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In vitro antiplasmodial activity, LC-MS analysis, and molecular docking studies of bioactive compounds from *Tetrapleura tetraptera* (Fabaceae) fruits

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ABSTRACT

Malaria continues to be a major public health concern, particularly for children and pregnant women in areas where the disease is endemic. Developing safe and efficient antimalarial therapies to fight the disease is essential. Medicinal plants represent a potential source for the development of new antimalarial drugs. *Tetrapleura tetraptera* is a plant native to West Africa and traditionally used to treat several diseases including Malaria. Here, we investigated the antiplasmodial activities of *T. tetraptera* fruit extracts against the chloroquine-sensitive (*Pf*3D7) and chloroquine-resistant (*Pf*DD2) strains of *Plasmodium falciparum in vitro* using SYBR green assay. In addition, the antioxidant potential of the fruit extracts was also determined. LC-MS analysis was carried out to identify the bioactive compounds in the extracts. Molecular docking studies provide significant prima facie evidence of inhibition hence, to evaluate the potential inhibition of *Plasmodium falciparum* dihydroorotate dehydrogenase (*Pf*DHODH), a validated malaria drug target, the identified compounds were docked against *Pf*DHODH. Strong antiplasmodial activities were demonstrated by the ethyl acetate and ethanolic extracts of *T. tetraptera* fruit, with IC_{50} values of 16.12 ± 0.04 μg/mL and 2.06 ± 0.02 μg/mL against the *Pf*3D7 strain, respectively. In the DPPH radical scavenging experiment, the ethanolic extract revealed considerable antioxidant activity

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with an EC_{50} value of 0.21 ± 0.82 mg/mL. Seven bioactive compounds were identified in the extract using LC-MS analysis. N-Methyl-1H-indole-3-propanamide (I), Tazolol (II), and Isopentyl salicylate (III) were identified as potential inhibitors of *Pf*DHODH with high binding affinities ranging from -32.08 to -30.69 kcal/mol. The potential lead compounds also interacted with Gly181, Leu531, and Arg265, which are critical amino acid residues in the catalytic activity of *Pf*DHODH. These findings support the traditional use of *T. tetraptera* fruit extracts for the treatment of malaria and as promising avenues for antimalarial drug development.

Introduction

Malaria poses a significant health threat, particularly to vulnerable populations such as pregnant women and children [1]. Pregnant women in malaria-endemic regions face an increased risk of severe complications, including anemia and preterm birth, while young children, especially those under the age of five, bear the brunt of malaria's impact. A recent malaria report released by the World Health Organization (WHO) in 2021 revealed that there were approximately 241 million cases of malaria worldwide. The burden of malaria is particularly significant in the African region, accounting for a majority of global malaria cases and deaths. Specifically, 96 % of malaria-related deaths were reported in Africa, with children under the age of 5 comprising 80 % of these fatalities [2]. Efforts to combat this infectious disease require effective antimalarial drugs that are safe and accessible [3]. Malaria manifests with a range of symptoms, including high fever, chills, headache, and body aches [4]. Pregnant women and children may experience additional challenges, such as increased susceptibility to anemia and decreased immunity [5]. Timely recognition of these symptoms is crucial for prompt diagnosis and appropriate treatment, reducing the risk of severe illness and long-term consequences.

Artemisinin-based combination therapies (ACTs) are recommended as the first-line treatment for uncomplicated malaria, clearing the parasites from the bloodstream. In severe cases, hospitalization and intravenous antimalarial medications may be required. However, the emergence of drug-resistant strains highlights the need for continuous efforts in antimalarial drug discovery [6].

Plasmodium falciparum is dependent on de novo pyrimidine biosynthesis, which was discovered when the parasite's entire genome was sequenced [7]. As a result, the parasite is also vulnerable to the inhibition of dihydroorotate dehydrogenase (DHODH), a flavin-dependent mitochondrial enzyme that catalyzes the fourth and rate-limiting step in the de novo pyrimidine biosynthesis pathway [8]. As shown by previously reported X-ray co-crystal structures of human and plasmodial DHODHs bound to selective inhibitors, there are sizable differences between DHODHs from different species, both in the overall sequence and among the amino acids forming the inhibitor binding site [7]. One potential target for antimalarial drug development is *P. falciparum* Dihydroorotate Dehydrogenase (*Pf*DHODH), an enzyme crucial for pyrimidine biosynthesis in the malaria parasite. Inhibiting *Pf*DHODH disrupts the parasite's ability to replicate and survive [9]. Molecular docking studies offer a computational approach to evaluate the binding affinity and interactions between potential antimalarial compounds and *Pf*DHODH, aiding in the identification of lead compounds for further development [10,11].

Tetrapleura tetraptera, a robust perennial tree in the Fabaceae family, thrives in West Africa's tropical deciduous forest [6]. With its single stem, spreading branches, and dark green foliage, it stands 15–20 m tall [6]. The tree bears distinct fruits emitting a pungent odor known for its insect-repelling properties [6]. Referred to as "Prekese" in Asante (Ghana), *T. tetraptera* is popular in Africa, and its fruits are widely utilized in traditional medicine [12]. Different parts of the plant (leaves, fruits, roots and stem bark) are utilized for various reasons, and occasionally multiple parts are combined for treatment in order to obtain the desired effect [6]. Various researchers have noted that extracts from the plant contain bactericidal, fungal, schizonticidal, plamosicidal, molluscicidal, and helminticidal activity [6]. These fruits possess notable antioxidant properties, reducing oxidative stress associated with malaria [6].

