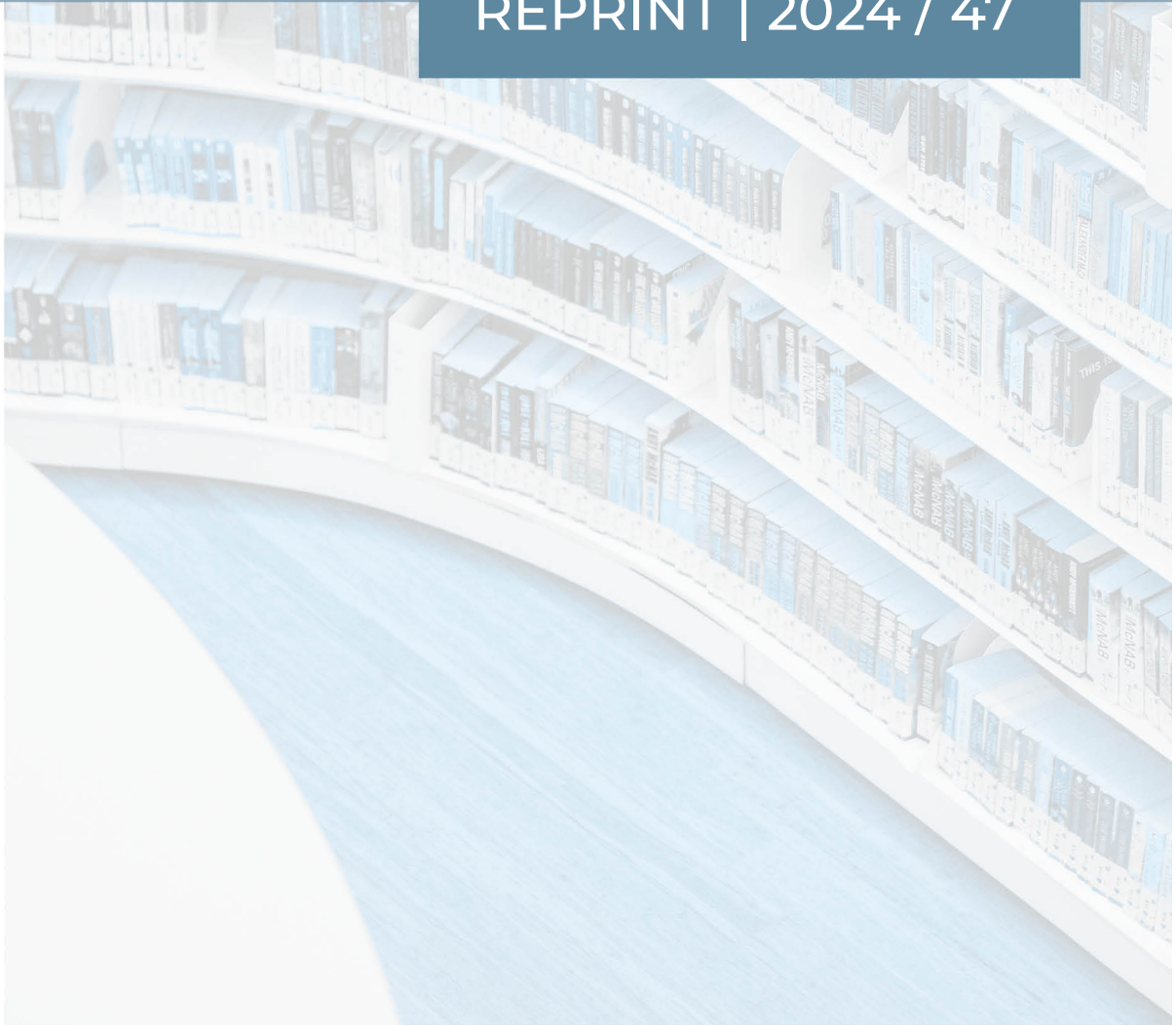


METABOLITE PROFILING OF ARTEMISIA AFRA AND ARTEMISIA ANNUA EXTRACTS REVEALS DIVERGENT EFFECTS ON PLASMODIUM FALCIPARUM

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

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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.

Journal Pre-proof

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, the World Health Organization currently recommends the use of Artemisinin-based Combination Therapies (ACTs) for the treatment of *P. falciparum* malaria (World Health Organization, 2015). ACTs are comprised of at least two mechanistically dissimilar drugs, including one fast-acting artemisinin derivative, mitigating the development of resistance and recrudescence by requiring the parasite to navigate two evolutionary hurdles (Haldar et al., 2018). Despite the reliance on ACTs, the emergence of partial artemisinin resistance along with partner drug resistance is a major concern that severely threatens malaria elimination and eradication efforts. Reports of partial artemisinin resistance are widespread throughout Southeast Asia (Fairhurst and Dondorp, 2016) and are increasingly common in Africa, including recent reports from Chad, Eritrea, Kenya, Mali, Uganda, and Rwanda, demonstrating that resistance to the first-line anti-malarial treatment has reached Africa, where the burden and mortality are the highest (Issa et al., 2023). Therefore, there is a need for improvements to malaria disease management that will continue to reduce mortality, curb transmission, and delay the further emergence and spread of resistance.

Currently, 80% of the human population worldwide relies on ethnomedical alternatives in primary healthcare, especially in malaria-endemic countries, where the availability, low cost, and traditional usage of certain plants remains important (Mamede et al., 2020; Shinyuy et al., 2023). The *Asteraceae* plant family has a long history in this context and is well known due to the discovery of artemisinin from *Artemisia annua*, which is native to temperate Asia (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 and van der Kooy, 2019; Moyo et al., 2019; Shinyuy et al., 2023). The mechanism of action of *A. afra*, however, remains unclear since artemisinin is thought to be either absent or present in very low amounts in this species, meaning the compounds responsible for the antiparasmodial activity remain unknown (Maciuk et al., 2023; Shinyuy et al., 2023).

