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In vitro antiplasmodial activity of six plants against chloroquine-sensitive and resistant strains of *Plasmodium falciparum*

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Abstract

Introduction: The effectiveness of the first-line malaria treatment has been affected by drug resistance and adverse side effects leading to a limited number of treatment options. This calls for the search for alternative antimalarial agents. The study evaluated the *in vitro* antimalarial activity of six plants frequently used in herbal antimalarial products in Ghana against chloroquine-sensitive strain (3D7) and chloroquine-resistant strain (DD2) of *Plasmodium falciparum*. **Method:** Aqueous extracts were prepared from the plants by decoction and freeze-dried. A fluorescence-based SYBR Green assay was used to evaluate the antimalarial activity of the extracts against *Plasmodium falciparum* strains 3D7 and DD2. Also, the cytotoxic effects (CC_{50}) of the plant extracts against red blood cells were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) rapid calorimetric assay technique. **Results**: *Alstonia boonei*, *Cryptolepis sanguinolenta,* and *Azadirachta indica* were the most effective mono-herbal extracts with IC_{50} of 8.64 µg/mL, 6.12 µg/mL, and 5.22 µg/mL respectively against 3D7 lab strain and 8.47 µg/mL, 5.12 µg/mL and 5.22µg/mL respectively against DD2 lab strain. The aqueous extracts of *Paullinia pinnata, Citrus aurantiifolia,* and *Tetrapleura tetraptera* exhibited moderate activity against both lab strains with IC_{50} values of 24.72 µg/mL, 34.89 µg/mL and 14.94 µg/mL respectively against 3D7 strain and 14.84 µg/mL, 31.01 µg/mL and14.74 µg/mL respectively against DD2 strain. All plant extracts exhibited no cytotoxicity against RBC (>100 µg/mL, except *Cryptolepis sanguinolenta* with CC_{50} 92.7 µg/mL). Moreover, except *Paullinia pinnata, Citrus aurantiifolia* and *Tetrapleura tetraptera* (with low selectivity index: SI < 10), all the plants displayed a good selectivity index (SI>10). **Conclusion**: All six frequently used antimalarial plants in monotherapy possess significant antimalarial activity against *Plasmodium falciparum* (3D7) and (DD2) strains. The data obtained from this study support the folkloric and frequent use of these plants in several herbal antimalarial products on the Ghanaian market.

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Introduction

Malaria is a major tropical disease with high morbidity and mortality, affecting about 3 billion of the world's population, particularly those residing in Asia, Latin America, and Sub-Saharan Africa. The sub-Saharan Africa region carries a disproportionate share of 90% of the global malarial load and accounts for 92% of the total malaria deaths(Wang, 2022).

A major setback in the treatment of malaria has been the development of resistance by the parasite to most single-molecule allopathic drugs used as well as the cost of its management. Also, pharmaceuticals of herbal origin are acquiring more relevance on the global market due to increased awareness of the side effects of synthetic drugs and Africa, especially which has compelled reputable pharmaceutical corporations to introduce herbal based formulations to the market (Chaitanya, 2021).

Herbal medicines have a long history, almost as far as human history. The majority of these plant-derived pharmaceuticals were discovered through traditional treatments and Indigenous people's folk knowledge, and some of them could not be replaced despite enormous advances in synthetic chemistry (Rizvi et al., 2022).

People around the world have developed their techniques for prevention and treatment of diseases (Aslam et al., 2016). In Asian and African nations, about 80% of people depend on native therapeutics for their main healthcare (Karole et al., 2019). Malaria is a disease caused by a plasmodium parasite, transmitted by the bite of infected mosquitoes (White, 2022) . There are 229 million cases of malaria worldwide and more than 400,000 people die from the disease each year, according to the World Health Organization (2020, 1997). In the WHO African Region, *Plasmodium falciparum* is the most chronic and lethal species infecting people, accounting for 96% of malaria cases and fatalities in 2020 in the 29 endemic African nations (Essoh et al., 2022). The rise of drug-resistant parasites in some regions of the world threatens the effectiveness of artemisinin-based combinations, which have been a successful weapon in efforts to eradicate malaria (Ouji et al., 2018).