In the pursuit of discovering new lead compounds for the development of novel medications, the phytochemical analysis of plant extracts and the identification of bioactive compounds play a crucial role [6]. Among the analytical techniques employed, liquid chromatography-mass spectrometry (LC-MS) holds significant importance in the identification and characterization of compounds present in plant samples [13].

Plants are rich sources of diverse phytochemical compounds, often referred to as secondary metabolites, which exhibit various health-enhancing properties [14]. These compounds can possess individual, additive, or synergistic actions, making them effective in treating specific illnesses [15].

LC-MS analysis enables the comprehensive profiling and characterization of the chemical composition of *T. tetraptera* fruit extracts. This analytical technique provides valuable insights into the phytochemical constituents responsible for the observed biological activities [10]. By correlating the presence of specific compounds with their antimalarial potential, researchers can identify promising candidates for further investigation.

This study aimed to delve into the therapeutic potential of *Tetrapleura tetraptera* fruits as a promising source of novel antimalarial agents while capitalizing on the antiplasmodial and antioxidant properties of *T. tetraptera* fruits. Employing liquid chromatography-mass spectrometry (LC-MS) for compound identification, the compounds present in the extract were identified and docked against *Pf*DHODH, to examine the inhibitory potential of the identified compounds. Insights drawn from this comprehensive and interdisciplinary approach could potentially contribute to the creation of novel, potent, and accessible antimalarial drugs, thus marking significant progress towards the global goal of malaria eradication.

Materials and methods

Materials

Sigma-Aldrich supplied ethyl acetate, dichloromethane, ethanol, petroleum ether, and methanol (USA). L-glutamine, SYBR Green I, and gentamycin were among the other reagents purchased from Sigma-Aldrich (USA). Both Rosewell Park Memorial Institute 1640 (RPMI) and Albumax II were purchased from Sigma Aldrich (USA). All additional chemicals and reagents came from reputable vendors. Both the chloroquine-sensitive strain (3D7) and the chloroquine-resistant strain (DD2) of *Plasmodium* parasites were received from the Department of Immunology at the Noguchi Memorial Institute for Medical Research for use in this experiment (NMIMR).

Sample preparation and extraction

T. tetraptera fruits were obtained at the Makola market (5.5555° N latitude and −0.2096° W longitude) in Accra, Ghana. A botanist from the University of Ghana's Department of Plant and Environmental Biology performed the authentication. The fruits were thoroughly cleaned before being dried in the oven for four hours at 65 °C [16]. Until needed, the dried fruits were pulverized and stored at 4 °C in an airtight container. For the extraction process, 50 g of the powdered fruit was first extracted using 500 mL of 99 % ethyl acetate (EtoAc) over the course of three days, resulting in an ethyl acetate extract. In a separate extraction, 50 g of the fresh powdered fruit was also extracted with 500 mL of ethanol (EtOH) for three days, leading to an ethanolic extract. The obtained crude extracts were then concentrated using a rotary evaporator.

Fractionation of the ethanolic crude extract

The active crude extract, derived from ethanol, underwent a sequential extraction process using a series of solvents. This process followed a specific order: starting with petroleum ether (S1), then dichloromethane (S2), followed by methanol (S3), and concluding with ethanol (S4) [30,31].

In vitro drug assay

Preparation of compounds

In order to attain a stock concentration of 1000 µg/mL, one milligram (1 mg) of each extract and sub-fractions were weighed and added to one milliliter (0.5 % DMSO). Before being used, the stock solution was stored in a −20 freezer after being vortexed to dissolve the compounds and filtered using a 0.2 µm pore filter unit. To obtain a workable solution of 100 µg/ml, the stock solutions were diluted ten times. The following concentrations of this working solution were obtained by serially diluting it nine times: 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.13 µg/mL, 1.56 µg/mL, 0.78 µg/mL, and 0.39 µg/mL [17].

Parasite culturing and preparation

The 3D7 chloroquine-sensitive strain of *P. falciparum* and the DD2 chloroquine-resistant strain, both obtained from the Department of Immunology of the Noguchi Memorial Institute for Medical Research, University of Ghana, were used to test the effectiveness of various compounds on both sexual and asexual parasite stages. The complete medium (CM) (10.44 g/liter RPMI 1640, 5.94 g/liter HEPES, 5 g/liter AlbuMAX II, 50 mg/liter hypoxanthine, and 2.1 g/liter sodium bicarbonate) was used to sustain continuous *P. falciparum* asexual cultures *in vitro* using a modified technique. Once a parasitemia of more than 5 % ring stages was attained, the parasites were maintained in the incubator with daily media changes while being cultured in O^+ RBCs. To obtain a synchronized ring stage, the culture was then treated with 5 % sorbitol. For the next few days, parasite growth was monitored by calculating the percentage (%) parasitemia using Giemsa-stained slides and a Light microscope at 100x magnification, until parasitemia of more than 5 % was observed. Uninfected blood was used to prepare a 14 ml parasite suspension with 2 % hematocrit and 1 % parasitemia in a full culture medium for plating [18].

