracts and the mechanisms by which they interrupt the *Plasmodium* life cycle may lead to new, effective antimalarial therapeutic alternatives. In this context, we investigated the antiplasmodial effects of extracts from *A. afra* and *A. annua* using several approaches, including quantifying artemisinin content, characterizing the plant's phenolic profile, and using metabolomics to measure the effect of these extracts on the metabolism of *P. falciparum* cultured *in vitro*. This objective whose findings were published in *Phytomedicine* (**Appendix III**) was made possible thanks to Dr. Lucia Mamede, through her collaboration with Professor Manuel Llinás (Huck Center for Malaria Research, CmaR, Pennsylvania State University, USA), part of this work achieved success. In this study we showed that *A. afra* and *A. annua* demonstrated distinct phenolic compositions as well as different levels of artemisinin. Phenolic compounds are widely present in this genus of plants and are often considered as active antiplasmodial agents (Maciuk et al., 2023; Shinyuy et al., 2023). We also found that the extracts from *A. annua* and *A. afra* elicit two separate parasitic metabolic responses which point to divergent modes of action. Our findings indicate that the antiplasmodial activity of *Artemisia annua* is primarily attributable to its artemisinin content, consistent with the well-documented effects of this compound on haemoglobin degradation and redox metabolism within the parasite. Artemisinin's mechanism of action involves the generation of reactive oxygen species and disruption of heme detoxification, ultimately leading to parasite death. In contrast, *Artemisia afra* appears to exert its effects through alternative pathways, potentially targeting lipid precursors and signalling mechanisms. This suggests that constituents other than artemisinin, such as flavonoids, coumarins, and phenolic acids, may play a significant role in its antimalarial activity. These distinct mechanisms of action underscore the value of investigating whether combining the two extracts might produce synergistic effects, rather than merely additive ones. Our findings shows that the methanolic

extract of *Artemisia afra* enhances and amplifies the antiplasmodial activity of the acetone extract of *Artemisia annua* across a wide concentration range. Consistent synergy was detected across multiple analytical models, with the Bliss independence model revealing particularly strong and biologically relevant interaction patterns (**Figure 45 and 46**). These results align with previous evidence indicating that *A. afra* and *A. annua* act through distinct yet complementary mechanisms ([Mamede et al., 2025](#)), thereby meeting the conceptual assumptions that underpin the Bliss framework.

After detailing the phytochemical diversity, the impact of post-harvest handling methods, characterisation of molecules with antiplasmodial properties and specific for *A. afra*, developing a formulation and understanding how extracts of *A. annua* and *A. afra* interact with plasmodium parasites, we shifted attention to *Erigeron bonariensis*. *Erigeron bonariensis*, just like *A. afra* and *A. annua*, is a member of the Asteraceae family with ethnopharmacological relevance. *E. bonariensis* has been used in resource limited regions including the Adamawa region of Cameroon and many other African countries as a traditional remedy for the management of malaria ([Lahngong et al., 2025](#)). However, this is without scientific evidence on the activity and safety of the herbal product. In this study, the objective was to evaluate the safety profile of *E. bonariensis* by examining both acute and subchronic exposure to its ethanol extract in an appropriate animal model, while also assessing its antiplasmodial and gametocytocidal activities. Crude extracts were prepared as previously described and subjected to in vitro assays to determine their efficacy against both sexual and asexual stages of *Plasmodium falciparum*, specifically the chloroquine-sensitive NF-54 and 3D7 strains. The findings indicate that n-hexane and methanol extracts exhibit strong activity against the asexual Pf3D7 strain, while the ethyl acetate extract shows only weak activity. Notably, all extracts display significant gametocytocidal effects on the *P. falciparum* NF-54 strain (**Table 14**). This activity is likely linked to the diverse phytochemical profile of EB, particularly its flavonoids and phenolic acids ([Barhoumi et al., 2013](#)). Phenolic compounds are known to induce oxidative stress, disrupting the parasite's life cycle and reducing its virulence ([Mamede et al., 2020](#)). Furthermore, quercetin and kaempferol are naturally present in EB ([Barhoumi et al., 2013](#)) and have been reported to inhibit *P. falciparum* growth ([Shinyuy et al., 2023](#)). The antimalarial effect may also stem from