In vitro, antimalarial assay necessitates continuous cultivation of *P. falciparum* and any other lab strains. It enables a quantitative evaluation of intrinsic drug sensitivity that is based on microscopic parasitemia evaluation and inhibitory concentration(IC) determination (Chetia, 2019). By comparing the inhibition of parasite growth in drug-exposed cells to *Plasmodium* drug-free control cultures, antimalarial medication activity is assessed, and Sigmoid dose-response curves are produced to evaluate the efficacy of compounds in terms of IC after testing the active substances in the primary tests in serial drug dilution. Microscopic tests have been replaced by other techniques such as the hypoxanthine 3 incorporation method (microculture radioisotope technique), the flow cytometry assay (SYBR Green I-based), colorimetric ELISA tests (pfLDH assay, pfHRP2 assay), and fluorescence assay (pfGFP-based, SYBR Green I-based). These approaches are generally straightforward, necessitate fewer time-consuming test steps, and offer high throughput, but they call for pricey equipment.

The research was carried out based on most frequently used plants in herbal antimalarial products on the Ghanaian market as reported in our previous work (Nortey et al., 2023), which are *Alstonia boonei* (leaves), *Azadirachta indica (leaves)*, *Citrus aurantiifolia* (leaves), *Cryptolepis sanguinolenta* (roots), *Paullinia pinnata* (leaves) and *Tetrapleura tetrapetra* (fruits). These plants were collected and screened against chloroquine-sensitive (3D7) and resistant (DD2) strains. All the plants have previously been evaluated for their antiplasmodial activity and antimalarial bioactives identified, however, a few of the plants have yet to be assayed against the 3D7 and DD2 strains (Table 1). *Plasmodium falciparum* has developed resistant strains to chloroquine due to a mutation in the *Pfc*rt gene present in the parasite's food vacuole (Turschner & Efferth, 2009). They possess a neutral threonine instead of the positively charged Lysine at position 76 of the *Pfc*t gene. This mutation causes reduced accumulation of chloroquine within the food vacuole and allows chloroquine to diffuse away from the food vacuole by a steep downward concentration gradient (Sanchez et al., 2004) (Cooper et al., 2002) (Sanchez et al., 2004).

This study sought to provide the scientific basis for their selection in the formulation of polyherbal preparations and compare their antiplasmodial effects against 3D7 and DD2 strains of *Plasmodium falciparum*.

Table 1: Previous antimalarial reports on the plants against chloroquine sensitive(3D7) and chloroquine resistant strains (DD2) of *Plasmodium falciparum* strains

Plants	Known antimalarial phytochemicals present	Extraction method	*In vitro* assay (IC_{50}, µg/mL)		References
			3D7	DD2	
Paullina pinnata	Alkaloids, terpenoids flavonoids, saponin, and coumarins	N/A	N/A	N/A	(Fred-Jaiyesimi & Anthony, 2011)
Citrus aurantiifolia	Flavonoids, coumarins, terpenoids and saponins	Maceration (aqueous)	71.31	N/A	(Bapna et al., 2017) (Laksemi et al., 2023) (Ettebong et al., 2019)
Azadirachta indica	Flavonoids, saponin, coumarins, terpenoids (Nimbin, azadirachtin) and alkaloids	Maceration (Ethanol)	7.52	N/A	(Deshpande et al., 2014)
Alstonia boonei	Alkaloids, triterpenes, flavonoids, saponins, terpenoids and coumarins	N/A	N/A	N/A	(Omoya & Oyebola, 2019)
Tetrapleura tetraptera	Flavonoids, saponins, alkaloids, saponins, coumarins and terpenoids	Maceration (Ethanol)	13.0	10.1	(Nsofor et al., 2023) (Lekana-Douki et al., 2011)
Cryptolepis sanguinolenta	Alkaloids (cryptolepine and cryptoquindoline), saponins, flavonoids, terpenoids and coumarins.	N/A	N/A	N/A	(Opoku-Agyemang et al., 2022)

N/A, No report available

Materials and methods

Collection and processing of plant materials.