Phenolic compounds are widely present in this genus of plants and are often considered as active antiparasmodial agents (Maciuk et al., 2023; 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 comparable *A. annua* extracts (Shinyuy et al., 2023). Furthermore, *P. falciparum* transmission blocking activity by *A. afra* was demonstrated *in vitro* along with the isolation of two guaianolide sesquiterpene lactones potentially involved in this effect (Moyo et al., 2019). Both *A. annua* and *A. afra* infusions were shown to significantly diminish sporozoite *in vitro* infectivity of hepatocytes and impair hypnozoite re-activation, independent of artemisinin (Ashraf et al., 2022). This work highlights that currently unknown compounds (or combinations of compounds) from these traditionally used plants target multiple points in the malaria parasite life cycle. 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 study, we investigated the antiparasmodial 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*. *A. afra* and *A. annua* have demonstrated distinct phenolic compositions as well as different levels of artemisinin. 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, and that these are differentially correlated with a purified artemisinin-based metabolic effect.

Materials and Methods

Description and collection of plant material of *A. annua* and *A. afra*

Leaves and twigs of both plants were collected at the flowering stage from the town of Bafia, in the Center region of Cameroon (Coordinates; 4.7546, 11.2240), during the dry season. Voucher specimens (SCA 7145 and SCA 6374 for *A. annua* and *A. afra*, respectively) were deposited at the Limbe botanical gardens, Cameroon. The samples were authenticated by a botanist and voucher number assigned.

Preparation of crude extracts.

Acetone crude extracts were prepared by maceration using the protocol described by Mbah *et al.*, 2011 (Mbah *et al.*, 2012). Briefly, each plant material was oven dried at 30 °C and ground to fine powder, and each 0.8 g of powder was macerated at room temperature for 72 h using 1 ml acetone. The macerated mixture was filtered through a funnel with Whatman N001 filter paper, and the filtrate was concentrated by rotatory evaporation using the R-205 brand Rotavapor (BÜCHI, Flawil, Switzerland). Each crude extract was weighed and stored at 4 °C for subsequent use.

Antiplasmodial assays

Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* chloroquine-sensitive strain 3D7 were maintained and used in antiplasmodial assays conducted similarly as described elsewhere (Allman *et al.*, 2016). Briefly, dried acetone extracts were dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mg/ml and tested in triplicate (n=3) at a maximal concentration of 100 µg/ml in a 48h *in vitro* colorimetric growth inhibition assay using lactate dehydrogenase activity as a proxy for parasite biomass, according to the method described by Makler *et al.* (Piper *et al.*, 1993).

Plant extract profiling and artemisinin quantification

Plant extracts were analyzed to obtain their phenolic composition and to detect and quantify artemisinin, if present. Briefly, extracts were resuspended in methanol, filtered (0.45 µm), and 10 µl were injected into an Agilent Technologies 1200 series HPLC system to obtain a phenolic profile. A gradient of acetonitrile (A) and formic acid 0.1% (B) was used on a Luna PFP column (250 x 4.6 mm, 5µM) for a duration of 87 mins with a flow rate of 1 ml/min. HPLC detection was done through diode-array detection (DAD) at 330 nm. Based on the literature on the *A. annua* (ANE) and *A. afra* (AFE) compositions, the local library of compounds and preliminary work (data not yet published), the phenolic acid chlorogenic acid, the flavone luteolin, and coumarin derivatives scopoletin and esculetin were used to identify the presence of these phenolic compounds in the extracts (Supplementary data Figure 1) (Ferreira *et al.*, 2010; Shinyuy *et al.*, 2023).

Artemisinin was detected using two different methodologies: first by HPLC with the method as described by Diawara *et al.* (Diawara *et al.*, 2011), and by mass spectrometry. Derivatization was achieved with 0.2% (m/v) sodium hydroxide at 50°C for 30 min followed by cooling with 1 ml ethanol for 10 min before completion with acetic acid (0.2N). Samples were filtered through a 0.45 µm Acrodisc syringe filter into vials before 20 µl were injected in the HPLC system. The isocratic method consisting of phosphate buffer (pH 6.3) (A) and methanol (B) ran for a total of 25 min with a 1 ml/min flow on a Luna PFP column (250 x 4.6 mm, 5 µM). HPLC artemisinin detection was done through a diode array detector (DAD) at 260 nm. Quantification was achieved through this method as described

previously using standards between 10 and 60 µg/ml (Supplementary data Figure 2A) (Diawara et al., 2011).

Detection of artemisinin was also performed by direct-infusion high-resolution-MS (DI-HRMS) of the plant acetone extracts. To do so, 10 mg/ml of AFE and ANE were prepared as well as 1 mg/ml of artemisinin and filtered (0.45 µm) before injection. DI-HRMS was performed on a Thermo Scientific LTQ XL Orbitrap mass spectrometer of the MASSMET Platform (Thermo Scientific, Bremen, Germany). Data were acquired in positive ion mode using full-scan MS with a mass range of 100-1000 *m/z*. The orbitrap operated at 30000 resolution (FWHM definition). All experimental data were acquired following daily external calibration. The following electrospray inlet conditions were applied: 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; capillary voltage, 45 V; tube lens, 80 V.