conyzine, an alkaloid isolated from EB (Opiyo, 2023), which could impair parasite metabolism and protein synthesis, ultimately causing death. In addition to its antiplasmodial property, EB has shown to be relatively safe due the absent overt signs of toxicity, meanwhile behavioral and physical parameters such as grooming, locomotion, and physical appearance remained normal with an LD₅₀ > 5000 mg/kg BW (Table 15). Additionally, they were no remarkable changes in biochemical parameters, hematological and kidney profile of EB after a subchronic exposure in mice (Table 16 and 17).

8.2 Conclusion

The distinct phytochemical profiles of *A. annua* and *A. afra* underscore the importance of exploring both species beyond their traditional use. The results show both intra-species and inter-species variations in the phytochemical constituents which fluctuate considerably with season and geographical region and have implications for the anti-plasmodial potential. Therefore, selection of a suitable *Artemisia* sample for use as a potential antimalarial treatment must take into consideration its geographical origin, the period of collection, season, and remote sensing data.

The proper drying of medicinal plants is crucial for preservation, but protocols must be carefully optimized to avoid loss of phytochemical content. The findings of this study demonstrate that post-harvest handling practices significantly influence the secondary metabolite profiles of *Artemisia annua* and *Artemisia afra*. However, the results support sun-drying as an effective method for preserving and enhancing the therapeutic potential of *A. afra* and *A. annua* in the fight against malaria. The systematic characterization of bioactive compounds from *A. afra* may lead to the discovery of additional antimalarial agents, contributing to the broader goal of malaria eradication. This study successfully identified two compounds exhibiting significant activity against both asexual and sexual stages of *Plasmodium falciparum*. These results underscore their promise as candidates for chemotherapy and transmission-blocking strategies, subject to further optimization and drug development. Moreover, this study is the first to report the presence 3-methoxy-5-heneicosylphenol and 4-ethenyl-2,5-dimethyl-5-hexene-2,3-diol within the genus *Artemisia*. These findings underscore the potential of incorporating *Hibiscus sabdariffa* into

formulations with *Artemisia afra* as an innovative approach to improve treatment compliance without compromising therapeutic benefits or safety. The dose-dependent suppression of parasitemia and the curative effects observed underscore the efficacy of the combination. The positive *in vitro* and *in vivo* outcomes, coupled with the absence of significant toxicity, pave the way for further research and development of these herbal combinations as alternative and complementary approaches to combat malaria.

Additionally, our findings suggest that the activity of *A. annua* is attributed to its artemisinin content, which aligns with the effects of this compound on hemoglobin and redox metabolisms. meanwhile *A. afra*, may affect lipid precursors and signaling, indicating extract constituents other than artemisinin are responsible for its activity.

Moreso, these findings equally demonstrate that EB possess considerable *in vitro* antiplasmodial and gametocytocidal properties, providing both parasite inhibition and transmission interruption. This activity coupled with the safety profile of EB supports its traditional use as a culturally sound alternative for managing malaria.

8. 3 Perspectives

- To fully characterise the chemical composition of infusions of both plants to allow a complete standardization and to understand the mechanism of action of these infusions against the sexual and asexual *Plasmodium* parasites.
- Further investigations into alternative post-harvest handling techniques and correlation to the antiplasmodial activity are warranted to generate more comprehensive data for optimizing the quality and efficacy of *A. annua* and *A. afra*.
- Further investigations, potentially leading to the development of standardized herbal formulations for antimalarial interventions.
- Additional experiments such as *in vivo* tests, enzymatic studies, and lipidomics will prove useful to confirm the antiplasmodial mode of action of *A. annua* and *A. afra*.