Extract/Fraction plating and assay

A 96-well coastal plate was used to plate 100 microliters of each of the nine dilutions (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.13 µg/mL, 1.56 µg/mL, 0.78 µg/mL, and 0.39 µg/mL) in duplicate. As a conventional antimalarial control medication, 15 ng/mL of artesunate was serially diluted and plated alongside the compounds. From the second through the tenth treated wells, 100 parasite mixtures with 2 % hematocrit and 1 % parasitemia were introduced. The plates were arranged in a modular chamber and gassed for 5 min with a gas mixture of 5 % Oxygen, 5 % Carbon dioxide, and 90 % Nitrogen before being kept at 37 °C for 72 h. One hundred microliters of parasite mix without any medication were added to the 11th wells as a negative control [18].

SYBR green assay

After 72 h, the plates were collected, and the assay was stopped by adding 100 µL of lysing buffer containing SYBR Green to each well. This was done carefully and gently to prevent the formation of bubbles. The assay was then read using a FLUOstar OPTIMA Fluorometer plate reader with control software version 2.20 at 470 nm and 520 nm wavelengths after the plates had been incubated in the dark for 30 to 60 min [18].

Antioxidant assay (DPPH method)

The free radical scavenging activity by the DPPH technique with various modifications was used to assess the antioxidant activity of the plant extracts [16]. Equal quantities of each extract's varied concentrations (concentration range: 0–5 mg/mL) were mixed with a methanolic solution of DPPH (0.5 mM). At a wavelength of 517 nm, the absorbance was measured after 20 min of incubation at room temperature (Tecan Infinite M200 Pro plate reader, Austria). The following formula was used to determine each extract's effective concentration at 50 % (EC_{50}) value: The formula for% antioxidant activity is [(A0A1)/A0 x 100]. In this instance, A0 denotes the absorbance of the negative control (methanol), and A1 denotes the absorbance of the test sample when DPPH is present. As a reference point, ascorbic acid was used. The experiments were carried out in triplicate. The extract concentration that can result in 50 % free radical scavenging activity or the half-maximum effective concentration (EC_{50}) value, was obtained [16]

Cytotoxicity analysis on RBCs

The toxicity of the plant extracts to red blood cells was screened using a modified version of the tetrazolium-based colorimetric test [19]. A 96-well microtiter plate was divided into three different wells, and each well contained 100 microliters of the crude and aqueous extracts, with concentrations ranging from 6.25 μg/mL to 100 μg/mL. Each well was then filled with 100 μL of uninfected red blood cells. Plant extracts, culture media, and uninfected red blood cells were removed from the optical densities by running control experiments for each parameter independently alongside the main assays. Next, the plates were incubated at 37 °C for 72 h in a humidified incubator with 5 % O_2 and CO_2 before 20 μl of a 2.5 mg/ml MTT (in phosphate-buffered saline) solution was added to each well and the plate was incubated once more for 4 h. To dissolve any formazan that may have developed, 200 μl of Triton X-100 in acidified isopropanol was applied to each well after incubation. The absorbances of the wells were then read at 570 nm on the plate reader after the plates were left at room temperature and in the dark for 24 h [19]

LC-MS analysis

Metabolite profiling was done by LC-MS to identify various bioactive compounds present in the ethanolic extract of *T. tetraptera* fruits using An Agilent Technologies-Infinity 1290 LC System with a 4226A Autosampler, 4204A Quaternary Pump, G1316C Temperature Control Compartment (Agilent Technologies, USA). A previously developed protocol was used for the characterization of the compounds with slight modifications [10].

Molecular docking simulation

The CDOCKER docking algorithm, a high-precision module reliant on the CHARMm36 forcefield, was employed in this research for molecular docking studies, leveraging its integration in Biovia Discovery Studio Client v19 [20]. We obtained the 1.75 Å resolution crystal structure of *P falciparum* 3D7 dihydroorotate dehydrogenase (*Pf*DHODH) [PDB ID: 7KZ4 [21] from the Protein Data Bank which served as our receptor. The crystal structure, bound inhibitor—DSM705 [21], served as a virtual standard control in our molecular docking simulation. We stripped the receptor of all crystal water molecules, sparing only the active water molecule known to establish a water-mediated hydrogen bond crucial for the stability of ligand binding at the active site [21]. Subsequently, we prepared the receptor using the Prepare Protein module in the Biovia Discovery Studio Client v19. The following settings were used: loop building was disabled, protonation was enabled with a protein dielectric constant value of 10, pH for protonation was set to 8.8, ionic strength at 0.145, and an energy cutoff of 0.9. We defined the binding site using the prepared receptor. The bound DSM705 helped delineate an 8.8 Å radius binding site sphere with coordinates: $x = 16.64$, $y = -33.29$, $z = 35.24$. To validate the docking setup's capacity for predicting experimentally significant compound binding conformations, DSM705 was re-docked into the binding pocket of the prepared receptor. The 3D conformers of the compounds identified in the ethanolic extract of *T. tetraptera* fruits, derived from the PubChem compound repository were docked into the prepared *Pf*DHODH 's binding pocket [21]. Ten docking poses were produced per compound, with each pose undergoing further scrutiny via the analyze ligand poses module in Biovia Discovery Studio Client v19. Any poses indicating one or more unfavorable interactions were eliminated from subsequent analysis. The compound poses with favorable interaction counts were then evaluated using binding energy scoring functions: *LigScore Dreiding 2* [22], *PLP2* [23], and *PMF04* [24]. We selected the optimal compound pose for each compound based on high binding energy scores across all scoring functions (*CDOCKER energy, LigScore Dreiding 2, PLP2*, and *PMF04*), complemented by a visual assessment of appropriate binding orientation. All protein-ligand interactions were performed using Biovia Discovery Studio Client v19 and UCSF ChimeraX [25].