HPLC-MS metabolomic characterization of *P. falciparum* trophozoites

The IC₅₀ values (concentrations inhibiting 50% of parasitic growth) of AFE and ANE determined by antiplasmodial assays were used to adjust extract concentrations in metabolomics assays, performed as described by Allman *et al.* (Allman et al., 2016). Briefly, the *P. falciparum* cultures were microscopically verified for presence of sufficient numbers of healthy trophozoite stage parasites before they were purified via magnet activated cell sorting (MACS). The sample of ~95-100% pure trophozoite-infected erythrocytes was distributed in 6-well plates at ~0.2% hematocrit and incubated for a one-hour recovery period. After recovery, the parasites were exposed to extracts or compounds at 10 x IC₅₀ for 2.5 h. Artemisinin (Sigma-Aldrich 361593, Machelen, Belgium), atovaquone (Sigma-Aldrich A7986), chloroquine (Sigma-Aldrich C6628) and quinine (Sigma-Aldrich 22620) were used as metabolite profile controls. After pelleting and washing in ice cold PBS pH 7.4, infected red blood cells were resuspended in 1 ml of 90% methanol with 0.5 µM [¹³C₄,¹⁵N₁] aspartate as an internal control. Supernatants were dried under nitrogen flow and stored at -70°C before being resuspended to 10⁶ cells/µl in HPLC-grade water with 1µM chlorpropamide (Alfa Aesar J64110, Karlsruhe, Germany) as an additional internal control for reverse phase HPLC-MS analysis on a Thermo Exactive Plus Orbitrap. Subsequently, 10 µl were injected through a C18 column (Waters XSelect HSS T3 2.5 µm, catalog no. 186006151) and ran using a 25 min gradient of 3% aqueous methanol, 15 mM acetic acid, 10 mM tributylamine, 2.5 µM medronic acid ion pairing agent (A) and 100% methanol (B). Detection was performed in negative-ion mode, using a scan range of 85 to 1,000 *m/z* and a resolution of 140,000 at *m/z* 200.

After data acquisition, .raw files were converted to centroided .mzML format using MSConvert of the ProteoWizard package and then visualized, processed and annotated with EI-MAVEN. The internal standard intensity was used to evaluate technical reproducibility, and peaks were manually curated based on the proximity to the retention time with the targeted library, expected isotopic abundance, and the signal/blank ratio. Chlorpropamide standard abundance was used to correct the peak areas *per* metabolite for instrument variation during the run. Subsequently, the average blank signals were subtracted, and metabolites were filtered based on the relative standard deviation (RSD, Standard Deviation/Mean) of pooled quality control samples run regularly throughout HPLC-MS data collection and removed when they showed low repeatability across technical replicates (RSD > 25%). The remaining metabolites were analyzed in two ways: log₂ fold changes were displayed in a self-organizing map, or metaprint (Allman et al., 2016), for profile comparison, or loaded directly in MetaboAnalyst 5.0 for statistical analysis.

¹H-NMR metabolomic characterization of *P. falciparum* trophozoites

NMR spectra were acquired on a Bruker NEO Ultrashield Plus 700 MHz equipped with a helium cold probe (cryoprobe) with samples dissolved in buffered D₂O at pH 7.4 with TSP as internal reference. ¹H-NMR experiments were performed with a CPMG sequence with 128 scans collected over a spectral width of 20 ppm. All spectra were phased and baseline-corrected manually using TopSpin v4. Spectra were stacked, aligned and integrated between ̈́0.5-9.5 ppm using MestReNova v14. Spectra were divided into buckets of 0.04 ppm, integrated to the sum of intensities and normalized to the total spectrum intensity. This transformation into a data matrix breaks the peaks into bins of a set width interval (in ppm *per* intensity) to reduce the data dimensionality and represent the original data. The bin tables were analyzed using MetaboAnalyst v5.0 and R (packages MBXUCL, PepsNMR and limpca) (Thiel et al., 2023). The NMR spectra were annotated using Chenomx NMR Suite 9.0 database and the Human Metabolome Database (HMDB), as according to literature. ANOVA2 was also implemented with an FDR correction to classify ¹H-NMR bins as significantly different in abundance between different treatments (AFE, ANE and artemisinin). To do so, treatments were paired and subset t-tests performed.

Results

A. afra and *A. annua* have different phenolic compositions

Plant extracts are highly complex and are composed of numerous compound classes. Amongst the most abundant are the phenolic derivatives, which are present in virtually every plant, demonstrating wide-ranging medicinal properties, including antiparasmodial activity (as reviewed elsewhere) (Mamede et al., 2020). Phenolic compounds, such as flavonoids and phenolic acids, have previously been reported to contribute to the antiparasmodial activity of *A. annua* and *A. afra* (Shinyuy et al., 2023). Using HPLC-based techniques, we measured the phenolic composition of *A. afra* (AFE) and *A. annua* (ANE) acetone extracts obtained by maceration for 72 h, filtered and concentrated as described previously (Mbah et al., 2012). First, AFE and ANE were analyzed by HPLC-UV with a reverse phase method to achieve a general overview of their phenolic composition (Figure 1A) according to the literature, the local library of compounds and preliminary work (data not yet published). Our results show that ANE contained a low amount of the phenolic acid chlorogenic acid which was absent in AFE. Luteolin and scopoletin, a flavone and a coumarine derivative, respectively, were identified in both extracts, although at different concentrations, and neither extract contained esculetin, as stated in the literature (Maciuk et al., 2023). Through this analysis, it is clear that there are aglycon forms of phenolic compounds with differing abundances between the two *Artemisia* species, confirming that, despite the time and place of collection being similar, the metabolomes of these two *Artemisia* species are inherently different.

In vitro activity of *A. afra* and *A. annua* extracts

To determine the antiparasmodial potency of the *A. afra* extract (AFE) and *A. annua* extracts (ANE), the IC₅₀ was calculated using an established lactate dehydrogenase (LDH) colorimetric revelation assay (Piper et al., 1993) resulting in an IC₅₀ for AFE of 11.73 ± 2.15 µg/ml, 0.09 ± 0.01 µg/ml for ANE, and 5.58 ± 1.14 ng/ml for artemisinin (Figure 1B). Notably, the activity of the ANE is in agreement with previous reports (Jonville et al., 2008). However, these assays cannot distinguish whether the antiparasmodial activity of these extracts is associated with the presence of artemisinin, which has a nanomolar IC₅₀ (Jonville et al., 2008), or with other inhibitory compounds, such as phenols (Shinyuy et al., 2023).