The leaves of Azadirachta indica and Citrus aurantifolia were collected and harvested locally in the Ablekuma area, Accra, Ghana. The leaves of Paullinia pinnata, Alstonia boonei, and fruit of Tetrapleura tetraptera were harvested locally in the Nkwatia area, Kwahu, Ghana. The dried roots of the Cryptolepis sanguinolenta plant were purchased from the Centre for Scientific Research, Mampong in March 2023. The collected specimen was authenticated by Miss Miriam Tagoe, the head of the School of Pharmacy Herbarium, Central University, and voucher number CUC/F/NK/003, CUC/L/NK/007, CUC/R/AM/002, CUC/L/A/009, CUC/L/NK/008 and CUC/L/A/006 were assigned to the Tetrapleura tetraptera, Alstonia boonei, Cryptolepis sanguinolenta, Azadirachta indica, Paullinia pinnata, and Citrus aurantiifolia respectively and the samples deposited at School of Pharmacy Herbarium, Central University, for future reference.

Sample extraction

The leaves of *A. indica, P. pinnata, C. aurantifolia*, and *A. boonei* were washed gently under running water and air-dried for about 7 days. The roots of *C. sanuginolenta* were sliced into pieces, washed under running water, and air-dried for about 7 days. The dried pods of *T. tetraptera* were washed under running water, chopped into sizeable amounts, and washed again, then air dried for about 5- 10 days. The aqueous extract of the leaves, roots, and fruits of *A. indica*, *P. pinnata, C. aurantifolia, A. boonei*, *C. sanguinolenta*, and *T. tetraptera* respectively were obtained by decoction according to the Ghana Herbal Pharmacopeia. The leaves were boiled with water for about twenty minutes and bark for about thirty (Busia.ed, 2007). The cooled extracts were filtered and freeze-dried at the Noguchi Memorial Research Institute. The freeze-dried powdered *T. tetraptera*, *A. boonei*, *C. sanguinolenta*, *A. indica, P. pinnata,* and *C. aurantifolia* were assigned TTE, ABE, CSE, AZE, PPE and CAE code names respectively. Plant extracts were stored in a refrigerator until needed for use.

Phytochemical test

Phytochemical components of the respective plants were identified using conventional techniques outlined (Evans, 2009).

In vitro antiplasmodial assay

Preparation of extract and Parasite

The extracts and parasites were prepared as described in previous work by (Amengor et al., 2024). A working solution was serially diluted using Roswell Park Memorial Institute Medium 1640 (RPMI 1640) to obtain the following concentrations: 100 µg/ml, 50 µg/mL, 25ug/ml, 12.5 µg/mL, 6.25 µg/mL, 3.13 µg/mL, 1.56, 0.78 µg/mL, and 0.39 µg/ml. The parasites were obtained from the Immunology department of Noguchi Memorial Institute for Medical Research, University of Ghana. P. falciparum asexual cultures were maintained as described in (Trager & Jensen, 1976). The parasites were plated based on the description in (Gathirwa et al., 2008). This procedure was repeated for all extracts.