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Chapter Nine

Appendices

9.1 List of publications

9.1.1 Publications associated with this work

Shinyuy, L.M.; Loe, G.E.; Jansen, O.; Mamede, L.; Ledoux, A.; Noukimi, S.F.; Abenwie, S.N.; Ghogomu, S.M.; Souopgui, J.; Robert, A.; et al. Secondary Metabolites Isolated from *Artemisia afra* and *Artemisia annua* and Their Anti-Malarial, Anti-Inflammatory and Immunomodulating Properties—Pharmacokinetics and Pharmacodynamics: A Review. *Metabolites* 2023, 13, 613. <https://doi.org/10.3390/metabo13050613>

Shinyuy, L.M.; Loe, G.E.; Jansen, O.; Ledoux, A.; Palmaerts, B.; Mamede, L.; Boussif, N.; Bonnet, O.; Enone, B.S.; Noukimi, S.F.; et al. Exploring the Phytochemical Diversity and Anti-Plasmodial Potential of *Artemisia annua* and *Artemisia afra* from Different Geographical Locations in Cameroon. *Molecules* 2025, 30, 596. <https://doi.org/10.3390/molecules30030596>

Shinuy, L.M., Thomas M. Schaefer, Wanyu Y. Bertrand, Carla Hamann, Lais P. de Carvalho, Mbua J. Vekima, Olivia Jansen, Allison Ledoux, Robert A. Shey, Smith B. Babiaka, Jana Held^{2,8}, Moses N. Ngemenya⁶, Germain S. Taiwe⁵, Stephen M. Ghogomu⁴, Gisèle E. Loe³, Michel Frederich¹. Toxicity profile, antiplasmodial and gametocytocidal properties of *Erigeron bonariensis* L. (anc. *Conyza* *bonariensis* (L.) Cronquist). <https://doi.org/10.1016/j.jep.2025.120609>

Lucia Mamede, Gabriel W. Rangel, **Lahngong Methodius Shinyuy**, Naïma Boussif, Marie-France Herent, Bernadette Govaerts, Olivia Jansen, Allison Ledoux, Pascal De Tullio, Joëlle Quetin-Leclercq, Manuel Llinás, Michel Frédérick. Metabolite profiling of *Artemisia afra* and *Artemisia annua* extracts reveals divergent effects on *Plasmodium falciparum*. *Phytomedicine*, Volume 136, 2025, 156361, ISSN 0944-7113, <https://doi.org/10.1016/j.phymed.2025.156361>.

9.1.2 Articles associated with this work in preparation for publication

Methodius S. Lahngong, Allison Ledoux, Olivia Jansen, Delphine Etienne, Sandra F. Noukimi, Suh N. Abenwie, Mary T. Efeti, Dorcas M. Lukasa, Robert A. Shey, Annie Robert, Jacob Souopgui, Stephen M. Ghogomu, Gisèle E. Loe, Michel Frederich. *Post-harvest Handling Influences the Secondary Metabolite Contents and Anti-plasmodial Properties of Artemisia annua and Artemisia afra* (Submitted to Journal of Ethnopharmacology)

Methodius S. Lahngong, Olivia Jansen, Lais P. de Carvalho, Allison Ledoux, Olivier Bonnet, Thomas M. Schaefer, Mary T. Efeti, Robert A. Shey, Annie Robert, Jacob Souopgui, Stephen M. Ghogomu, Gisèle E. Loe, , Jana Held, Michel Frederich. *Anti-plasmodial and Gamecytotoxic Compounds From Artemisia afra. Characterisation and Molecular Docking Studies.*

Methodius S. Lahngong, Olivia Jansen, Allison Ledoux, Nying S. Limnyuy, Ekwen L. Ndangoh, Nervis N. Boning, Sandra F. Noukimi, Suh N. Abenwie, Wanyu Y. Bertrand, Annie Robert, Denis Zofou, Germain S. Taiwe, Tiencheu Bernard, Moses N. Ngemeya, Jacob Souopgui, Stephen M. Ghogomu, Gisèle E. Loe, Michel Frederich. *Sensory Analysis, Phytochemical Profile and Antimalarial Properties of a Formulation of Artemisia afra and Hibiscus sabdariffa.*