HPLC-UV and DI-HRMS reveal a difference in abundance of artemisinin between *A. annua* and *A. afra*

In order to explore the mechanism of the *P. falciparum* killing activity of AFE and ANE, both the phenolic composition and artemisinin content of the extracts were measured. Analysis of the AFE and ANE by derivatization and HPLC-UV indicated the presence of artemisinin in ANE (Supplementary data Figure 2B), with a concentration of 19.3 mg/g of artemisinin per dry extract. On the other hand, AFE had negligible signal at the same retention time, falling below the limit of detection (2.73 µg/ml). It is possible that AFE may contain trace amounts of artemisinin, though these would represent less than 2.73 mg/g artemisinin in dry plant. Direct-infusion high-resolution-MS (DI-HRMS) profiling of purified artemisinin resulted in artemisinin-specific ions detected at m/z 305.13559 ($[M+Na]^+$) and m/z 587.28125 ($[2M+Na]^+$) (Supplementary Figure 3). DI-HRMS spectra obtained from ANE and AFE demonstrate notable differences (Supplementary Figure 4). Although ANE has significant artemisinin fragments at the $[M+Na]^+$ and $[2M+Na]^+$ mass windows, AFE displayed significantly reduced intensities relative to ANE, confirming the presence of low-levels of artemisinin in AFE (Figure 1C). These results suggest that other, non-artemisinin compounds contribute to the antimalarial activity of this *A. afra* extract.

P. falciparum trophozoites have different metabolic responses to *A. afra* and *A. annua* extracts To explore the effect of AFE and ANE on parasite metabolism, HPLC-MS metabolite profiling was performed on magnetically purified late-stage *P. falciparum* trophozoites that were incubated for 2.5 h with either plant extract at 10 X IC_{50} along with purified antimalarial drugs at 10 X IC_{50} for 2.5 h as controls. Parasite metabolites were extracted with 90% methanol, processed, and analyzed using both HPLC-MS and NMR. The HPLC-MS data demonstrate that *P. falciparum* parasites exposed to ANE display similar metabolic responses to treatment with artemisinin including a marked decrease in hemoglobin-derived peptides. Student's t-test shows that the ANE and artemisinin had no significant metabolic profile differences (p-value < 0.05). This metabolite profile is not seen with exposure of parasites to AFE (Figure 2A, 2B). In contrast, AFE exposure results in a relative decrease in metabolites involved in folate biosynthesis, an increased abundance of hemoglobin-derived peptides and amino acids, and a lower abundance of glutathione metabolism and lipid metabolism-related metabolites, e.g. acetyl-CoA and sn-glycerol 3-phosphate (Figure 2B). Of the 22 amino acids involved in aminoacyl-tRNA biosynthesis, 12 out of 15 detected are significantly different between AFE and ANE treatments. These differences support the disruption to hemoglobin metabolism by seen by ANE since hemoglobin is known as the main source of amino acids in this parasite (Creek et al., 2016; Okombo et al., 2022). When comparing the metabolic response of AFE to purified artemisinin (Figure 2C), glutathione, glutathione disulfide, and NADPH show the biggest differences (p-value < 0.05). This confirms that glutathione metabolism is affected by artemisinin treatment, which is recapitulated when comparing AFE and ANE (Figure 2D) (Siddiqui et al., 2017).

ANE, AFE and artemisinin treated parasite samples were also analyzed using 1H -NMR to compare changes in metabolism. A PCA analysis revealed a major separation (PC1 of 44.55%) between AFE treatment and ANE and artemisinin (Supplementary data Figure 5). This was further confirmed by ANOVA-Simultaneous Component Analysis+ (ASCA+) (Thiel et al., 2023), which identified that the largest variance (45.67%, p-value < 0.01) was attributed to the treatment conditions, while the assay-induced variability was linked to only 10.85% of the data variability (p-value = 0.04). The ASCA+ score plot for the treatment effect separates AFE through PC1 (88%) from ANE and artemisinin (Figure 2E), confirming that AFE treatment is similar to artemisinin, but different from ANE treatment.

ANOVA2 provided a list of statistically significant metabolites in the 1H -NMR data to distinguish treatment groups. Three amino acids (leucine, valine, histidine), glutathione, NAD⁺, ADP, O-

phosphocholine, myo-inositol, glucose, N-acetylglucosamine, and glycine were found to be significantly different in abundance between AFE and ANE treatment (Figure 2F). Biochemical pathway analysis of this list of metabolites reveals changes to aminoacyl-tRNA biosynthesis linked with amino acid changes like glycine, which is also a precursor for glutathione by ANE and artemisinin treatments. Additionally, two lipid precursors, O-phosphocholine and myo-inositol, are significantly different in abundance in parasites treated with AFE when compared to ANE or artemisinin. O-phosphocholine and myo-inositol are involved in several pathways, such as glycerophospholipid metabolism or phosphatidylinositol signaling. This is particularly interesting because myo-inositol is only significantly different when comparing AFE to ANE, suggesting that phenolic compounds found in AFE in the absence of artemisinin may exert this metabolic effect on malaria parasites.

An overview of the metabolic pathways being affected by malaria parasite treatment with AFE and ANE extracts (Figure 3) identifies glutathione metabolism as most impacted, followed by glyoxylate and dicarboxylate metabolism. These pathways are connected by the amino acids glutamate and glycine, which are precursors of glutathione. Taken together, our LC-MS and ¹HNMR results suggest disruptions to hemoglobin degradation and redox-derived modes of action by ANE and artemisinin. Treatment with the AFE plant extract, on the other hand, has a distinct profile associated with changes in lipid and carbon metabolism that may be due to the phenolic compounds detected.