Extract plating and SYBR green assay

With artesunate (ATS) as the standard drug, 100mL of each of the already prepared concentrations in 2.4.1 were plated in duplicates in an already labeled (1 to 11) 96 well coastal plate. 15ng/mL of this standard drug was serially diluted and plated alongside the extracts. 100 µL of parasite mix with 2% hematocrit and 1% parasitemia were added to each treated labeled well from the 2 to 10 (Test wells). A parasite +2% hematocrit +1% parasitemia mix only was added to well no. 11 as a negative control. The procedure was repeated for the rest of the extracts. The plates were carefully arranged in a modular Chamber and gassed for 5min with a gas mixture as described in (Amengor et al., 2024). The plates were subsequently maintained at 37 °C for 72 hrs. After 72hrs, the plates were removed and assayed by the addition of 100 µL lysing buffer containing SYBR Green to each of the wells. The mixture in the plates was carefully mixed and incubated in the dark for 30-60 minutes. The plates were then read using a FLUOstar OPTIMA Fluorometer plate reader (software version 2.20) at 470nm and 520nm wavelengths.

Cytotoxicity studies

The extracts were tested for toxicity to RBCs using a modified version of the tetrazolium-based colourimetric technique. 100 µL of each extract was prepared to obtain concentrations ranging from 6.25 µg/mL to 100 µg/mL. These concentrations were instilled in triplicate into the wells of a 96-well microtiter plate. 100 μL of uninfected RBCs were subsequently put into each well. The plates were incubated and maintained and the optical densities of wells were measured based on the description in described in (Amengor et al., 2024) (Ikem et al., 2020). Extracts, culture medium, and uninfected RBCs were subtracted from the optical densities by running control experiments for each parameter independently alongside the main experiment. Artesunate which is a known antimalarial drug was used as a positive control in the cytotoxicity assay

Statistical Analysis

Each product was tested in a duplicate and the median inhibitory dose (IC_{50}) and median cytotoxic concentration (CC_{50}) values of the extracts against asexual Plasmodium falciparum was estimated from dose-response curves by non-linear regression analysis using Graph pad Prism version 7.0 Software (Graph Pad Software, San Diego, CA, USA).

Results and discussion

Phytochemical screening of aqueous extracts of plant samples.

Phytochemicals are active biologically compounds found in plants (Agidew, 2022). Some of the significant phytochemicals are coumarins, tannins, saponins, alkaloids, and glycosides. Table 2 contain the phytochemical analysis of the aqueous extracts of the plant samples.

Antiplasmodial activity

The sequel of *in vitro* antiplasmodial assay of aqueous extracts of the six selected medicinal plants against *Plasmodium falciparum* 3D7 and DD2 lab strains are graphically represented on Fig. 1. IC_{50} values of the 3D7 lab strain were generally higher than those of DD2 lab strain.

Table 2: Phytochemical screening results of aqueous extracts of plant samples.

Phytochemical	CSE	AZE	PPE	TTE	CAE	ABE
Saponins	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Hydrolysable Tannins	-	+	+	+	+	+
Condensable tannins	+	-	-	-	-	-
Terpenoids	+	+	+	+	+	+
Alkaloids	+	+	+	-	-	+
Flavonoids	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+
Anthracene glycosides	+	-	-	-	-	+
Coumarins	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Hydrolysable Tannins	-	+	+	+	+	+
Condensable tannins	+	-	-	-	-	-
Terpenoids	+	+	+	+	+	+

KEY: + – phytochemical detected

- – phytochemical not detected.

Cryptolepis sanguinolenta extract (CSE), *Azadirachta indica* extract (AZE), *Paullinia pinnata* extract (PPE), *Tetrapleura tetreptera* extract (TTE), *Citrus aurantifolia* extract (CAE), *Alstonia boonei* extract (ABE)

Cytotoxicity

In these studies, the in vitro cytotoxicity was expounded as follows; low selective index <10 and high (or good) selective index >10 (Obbo et al., 2019). The outcome showed that the aqueous extracts of PPE, TTE, and CSE indicated a low selective index. AZE, CSE and ABE exhibited high selective index as seen in Table 3 below.