Methodius S. Lahngong, Allison Ledoux, Michel Frederich. *Synergistic interactions between Artemisia afra and Artemisia annua: Rationale for combining Artemisia afra and Artemisia annua*

9.1.3 Other publications

Babiaka, S.B.; **Lahngong, M.S.**; Simoben, C.; Ongarora D.S.B.; Chi, G.F.; Tshitenge, D.T.; Karpoomath, R.; Rylands, M.; Mbah, J.A.; Abuga, K.O.; Loveridge, E.J and Frédérich, M. Iboga-Indole Alkaloids as Potential Plasmodial Inhibitors: Identification, Biosynthesis, and Molecular Docking Studies. Chem Biodivers. 2025 Dec;22(12):e01035. <https://doi.org/10.1002/cbdv.202501035>

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9.2 Scientific conferences and symposium

9.2.1 List of oral communication

MS Lahngong, A. Ledoux, O. Jansen, and M. Frederich. "Effect of Seasonal and Geographical Location on the Secondary Metabolite Contents of *Artemisia annua* and *Artemisia afra*. Anti-plasmodial Properties." Paper presented at AFERP Young Researchers Seminar, 2025. <https://hdl.handle.net/2268/330403>

L. Mamede, Gabriel W. Rangel, **M. S. Lahngong**, Marie-France Herent, Naima Boussif, O. Jansen, A. Ledoux et al. "METABOLITE PROFILING OF *Artemisia afra* AND *Artemisia annua* extracts reveals divergent effects on *Plasmodium falciparum*." Paper presented at Belgian Society of Pharmaceutical Sciences (BSPS) forum 2025. <https://hdl.handle.net/2268/330395>

MS Lahngong, A. Ledoux, and M. Frederich. "Exploring the Phytochemical Diversity and Anti-plasmodial Potential of *Artemisia annua* and *Artemisia afra* from Different Geographical Locations in Cameroon." Paper presented at CIRM-DAY 2024, Liege, Belgium, 31 January 2024. <https://hdl.handle.net/2268/328498>

MS Lahngong, Gisele Etame Loe, A. Ledoux, O. Jansen, Souopgui Jacob, Stephen M Ghogomu, and M. Frederich. "Antimalaria Properties and Toxicity of Extracts of *Artemisia annua*, *Artemisia afra*, *Hibiscus sabdariffa* and Extract Combinations. Development of Galenic Formulation." Paper presented at Malaria symposium: Malaria control, prevention and treatment; from the laboratory to the population, April 19, 2023. <https://hdl.handle.net/2268/328465>

9.2.2 List of posters

M. S. Lahngong, A. Ledoux, O. Jansen, B. Palmaerts, kristiaan demeyer, Ghogomu M. Stephen, Souopgui Jacob, Gisele Etame Loe, E. Hallot, and M. Frederich. "Effect of season and geographical location on the secondary metabolite contents and antiplasmodial properties of *Artemisia afra* and

Artemisia annua from different geographical locations in Cameroon." Poster session presented at International congress on Natural product research, 17 July 2024. <https://hdl.handle.net/2268/328534>

M. S. Lahngong, Nying S. Limnyuy, Ekwen Luis, Gisèle E. Loe, Vickima Joel, Yuwong B. Wanyu, Robert Shey, Stephen M. Ghogomu, and M. Frederich. "Effect of *Hibiscus sabdariffa* on the in vivo anti-plasmodial properties and toxicity of *Artemisia afra* and *Artemisia annua*." Paper presented at Faculty of Science EXPO 2024, 16 May 2024. <https://hdl.handle.net/2268/328486>