Discussion

In light of the diverging reports in the literature regarding the antiparasmodial activity of *A. afra* and *A. annua* extracts, this study was conducted to provide a comparison of their metabolite composition and to capture the metabolic response of *P. falciparum* parasites to these plant extracts. This study uses several complementary methods to evaluate the direct effects of these plant extracts on the parasite. In particular, we focused on phenolic composition, the abundance of artemisinin, and using comprehensive metabolomics approaches to measure metabolic changes in asexual *P. falciparum* trophozoites upon extract exposure *in vitro*.

Importantly, these extracts were not prepared in the same manner in which the plants would be traditionally consumed. For this study the leaves and twigs were extracted with acetone as opposed to a traditional infusion (du Toit and van der Kooy, 2019; Shinyuy et al., 2023). Based on the polarity difference between the two extraction solvents, a different phenolic composition is to be expected, and indeed, the HPLC profile showed mostly aglycon forms of flavonoids, coumarins, and phenolic acids (Ferreira et al., 2010; Mouton et al., 2013). As such, the extract composition and effects cannot be directly linked to the effects of traditional infusions. Still, non-polar extractions of *A. annua* have been associated with antiparasmodial activity *in vitro* and a significant artemisinin content (Mouton et al., 2013).

P. falciparum growth inhibition assays were conducted *in vitro* to establish the antimalarial potency of each extract revealing that ANE has the higher potency, with over one hundred-fold decrease in IC₅₀ compared to AFE. The antiparasmodial activity of ANE has been shown to be almost exclusively (> 90%) attributed to the concentration of artemisinin; hence, its strong antiparasmodial activity was to be expected (Maciuk et al., 2023). However, compounds other than artemisinin may also be responsible for the activity of *A. afra*, especially since AFE, which has reduced artemisinin content, has also been found to be a promising antiparasmodial based on previous screens (Jonville et al., 2008; Maciuk et al., 2023). In this context, the IC₅₀ is a unidimensional measure that indicates the *in vitro* activity but fails to inform on the mode of action, timing, or life stage affected, as the assay is typically performed over a complete 48 h asexual blood stage cycle (Jonville et al., 2008).

Our results indicate that both AFE and ANE contain phenolic compounds and derivatives that are distinct to each extract. Flavonoids and other phenolic compounds have previously been found to demonstrate synergy with artemisinin, which potentiates their antiplasmodial activity and expands the ways that parasite development can be inhibited, including targeting additional kinases, apicoplast metabolism, and artemisinin-enhanced heme interactions (Ferreira et al., 2010; Maciuk et al., 2023). The flavonoid luteolin was found in both extracts and is thought to be partially responsible for the antiplasmodial activity of these plants, though its antimalarial potential (μM IC_{50} range) is below that of artemisinin (nM IC_{50} range) (Maciuk et al., 2023; Weathers et al., 2014). Additionally, phenolic compounds have multiple putative antiplasmodial modes of action, *i.e.*, inhibition of heme detoxification (flavonoids), inhibition of fatty acid biosynthesis (flavonoids and phenolic acids), and/or redox cycling (Maciuk et al., 2023; Mamede et al., 2020). Any of these compounds could participate in the antiplasmodial activities of ANE and AFE because they are readily detected in the extracts (Feng et al., 2020; Maciuk et al., 2023).

The artemisinin content of ANE and AFE extracts was measured through two different means. HPLC with UV detection after derivatization revealed that ANE has a high content of artemisinin, while AFE levels were below the limit of detection. Because of the possibility that artifacts resultant from derivatization could lead to variability in the detection of artemisinin, and the relatively low limit of detection associated with the method ($2.73 \mu\text{g/ml}$), we also used DI-HRMS. Again, artemisinin was found only in trace amounts in AFE, while ANE showed a higher abundance. It remains unclear whether *A. afra* and *A. annua* share a biosynthetic process to produce artemisinin and related sesquiterpenes or if their presence in AFE may be due to contamination (Maciuk et al., 2023). To date, a shared process to synthesize artemisinin has not been demonstrated between these plants, and, despite best efforts during collection, contamination can never be fully excluded.

To better understand the mechanism of action of these extracts, samples were analyzed using both LC-MS, and ^1H -NMR metabolomics. The metabolic response of *P. falciparum* to the AFE and ANE were starkly different (Figure 2A and 2B). ANE-treated parasites had no statistical differences compared to artemisinin-treated parasites, suggesting a predominantly artemisinin-based effect on parasite metabolism for ANE, as reported previously (Maciuk et al., 2023). The metabolic response of *P. falciparum* to treatment with AFE, ANE and artemisinin are also markedly distinct from the response to chloroquine. However, the pattern of decreased abundances of hemoglobin-derived peptides and amino acids (*e.g.* glutamate, histidine, valine, among others) seen with ANE and artemisinin treatments resembles the effects seen with quinine treatment, indicating similar impacts on hemoglobin digestion (Figure 2A). However, the mode of action of ANE against *Plasmodium* parasites may be more pleiotropic in nature as compared to purified artemisinin given the presence of additional flavonoids in the extract.

We detected AFE-associated changes in carbohydrate metabolism and lipid metabolism. AFE-treated parasites show marked changes in anaerobic glycolysis, which reflect modifications to TCA cycle and propanoate metabolism. Additionally, phospholipid metabolism linked with fatty acid synthesis is more affected by AFE treatment, significantly changing the availability of membrane precursors, and choline and myo-inositol signaling. These effects of AFE treatment may indicate disruptions to parasitic fatty acid metabolism and membrane remodeling (Beri et al., 2019). Flavonoids are thought to inhibit the entry of myo-inositol into the iRBC, which would hamper parasite development by interfering with phospholipid synthesis and osmoregulation (Mamede et al., 2020). Both AFE and ANE contain flavonoids, and studies with an *A. annua* flavonoid, casticin, have shown synergy with artemisinin and inhibition of myo-inositol import into iRBCs, further demonstrating how such interactions may disrupt parasite viability (Weathers et al., 2014).