Statistical analysis

Each product was tested in a triplicate and herbal product concentration that inhibits the asexual *P. falciparum* parasite by 50 % (IC_{50}) or concentrations at which 50 % cytotoxic effect occurred on RBCs (CC_{50} values) were 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

In-vitro anti-plasmodial and cytotoxic activity of extracts of T. tetraptera fruit

The antiplasmodial activity of herbal extracts against asexual parasites that cause the disease has been classified as "good" (IC_{50} 10 μg/mL), "moderate" (IC_{50} values from 10 μg/mL to 50 μg/mL), "low" (IC_{50} values from 50 μg/mL to 100 μg/mL), and "inactive" (IC_{50} values > 100 μg/mL) [26].

The *in vivo* antiplasmodial potency of the ethanol fruit extract of *T. tetraptera against Plasmodium berghei has* been evaluated and confirmed [27]. Subsequently, both the ethanolic and ethyl acetate crude extracts from the fruit were evaluated for *in vitro* antiplasmodial activity against the chloroquine-sensitive P. falciparum 3D7 strain. The ability of *T. tetraptera* fruit extracts to inhibit the growth of *Pf*3D7 and *Pf*DD2 strains *in vitro* is demonstrated in (Fig. 1A) together with the resultant fractions from the ethanolic extract. When compared to the ethyl acetate extract (TT EA), the *T. tetraptera* (TT ETOH) ethanolic extract demonstrated greater antiplasmodial efficacy with IC_{50} values of 16.12 ± 0.04 and 2.06 ± 0.02 μg/mL in *Pf*3D7 strain respectively. Methanol extracts from stem bark and roots have been shown to effectively inhibit the growth of the *P. falciparum* 3D7 strain, demonstrating substantial antiplasmodial activity [6]. This outcome prompted subfraction analyses of the ethanolic extract to determine the fraction that might be responsible for the activity. The ability of fractions to inhibit parasite growth varied depending on dose, with dichloromethane fraction (S2) showing the greatest ability to do so in both strains (Fig. 1B and 1C). The lowest IC_{50} for the *Pf*3D7 strain was achieved by the ethanolic extract, dichloromethane fraction (S2), methanolic fraction (S3), and ethanolic fractions (S4). In contrast, the ethyl acetate extract and petroleum ether fraction (S1) had a moderate antiplasmodial activity with IC_{50} values between 10 and 20 μg/mL (Fig. 1B). Ethyl acetate extract (TT EA) and petroleum ether fraction (S1) demonstrated moderate antiplasmodial activity with IC_{50} values between 10 and 20 μg/mL, but dichloromethane fraction (S2), the methanolic fraction (S3), and ethanolic fractions (S4) had the least IC_{50} *Pf*DD2 and the strongest antiplasmodial activity (Fig. 1C).

While adult RBCs utilized in the study lack nucleic acids, the SYBR Green assay includes binding of the SYBR Green dye to double-stranded DNA, which becomes fluorescent when bound and can be detected [18]. The MTT assay was used to determine the selective activity of the extract as described in [18]. The MTT assay, in contrast, relies on the viability of the enzymes present in the RBCs to transform the yellowish tetrazolium into the dark purple complex formazan. The specific enzyme or enzymes responsible for the conversion are still unknown. The only way the conversion would be conceivable is if the extracts they are treated with do not cause the cells to die. The viability of red blood cells (RBCs) following exposure to TT ETOH, TT EA, and the partitioned fractions (S1, S2, S3, and S4) of ethanolic extracts from *T. tetraptera* fruits is presented (Table 1). At all of the highest concentrations (100 μg/mL), the extracts and fractions had a cell viability of greater than 50 %. The ethanolic extract had the highest selective Index (SI) value in the *Pf*3D7 strain when compared to the TT EA. Similarly, among the fractions, in both the *Pf*3D7 and *Pf*DD2 strains, fractions S2, S3, and S4 showed a greater SI value than S1 did (Table 1). The selectivity index shows how well the extracts and fractions are cytotoxic to Plasmodium parasite cells as opposed to healthy red blood cells.