Discussion

In this study, the antimalarial potencies of six most frequently use plants to treat malaria were evaluated against chloroquine sensitive and resistant *Plasmodium falciparum.* These plants are mostly used in antimalarial FDA approved herbal products on the Ghanaian market. Plant extracts are particularly essential in medicine, and their biologically active components are thought to be the reason for this(Dar et al., 2023). When taken orally, herbal remedies may have the desired healing effect, but they may also contain other components that could have harmful effects. Phytoconstituents are said to have a wide range of therapeutic advantages, hence it is always important to evaluate the phytoconstituents in plants with ethnomedical qualities (Shaikh & Patil, 2020). A vast source of novel and promising medicines can be found in

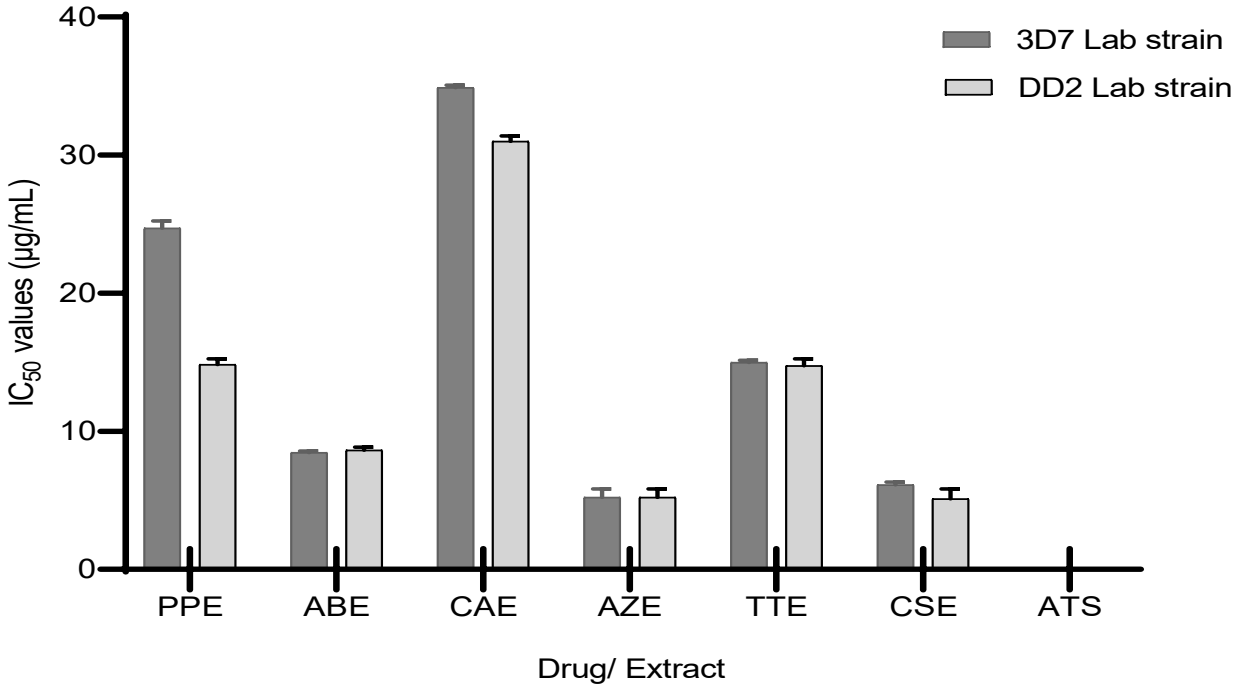

Fig. 1: IC_{50} of the various plant extracts against 3D7 and DD2 lab strains.
Key: *Cryptolepis sanguinolenta* extract (CSE), *Azadirachta indica* extract (AZE), *Paullinia pinnata* extract (PPE), *Tetrapleura tetreptera* extract (TTE), *Citrus aurantifolia* extract (CAE), *Alstonia boonei* extract (ABE) and Artesunate (ATS)