In contrast, an accumulation of choline had been previously reported in artesunate and artemisinin-treated parasites, as is the case in our study (Creek et al., 2016; Wang et al., 2022). Choline shares the same precursor with sn-glycerol-3-phosphate, an important metabolite that branches from anaerobic glycolysis that retains two roles: maintaining cytosolic redox balance through NAD^+ and transferring reducing equivalents into the mitochondrial electron transport chain (Biddau and Müller, 2016). The decrease in NAD^+ , NADPH, choline, sn-glycerol-3-phosphate, and their precursors, glucose and acetyl-CoA in AFE-treated samples are consistent with changes to carbon and lipid metabolism that prevent maintenance of these systems (Biddau and Müller, 2016). This is further confirmed by the higher fold change of pyruvate in these samples which, along with a reduced level of acetyl-CoA, could result in an inhibition of fatty acid synthesis and the expected downstream effects (Figure 3) (Biddau and Müller, 2016; Mok et al., 2021). This suggests a selective interference of fatty acid synthesis by AFE and is in line with the biological activity of flavonoids and phenolic compounds that have been shown to inhibit fatty acid biosynthesis related enzymes (FabG, FabZ and FabI) (Tasdemir et al., 2006). Therefore, the potential inhibition of fatty acid synthesis by the flavonoids and phenolic acids present in AFE cannot be excluded as one of its contributing antimalarial modes of action (Mamede et al., 2020). Future lipidomic or isotope tracing studies could help elucidate this potential effect more clearly.

Another effect of AFE and ANE on the metabolome of *P. falciparum* manifests in redox cycling agents, namely glutathione, glutathione disulfate, NADPH and NAD^+ . These pathways could be affected in at least two ways: first, lower biosynthesis of glutathione metabolites and lower recovery of cofactors (Biddau and Müller, 2016). Artemisinin-resistant strains have been shown to increase glutathione production to mitigate oxidative damage following artemisinin treatment (Siddiqui et al., 2017). Thus, the increases in glutathione observed following ANE and artemisinin treatment are expected (Siddiqui et al., 2017). However, the overall lower fold change in these metabolites following AFE treatment was unexpected. Nonetheless, this change may be a downstream consequence of other effects, such as changes to the metabolism of glutathione precursors like glycine and glutamate, along with the lower recovery of NAD(P)^+ following lower pyruvate to lactate conversion and fatty acid synthesis (Biddau and Müller, 2016). However, it is possible that other compounds in AFE may act as pro-oxidants in specific conditions, overcharging this parasitic defense system (Ferreira et al., 2010). Oxidative damage has been associated with lipid peroxidation and mitochondrial and plasma membrane changes. Such effects cannot be excluded without additional testing for lipid specific metabolites (Mamede et al., 2022).

Our results also align with the reported benefits of the use of *A. afra* and *A. annua* extracts to treat malaria (Maciuk et al., 2023; Weathers et al., 2014). *A. afra*, despite containing, at best, vestigial amounts of artemisinin, does not owe its activity to this sesquiterpene. Instead, *A. afra* appears to rely on other compounds, perhaps flavonoids, to interfere with the parasite and its lipid precursors. Others have reported that *A. afra* contains additional sesquiterpenes that are gametocytocidal (Moyo et al., 2019). On the other hand, the antiplasmodial activity of *A. annua* can be attributed mostly to the high abundance of artemisinin, with no statistically significant differences in metabolite abundances between the two treatments. Both the ANE and artemisinin also function by affecting glutathione and lipid metabolism possibly due to oxidative reactions (Figure 3). Interestingly, despite the long-term traditional use of this plant, artemisinin resistance had not been observed until the clinical use of artemisinin and its derivatives (Maciuk et al., 2023). Recent studies have found that the use of extracts from the whole *A. annua* plant can overcome artemisinin resistance, which suggest the activity of several compounds of disparate modes of action (Ashraf et al., 2022). Therefore, although artemisinin likely plays a central role in the antiplasmodial activity of the ANE plant extract, other compounds in the extract or perhaps their combined interaction are able to prevent resistance, retain efficacy, and

modulate other aspects of malaria at an inflammatory and immunological level (Ekiert et al., 2021; Shinyuy et al., 2023).

Conclusion/Perspectives

Both *A. afra* and *A. annua* extracts have been extensively reported for their traditional use against malaria (Maciuk et al., 2023). Whereas the latter has led to the discovery of artemisinin, the former retains antiplasmodial activity despite negligible amounts of artemisinin, based on the literature and the data from this study. Our findings suggest that the activity of *A. annua* is mostly due to its artemisinin content, which aligns with the effects of this compound on hemoglobin and redox metabolisms. *A. afra*, on the other hand, may affect lipid precursors and signaling, indicating extract constituents other than artemisinin are responsible for its activity. Additional experiments, *e.g.*, *in vivo* tests, enzymatic studies, and lipidomics will prove useful to confirm the link between these pathways and the antiplasmodial effects of these extracts. We conclude that both plant extract profiles are distinct and should be further explored for additional antimalarial compound identification toward the goal of malaria eradication.