In vitro antioxidant activity of the extracts of T. tetraptera fruits

Due to reactive oxygen and nitrogen species created by the host defense system during multiple processes, *P. falciparum*-infected

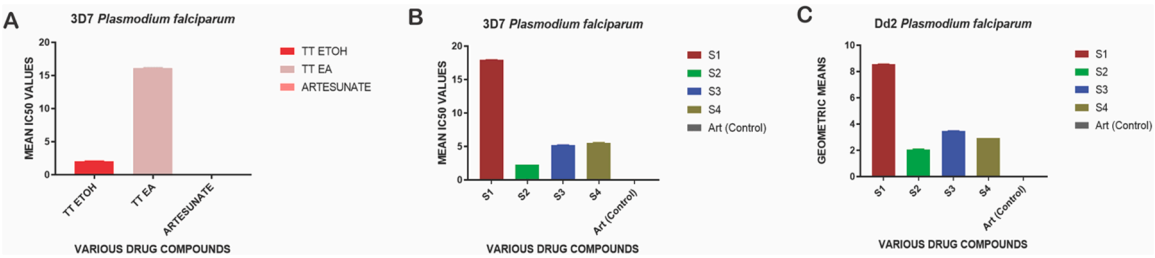

Fig. 1. Anti-plasmodial effects of *T. tetraptera* fruit extracts and extract fractions.
All experiments were performed in triplicate.
Panel A showcases the anti-plasmodial activity of the ethanolic (TT EtOH) and ethyl acetate (TT EA) extracts derived from *T. tetraptera* fruits. TT EtOH exhibits an IC_{50} value of 2.06 ± 0.02 μg/mL, while TT EA demonstrates an IC_{50} value of 16.12 ± 0.04 μg/mL against the chloroquine-sensitive strain (3D7).
In Panel B, the petroleum ether extract (S1), dichloromethane (S2), methanolic (S3), and ethanolic (S4) extracts obtained from the ethanolic extract of *T. tetraptera* fruits are evaluated. Against the chloroquine-sensitive strain (3D7), S1 shows an IC_{50} value of 17.93 ± 0.0121 μg/mL, S2 exhibits an IC_{50} value of 2.30 ± 0.0058 μg/mL, S3 demonstrates an IC_{50} value of 5.21 ± 0.0135 μg/mL, and S4 presents an IC_{50} value of 5.50 ± 0.071 μg/mL.
Panel C focuses on the same extracts and fractions tested against the chloroquine-resistant strain (DD2). S1 reveals an IC_{50} value of 8.56 ± 0.0076 μg/mL, S2 shows an IC_{50} value of 2.08 ± 0.0088 μg/mL, S3 exhibits an IC_{50} value of 3.48 ± 0.0077 μg/mL, and S4 presents an IC_{50} value of 2.95 ± 0.0043 μg/mL.
These results provide evidence of the anti-plasmodial activity of *T. tetraptera* fruit extracts and their fractions against both chloroquine-sensitive and chloroquine-resistant strains. The significant findings support the potential of *T. tetraptera* as a source of compounds for the development of anti-malarial therapies.

Table 1
Various IC50 values on RBCs and selectivity indices (SI) of the extracts and standard drug.

Sample	IC_{50} Value ± Std error (μg/mL)	Si Values [3D7]	Si Values [Dd2]
TT ETOH	≥100	≥48.5	N/A
TT EA	≥100	≥6.20	N/A
S1	≥100	≥5.57	≥11.68
S2	≥100	≥43.48	≥48.08
S3	≥100	≥19.19	≥28.74
S4	≥100	≥18.18	≥33.90
ART (Artesunate)	≥100	N/A	N/A

red blood cells are constantly exposed to oxidative stress [28]. The primary endogenous antioxidant source for scavenging free radicals in the body is GSH. Thus, it is essential for preserving homeostasis, especially in pathological circumstances. As a result, the extracts' antioxidant activity was assessed using the free radical scavenging assay. Red blood cells with infections are continuously subject to oxidative stress caused by both external and endogenous mechanisms. Hence, having antimalarial drugs that also have antioxidant properties is beneficial in conditions like malaria which heavily expose erythrocytes to oxidative stress. Hence, the extracts' total antioxidant levels were assessed [28].

The ethanolic extract (TTETOH) and ethyl acetate (TTEA) of *T. tetraptera* fruits were shown to have DPPH radical scavenging properties, with EC_{50} values of 0.21 ± 0.82 mg/mL and 3.93 ± 0.82 mg/mL, respectively (Fig. 2).

LC-MS

In this current work, LC-MS analysis was performed to identify various phytochemicals present in the ethanol extract of *T. tetraptera* fruits. Seven bioactive compounds belonging to their chemical group were tentatively identified using the mass data and MS spectra from Personal Compound Database Library. Other public databases such as ChemSpider [10], and Pubchem [10] were also used. The list of compounds and their molecular mass, molecular formula, CAS number, and their retention times are represented (Table 2) and the chromatogram (Fig. 4).

N-Methyl-1H-indole-3-propanamide belongs to the Indole alkaloids group, in particular, and has been extensively investigated for their potential as antimalarial agents. Examples include natural products such as quinine and artemisinin, which have been used for centuries in the treatment of malaria. These compounds exhibit potent antimalarial activity by interfering with various stages of the parasite's life cycle. Analogs of N-Methyl-1H-indole-3-propanamide have been reported to have antimalarial activities.