plants, which contain phytochemicals like steroids, glycosides, flavonoids, alkaloids, amino acids, saponins, tannins, and more (Majrashi et al., 2023). In addition to the health benefits associated with macronutrients and micronutrients, phytochemicals are biologically active, naturally occurring chemical compounds found in plants (Agidew, 2022). Alkaloids are responsible for most antimalarial properties of plants. The first successful antimalarial drug was the alkaloid quinine, extracted from the Cinchona tree (Hariyanti et al., 2022). There are studies on the mechanism by which some alkaloids effect antiplasmodial activity, typically amongst them is cryptolepine, found in Cryptolepis sanguinolenta. Flavonoids and saponins possess a number of medicinal benefits, including anticancer, antioxidant, anti-inflammatory, antimalarial, antioxidant, neuroprotective, antitumor, and anti-proliferative agents and antiviral properties (Korsah et al., 2021).

From the studies, significant levels of saponins, tannins, condensed tannins, glycosides, anthracene glycosides, alkaloids, terpenoids, coumarins, and flavonoids were present in *Cryptolepis sanguinolenta* aqueous extract. Similar phytochemical composition was observed in *Azadirachta indica*. Anthracene glycosides were undetected in *Paullinia pinnata* and *Tetrapleura tetraptera* aqueous extract. *Alstonia boonei* aqueous extract showed the presence of saponins, hydrolyzable tannins, tannins, terpenoids, coumarins, flavonols, alkaloids, and anthracene glycosides (Table 2). These results are comparable to previous studies reported on Table 2.

All the plants have previously evaluated for their antiplasmodial activity and their antimalarial bioactives identified, however few of the plants have been assayed against the 3D7 and DD2 strains (Table 1). Again, none of the previous reports compared the antiplasmodial potency of these plants to assist in their selection for formulations in polyherbal preparations. Although, herb-herb polytherapy may not always favor the most performing herbal monotherapy, this kind of comparative studies will serve as a guide for the plant's selection in combination therapy in further studies. The following parameters were used to evaluate the *in vitro* antiplasmodial of the plants: high antiplasmodial activity (IC50 ≤ 10 µg/mL), moderate antiplasmodial activity (IC_{50} of 11-50 µg/mL), low antiplasmodial activity (IC_{50} of 50-100 µg/mL), and inactive (IC_{50} of ≥100 µg/mL) as reported in (Cudjoe et al., 2020).The increasing order of the antimalarial potency of plants as seen on Fig. 1 were as follows; *Azadirachta indica> Cryptolepis sanguinolenta >Alstonia boonei> Tetrapleura tetraptera> Paullinia pinnata> Citrus aurantiifolia* against the two lab strains. *Alstonia boonei, Cryptolepis sanguinolenta, and Azadirachta indica*

exhibited good antimalarial activity in 3D7 and DD2 parasites. Alkaloids isolated from *Cryptolepis sanguinolenta* exhibited significant antiplasmodial activity against K1 chloroquine strain of *Plasmodium falciparum* by (Paulo et al., 2000). Similarly, antiplasmodial studies in *Azadirachta indica* and *Alstonia boonei* by (Deshpande et al., 2014) and (Bello et al., 2009) showed similar activity. Flavonoids and phenolic compounds in medicinal plants have been linked with antimalarial activity and could be the justification for the greater activity exhibited by these plant samples. *Citrus aurantiifolia*, *Paullina pinnata and Tetrapleura tetraptera* showed moderate antimalarial activity against the 3D7 parasite. Results from the studies against these aforementioned plants with moderate antiplasmodial activity were comparable to similar reports in (Lekana-Douki et al., 2011)(Maje et al., 2007) (Bapna et al., 2014). The 3D7 lab strain were observed to be less susceptible in the plants compared to the DD2 labs train. This observation was significant in *Paullinia pinnata* with IC_{50} values of 24.72±0.50 μg/mL and 14.84±0.14 μg/mL against 3D7 and DD2 respectively. *Azadirachta indica* showed similar potency (5.22±0.26 μg/mL) against both of the chloroquine sensitive and resistant strains. In a study by (Oseni & Akwetey, 2012) reported in their *in vivo* studies that the *Azadirachta indica* showed significant *in vivo* chemosuppresion (>70%). *Tetrapleura tetraptera* also showed almost the same activity against the two strains.