M. S. Lahngong, A. Ledoux, B. Palmaerts, Demeyer Kristiaan, J. Souopgui, Ghogomu Mbigah Stephen, Gisele Etame Loe, E. Hallot, and M. Frederich. "Exploring the Phytochemical Diversity and Antiplasmodial Potential of *Artemisia annua* and *A. afra* from Different Geographical Locations in Cameroon." Poster session presented at Conference: 71st International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, Dublin, Greece, 04 November 2023. <https://hdl.handle.net/2268/328462>

Appendice I: Senesory card of hedonic testing of food product

Evaluation card for hedonic rating test for food product

Project Title: Sensory analysis, phytochemical profiling and antimalarial properties of galenic formulation of *Artemisia afra* and *Hibiscus sabdariffa* (folere) as treatment options against malaria.

Name..... **Gender**

Age.....

Product.

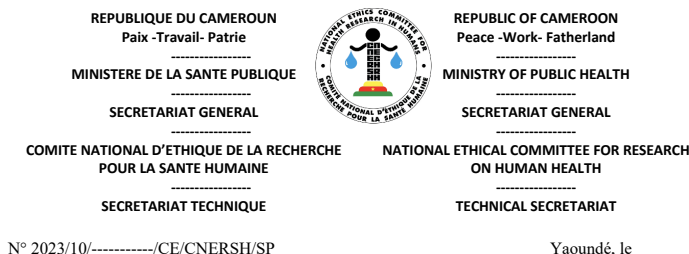
Saample code	Colour	Test	Flavour	Consstency/ texture	Bitterness	Overall acceptability	Remark, if any
F1							
F2							
F3							
F4							
F5							
F6							
F7							

Signarure.....

<u>Judgement</u>	<u>Score</u>
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike Moderately	3
Dislike very much	2
Dislike extreamely	1

<u>Judgement Score for bitterness only</u>		
Extremely	bitter	9
Too much	bitter	8
Moderately bitter		7
Slightly	bitter	6
Neither bitter neither normal		5
slightly normal and bitter		4
Moderately normal		3
Slightly normal		2
Normal		1

Appendice II: Ethical clearances



CLAIRANCE ETHIQUE

Le Comité National d’Ethique de la Recherche pour la Santé Humaine (CNERSH), en sa session extraordinaire du 27 octobre 2023, a examiné le projet de recherche intitulé : **«Contributions in emptying plasmodium reservoirs to accelerate malaria elimination in high transmission settings using Artemisia afra infusion : case study in Cameroon»** soumis par le Professeur Stephen M. GHOGOMU, Investigateur Principal, Université de Buéa.

Le projet est d’un grand intérêt scientifique et social. L’objectif général de cette étude est de faire face à l’émergence de la résistance à l’ACT chez *P. falciparum* dans les régions de l’Ouest, du Sud-Ouest, du Littoral et du Centre - Cameroun. La procédure de l’étude est bien documentée et claire. Cette étude se déroulera dans quatre régions du Cameroun à savoir l’Ouest, le Sud-Ouest, le Littoral et le Centre. Les risques liés à l’étude sont précisés ainsi que les mesures pour les éviter et les minimiser. La notice d’information et le formulaire de consentement éclairé, en français et en anglais, sont bien élaborés et simples à comprendre. Les mesures prises pour garantir la confidentialité des données collectées sont présentes dans le document. Les CVs des Investigateurs les décrivent comme des personnes compétentes, capables de mener à bien cette étude. Pour toutes ces raisons, le Comité National d’Ethique approuve pour une durée d’un an, la mise en œuvre de la présente version du protocole.

Les Investigateurs sont responsables du respect scrupuleux du protocole approuvé et ne devraient y apporter aucun amendement aussi mineur soit-il, sans avis favorable du CNERSH. Les investigateurs sont appelés à collaborer pour toute descente du CNERSH pour le suivi de la mise en œuvre du protocole approuvé. Le rapport final du projet devra être soumis au CNERSH et aux autorités sanitaires du Cameroun.