Tazolol also known as 1-Isopropylamino-3-(2-thiazoloxy)−2-propanol is a chemical compound with a unique molecular structure that combines an isopropylamino group, a thiazole ring, and a hydroxyl group. This compound exhibits many potential therapeutic properties [29]. In the context of antimalarial activity, thiazole-containing compounds have shown promising results against *P. falciparum*, the parasite responsible for malaria [29]. In terms of *in vitro* antimalarial activity, modifications to the N-aryl amide group connected to the thiazole ring are the most significant, producing compounds with high antimalarial potency and minimal cytotoxicity in HepG2 cell lines [29].

Isopentyl salicylate belongs to the chemical group salicylic acid. A derivate of salicylic acid, 6-(8′Z-pentadecenyl)-salicylic acid, was isolated from *Viola websteri* and shows a mild anti-malarial effect in mice, which could be used for malaria therapy [30].

Tartronate semialdehyde belongs structurally to the class of compounds known as aldehydes. Aldehyde derivatives have been investigated for their potential antimalarial activity, offering a promising avenue for the development of new antimalarial drugs. [31] examined a series of aldehyde derivatives and evaluated their antimalarial activity against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* [32]. The results showed that certain aldehyde derivatives exhibited significant inhibitory effects on the growth of the malaria parasite, with improved potency compared to the reference drug chloroquine. Small-molecule aldehydes and amides have also been discovered to be effective plasmodial cysteine protease inhibitors [32].

Flufenacet belongs to the class of herbicides known as acetamide herbicides. Pendimethalin; a dinitroaniline and a member of the class inhibits proliferation in *P. marinus, trypanosomatids,* and *P. falciparum* by inhibiting microtubules during parasite development [33].

Diazoxide is a pharmaceutical compound that belongs to the class of benzothiadiazine. These are structurally similar to the thiazine-derived 1,1-dioxothiazine scaffold. Thiazine derivatives have been discovered to have antimalarial properties [34].

Molecular docking

Our *in-vitro* findings presented here reveal that the extract of *T. tetraptera* fruits exhibits anti-malarial activity, particularly against the *P. falciparum* strain widely known to cause malaria in Sub-Sahara Africa [35]. Progress in understanding the molecular targets of malaria therapeutics, specifically those caused by the *P. falciparum* strain, has uncovered the enzyme dihydroorotate dehydrogenase as a prime target [21]. Notably, several potent inhibitors of this enzyme specific to the *P. falciparum* 3D7 strain have been identified, with detailed structural studies showcasing their binding to the enzyme [21]. With this knowledge, we formed the hypothesis that compounds within *T. tetraptera* fruit extract could be the active agents responsible for its anti-malarial effect, likely due to their inhibition of *Pf*DHODH. Utilizing the wealth of available structural data on *Pf*DHODH bound to various inhibitors [21], we investigated the binding potential of all seven compounds identified from *T. tetraptera* fruits using LC-MS to this enzyme through molecular docking

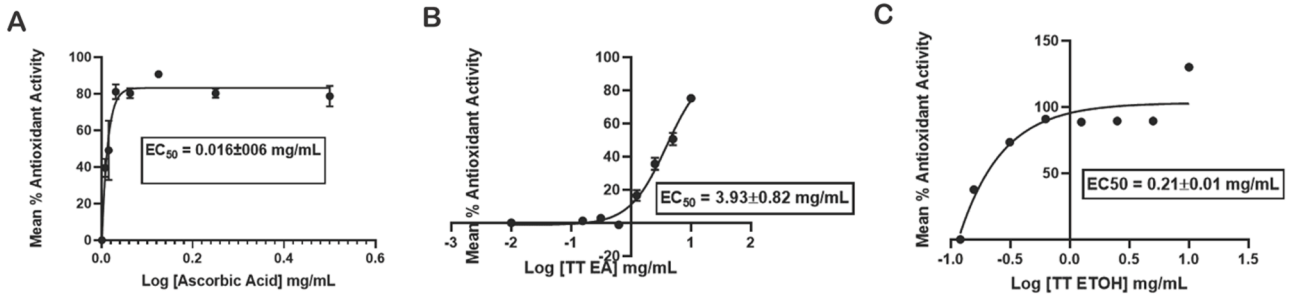

Fig. 2. Antioxidant activity of ethyl acetate and ethanolic extracts of *T. tetraptera* fruits.
Panel A presents the antioxidant activity of ascorbic acid, serving as a standard reference compound, with an EC_{50} value of 0.016 ± 0.006 mg/mL.
In Panel B, the ethyl acetate extract of *T. tetraptera* fruits (TT EA) exhibits an EC_{50} value of 3.93 ± 0.82 mg/mL, indicating its antioxidant potential.
Panel C showcases the antioxidant activity of the ethanolic extract of *T. tetraptera* fruits (TT EtOH), with an EC_{50} value of 0.21 ± 0.82 mg/mL.
These results highlight the antioxidant properties of the ethyl acetate and ethanolic extracts obtained from *T. tetraptera* fruits. The findings suggest that these extracts possess significant antioxidant activity, potentially contributing to their therapeutic applications and nutritional value.