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Figure legends

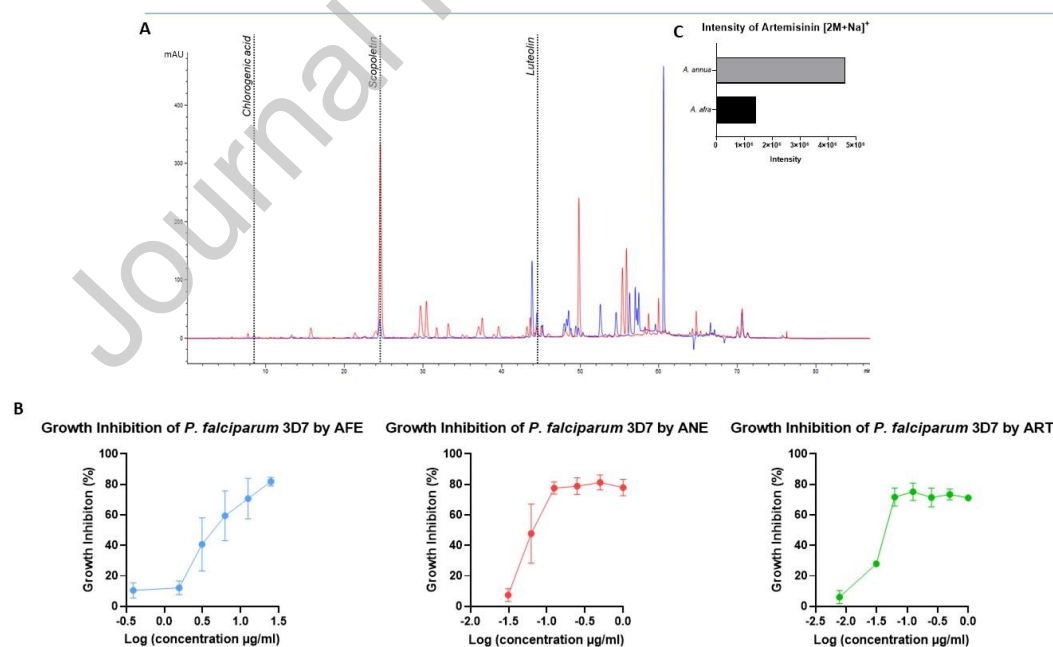


Figure 1 – A – HPLC-UV chromatograms of *A. afra* extract (AFA) (blue) and *A. annua* extract (ANE) (red) detected at 330 nm (dotted lines represent retention times of pure standards). B – Growth inhibition curves of AFE, ANE and artemisinin (ART) over 48h (error bars represent standard deviation). Results

are representative of three independent experiments. AFE maximal tested concentration was of 25 $\mu\text{g/ml}$, and ANE and ART was 1 $\mu\text{g/ml}$. C – Inset bar representation of $[\text{2M}+\text{Na}]^+$ intensity in ANE and AFE.

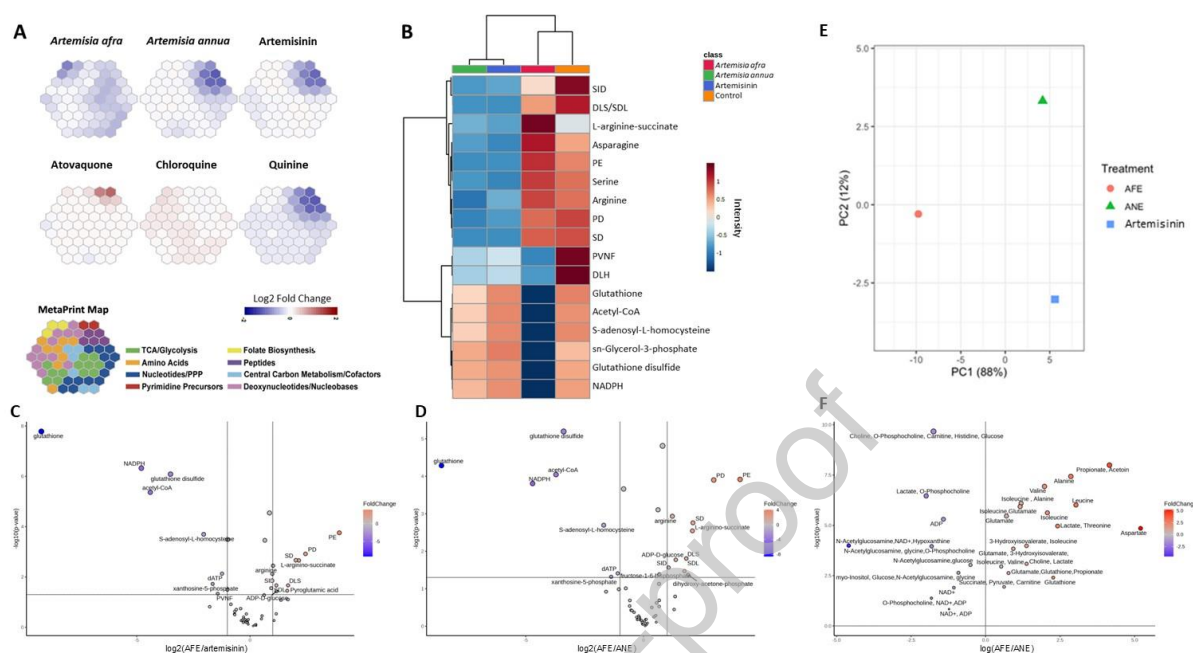


Figure 2: LC-MS and ^1H -NMR metabolomic analysis of the *in vitro* antiplasmodial effects of AFE, ANE, artemisinin, atovaquone, and control (DMSO). (A) Metaprint profiles based on LC-MS data in which metabolite clusters are associated to eight major metabolic pathways as attributed in the MetaPrint Map (Allman et al., 2016). (B) Heatmap based on LC-MS data of the metabolites scored as significant by ANOVA. (C+D) Volcano plots (fold change threshold: 2.0; unpaired Student's t test for each metabolite between triplicates samples across conditions, p-value threshold: 0.05) based on LC-MS data of comparisons (C) AFE/artemisinin and (D) AFE/ANE. Points represent metabolites: in gray (non-significant), red (positive fold), blue (negative fold). (E) Multivariate analysis ASCA+ via limpca R package: score plot for the treatment effect. (F) Volcano plot (fold change threshold: 1.0; unpaired Student's t test for each metabolite between triplicates samples across conditions, p-value threshold: 0.05) based on ^1H -NMR data AFE/ANE. (DLH – aspartyl-histidyl-leucine; DLS - leucyl-aspartyl-serine; PD - prolyl-aspartate; PE - prolyl-glutamate; PVNF – L-prolyl-L-valyl-L-asparaginyl-L-phenylalanine; SD - serylaspargic acid; SDL - seryl-aspartyl-leucine; SID - aspartyl-isoleucyl-serine)