RBCs are human cell line or cellular model used to study potential interactions of the blood with other substance. Morphological changes, subsequent disruption of RBC membrane integrity and hemolysis could be used to determine the cytotoxicity of various compounds (Podsiedlik et al., 2020). Cytotoxicity at the preliminary stage is important in finding the possible toxicity of plant extracts or biologically active compounds segregated from plants. In preclinical studies of a compound, ability of a compound to discriminate between the pathogen and host is very important (Elhassanny et al., 2020) (Alven & Aderibigbe, 2019).This discrimination is crucial for the safety and efficacy of the compound. *In vitro* determination of hemolytic properties is a usual and significant technique in assessing the cytotoxicity of chemicals and drugs and its advantages are being affordable, easily obtainable and simple to use (Sæbø et al., 2023). All the plants showed high selectivity for the pathogen hence low or no toxicity against RBC as seen in Table 3. Extracts with high selectivity index offer the potential of been a safer therapy (Obbo et al., 2019).

When the selective index (SI) > 10, it is usually accepted that the compound or extract is non- toxic. *Azadirachta indica, Cryptolepis sanguinolenta, Alstonia boonei* showed the highest selectivity index (SI > 10). Showing the basis for their frequent use in several herbal antimalarial products in Ghana as reported in (Nortey et al., 2023). However, *Paullinia pinnata,*

Table 3: Cytotoxicity (CC_{50}) result against red blood cells

Extracts	CC_{50} Values (μg/mL)	Selective Index
PPE	129.63	5.24
ABE	109.50	12.93
CAE	100.23	2.87
AZE	118.90	22.78
TTE	102.72	6.85
CSE	92.68	15.14
ATS	72.62	> 100

Key: *Cryptolepis sanguinolenta* extract (CSE), *Azadirachta indica* extract (AZE), *Paullinia pinnata* extract (PPE), *Tetrapleura tetreptera* extract (TTE), *Citrus aurantifolia* extract (CAE), *Alstonia boonei* extract (ABE), Artesunate (ATS)

Citrus aurantiifolia and *Tetrapleura tetraptera* exhibited low selectivity index (SI < 10). There is a need for further study on the safety profiles of these plants with low selectivity indexes.

Conclusion

It can be inferred from the studies that all the six frequently used antimalarial plants possess a significant antimalarial activity against the lab strains; chloroquine sensitive (3D7) and chloroquine resistant (DD2) strains. These data provide rationale for their frequent use in several herbal antimalarial products on the Ghanaian market. A study on the combination effects of their polyherbal usage is underway. The data reported will guide how these combinations can be carried out or if it is more economical for practitioners to use the plants in isolation. The safety of the various plants being reported is crucial in guiding their use.

Authors contributions

Conceptualization, Samuel Korsah and Nathaniel Nene Djangmah Nortey.; methodology, John Antwi Apenteng and Felix Kwame Zoiku.; formal analysis, Samuel Korsah.; investigation, Nana Adwoa Boamah-Danso, Kanati Perry and Prince Antwi.; resources, Miriam Tagoe, Jessica Korsah and David Ntinagyei Mintah.; writing—original draft preparation, Samuel Korsah and Nathaniel Nene Djangmah Nortey.; writing—review and editing, Samuel Korsah.; supervision, Samuel Korsah.; project administration, John Antwi Apenteng. Funding acquisition; this work was jointly funded by authors with no external funding. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication.

Declaration of generative AI and AI-assisted technologies in the writing process

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