La présente clairance peut être retirée en cas de non-respect de la réglementation en vigueur et des recommandations susmentionnées.

En foi de quoi, la présente clairance éthique est délivrée pour servir et valoir ce que de droit.

Ampliations

Le Président

MINSANTE


Pr. MBACHAM Wilfred

N.B : cette clairance éthique ne vous dispense pas de l’autorisation administrative de recherche (AAR), exigée pour mener cette étude sur le territoire camerounais. Cette dernière vous sera délivrée par le Ministère de la Santé Publique.

- N° d’enregistrement : IORG0007861-IRB00009439-FWA00016054 - setcominae@gmail.com

- Arrêté N° 0977/A/MSP/SESP/SG/DROS du 18 avril 2012 portant création, organisation et fonctionnement des Comités d’Ethique de la Recherche pour la Santé Humaine au sein des structures relevant du ministère en charge de la santé publique.

Appendix III: Publication

Phytomedicine 136 (2025) 156361



Contents lists available at ScienceDirect

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journal homepage: www.elsevier.com/locate/phyomed



Original Article

Metabolite profiling of *Artemisia afra* and *Artemisia annua* extracts reveals divergent effects on *Plasmodium falciparum*

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Keywords:

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Malaria
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Plasmodium spp.
Artemisia annua
Artemisia afra

ABSTRACT

Background: *Artemisia* spp. have been used for millennia in traditional medicine to treat a variety of ailments, including malaria. Extracts of *Artemisia afra* and *A. annua* remain widely used throughout Africa for healthcare purposes, notably to prevent and/or treat malaria. However, the modes of action of these plant extracts remain unclear, with contradictory reports regarding the presence and role of artemisinin in both plants.

Purpose: The aim of this study was to identify differences in the antimalarial mode of action of *A. afra* and *A. annua* by measuring their phenolic profiles and comparing their effect on parasite metabolism *in vitro*.

Methods: In this work, we analyzed the phenolic profile of *A. afra* and *A. annua* extracts through high-performance liquid chromatography (HPLC), detected and quantified artemisinin through HPLC and mass spectrometry (MS), and performed comparative HPLC-MS metabolomic analysis on *in vitro*-cultured *Plasmodium falciparum* trophozoites to elucidate the potential modes of action of these plant extracts.

Results: *A. afra* contained only trace amounts of artemisinin and elicited a different parasite metabolic response compared to *A. annua*, which contained significantly more artemisinin and correlated closely with the parasite response profile elicited by purified artemisinin. *A. annua* impacted parasite glutathione metabolism in agreement with the established redox activity of artemisinin, while *A. afra* had an effect on lipid precursors.

Conclusions: This study reveals that *A. afra* and *A. annua* have divergent effects on *Plasmodium falciparum* metabolism and provides support for ongoing efforts exploring the use of *A. afra* for the treatment of malaria.

Introduction

Malaria is the deadliest parasitic disease in the world, killing over half a million people annually, mostly children under five years of age and pregnant women, making this infectious disease a top priority for eradication (World Health Organization, 2023). Despite concerted global efforts to control, eliminate, and eradicate malaria, widespread antimalarial resistance of *Plasmodium falciparum*, the most deadly and prevalent *Plasmodium* species responsible for human malaria, has significantly hindered these efforts. To slow the emergence of resistance,

Abbreviations: ASCA+, ANOVA-simultaneous component analysis+; AFE, *Artemisia afra* -; ANE, *Artemisia annua*; ACTs, artemisinin-based combination therapies; DMSO, dimethylsulfoxide; DAD, diode-array detection; DI-HRMS, direct-infusion high-resolution-MS; HPLC, High-performance liquid chromatography; HMDB, human metabolome database; ¹H NMR, proton nuclear magnetic resonance; LDH, lactate dehydrogenase; MACS, magnet activated cell sorting; MS, mass spectrometry; RSD, relative standard deviation.

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