Table 2
List of compounds identified from ethanolic extracts of *T. tetraptera* fruits using LC-MS.

ID	Compound	Formula	Mass	RT	Diff (DB, ppm)
I	N-Methyl-1H-indole-3-propanamide	C12 H14 N2 O	202.1	9.084	−4.66
II	Tazolol	C9 H16 N2 O2 S	216.1	12.08	−2.87
III	Isopentyl salicylate	C12 H16 O3	208.1	8.578	0.26
IV	Tartronate semialdehyde	C3 H4 O4	104	2.27	−1.73
V	4-Chlorosalicylic acid	C7 H5 Cl O3	172	7.907	1.99
VI	Flufenacet	C14 H13 F4 N3 O2 S	363.1	0.895	−1.16
VII	Diazoxide	C8 H7 Cl N2 O2 S	230	6.665	4.43

simulation studies. In the present study, we utilized the 1.75 Å resolution X-ray structure of *Pf*DHODH (PDB: 7KZ4), bound with the inhibitor DSM705 [21], to assess whether the compounds identified in *T. tetraptera* extracts potentially interact with this enzyme. The binding pocket was demarcated as the region within 8.8 Å of the bound DSM705 molecule, a site recognized for its propensity to bind inhibitors (Fig. 3A). To ascertain the validity of our docking simulations in generating experimentally pertinent ligand conformations while maintaining the rigidity of the enzyme's solved secondary structure, we first isolated the chemical structure of DSM705 and re-docked it into the defined pocket. The resulting docked DSM705 exhibited a root-mean-square deviation (RMSD) of 0.36 Å from the crystallographically resolved structure of DSM705 (Fig. 3B). This result provides compelling evidence of the reliability of our docking simulation approach in forecasting experimentally relevant conformations.

In order to comprehensively evaluate the binding affinity of various compounds, we utilized an integrative approach. Rather than relying on a single scoring function, multiple scoring functions were utilized to provide a more comprehensive understanding of the binding affinity scores. The scoring functions employed included *CDOCKER energy* [20] *y, Ligscore2* [22], *Piecewise Linear Potential 2 (PLP2)* [23], and *Potential of Mean Force 04 (PMF04)* [24]. *CDOCKER energy* was first used to predict the binding affinities of the compounds. This scoring function is based on interaction energy plus ligand strain, with more negative values indicating stronger receptor-ligand binding affinities. Then, the binding energies of the docked poses were further assessed using *Ligscore2, PLP2*, and *PMF04*. *LigScore2* and *PLP2* are empirical docking scoring functions designed for quick and straightforward prediction of receptor-ligand binding affinities [22,23]. For *LigScore2*, higher scores correspond to stronger receptor-ligand binding affinities. On the other hand, *PLP2*, like *CDOCKER energy*, indicates stronger binding with more negative scores. *PMF04*, derived from the statistical analysis of 3D protein-ligand interaction structures, was also used [24]. This knowledge-based scoring function similarly indicates a stronger receptor-binding affinity with more negative scores. In terms of *CDOCKER energy*, N-Methyl-1H-indole-3-propanamide (I) exhibited the strongest binding affinity, followed closely by Tazolol (II) and Isopentyl salicylate (III) (Table 3). DSM705, despite being