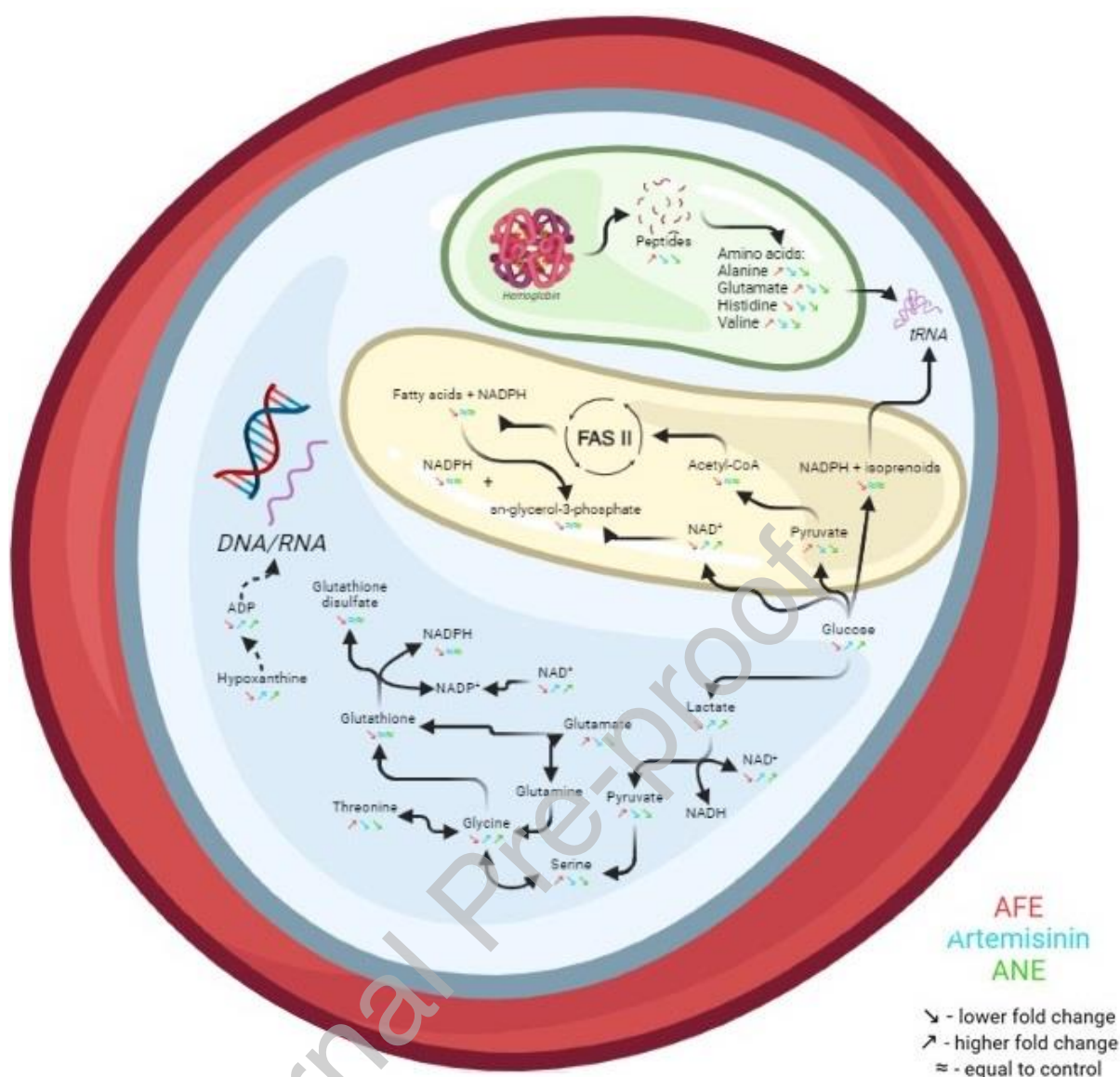


Figure 3 – Schematic view the affected pathways by AFE, ANE and artemisinin. FAS II – Fatty type II Acid Synthesis. (Arrows: red – AFE, blue – artemisinin, green – ANE; > - higher fold change, < - lower fold change, ≈ - equal to control)

Abbreviations

ANOVA-Simultaneous Component Analysis+ – ASCA+

Artemisia afra – AFE

Artemisia annua – ANE

Artemisinin-based Combination Therapies – ACTs

Dimethylsulfoxide – DMSO

Diode-array detection – DAD

Direct-infusion high-resolution-MS – DI-HRMS

High-performance liquid chromatography – HPLC

Human Metabolome Database – HMDB

¹H-NMR – Proton Nuclear Magnetic Resonance

Lactate Dehydrogenase – LDH

Magnet activated cell sorting – MACS

Mass spectrometry – MS

Relative standard deviation – RSD

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We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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X

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Credits

LM wrote the draft, all authors revised the draft. Experiments were carried out by LPM, GWR, LMS, NB, MFH and BG and supervised by OJ, AL, PDT, JQL, ML and MF. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Conflict of Interest

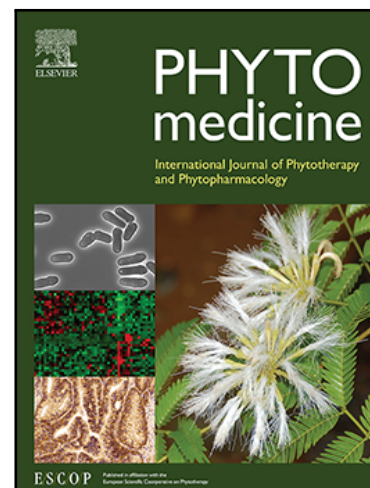
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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

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