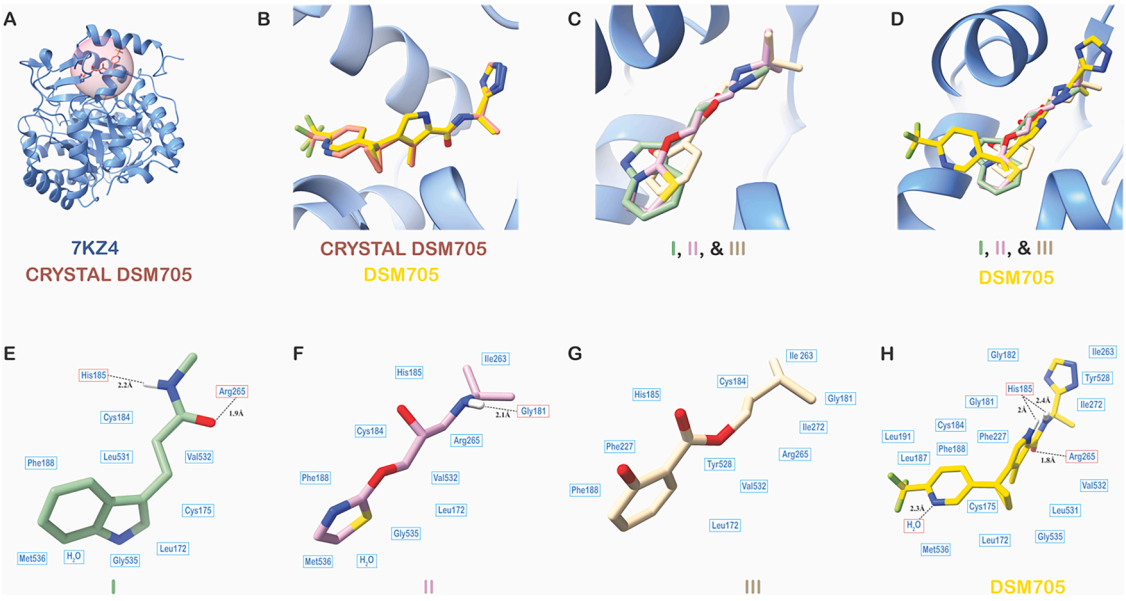

Fig. 3. Docking results of DSM705 and potential inhibitors of *Pf*DHODH. A: The defined binding pocket (sphere, colored pale red) of *Pf*DHODH (PDB ID: 7KZ4, colored cornflower blue). **B**: Superimposition of DMS705 (colored, salmon) in the crystal structure of *Pf*DHODH (colored cornflower blue) and the docked pose of DMS705 (colored, yellow). C & D: The binding poses of docked DMS705 (shown as a stick, colored yellow) and the potential inhibitors of *Pf*DHODH (colored cornflower blue). The docked potential inhibitor compounds are depicted as sticks and colored dark sea green (I), plum (II), and wheat (III). **E – H**: Detailed interactions of the docked potential inhibitors of *Pf*DHODH and DMS705 in the binding pocket. The critical residues for ligand binding are shown in a rectangle framed box; hydrogen bonds are black dashed lines, and the docked potential inhibitor compounds are depicted as sticks and colored dark sea green (I), plum (II), and wheat (III) as well as yellow (DMS705).

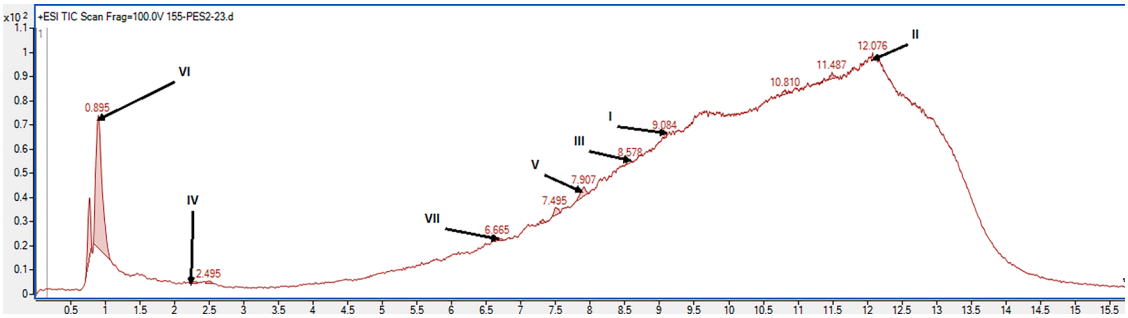

Fig. 4. chromatogram of ethanol extract of *T. tetraptera* fruits
Mass spectrum of identified compounds.

Table 3
In silico binding energies for all compounds docked into the *P. falciparum* 3D7 dihydroorotate dehydrogenase (PDB ID: 7KZ4).

ID	Name	Binding Affinity Energies (*Kcal/mol*)			
		CDOCKER energy	*LigScore2 Dreiding*	*PLP2*	*PMF04*
I	N-Methyl-1H-indole-3- propanamide	−32.08	5.09	−72.49	−30.15
II	Tazolol	−30.72	4.88	−71.63	−33.12
III	Isopentyl salicylate	−30.69	4.73	−67.87	−39.06
DSM705	DSM705	−28.86	7.25	−131.13	−115.99
IV	Tartronate semialdehyde	−22.86	3.21	−39.31	−21.17
V	4-Chlorosalicylic acid	−22.22	4.36	−64.89	−31.93
VI	Flufenacet	−20.20	4.95	−75.52	−57.85
VII	Diazoxide	−9.39	3.99	−37.77	−17.14

the benchmark compound, had a less negative (thus, less favorable binding) *CDOCKER energy* than these compounds (Table 3). When considering LigScore2, DSM705 had the highest score, indicating the strongest binding affinity. N-Methyl-1H-indole-3-propanamide (I), Tazolol (II), and Flufenacet (VI) also showed relatively strong binding affinities (Table 3). For *PLP2* and *PMF04* scores, DSM705 demonstrated significantly stronger binding affinities than the other compounds, as indicated by its much more negative scores. This is consistent with its role as a known inhibitor [21].

Previous studies have shown that a more negative binding energy (stronger binding affinity) does not indicate better inhibition [36, 37] hence N-Methyl-1H-indole-3-propanamide (I), Tazolol (II), and Isopentyl salicylate (III) plausible inhibitors of *Pf*DHODH as they interact with residues involved in the catalytic activity of *Pf*DHODH.

Despite the distinct chemical structures of N-Methyl-1H-indole-3-propanamide (I), Tazolol (II), and Isopentyl salicylate (III), these compounds demonstrate a commonality in their binding pose with the reference inhibitor, DSM705, suggestive of a shared binding mechanism (Fig. 3C, D). It's noteworthy that the presence of the 4-CF_3-pyridinyl moiety in DSM705 could account for the observed variations in binding affinities with these compounds, potentially due to its role in facilitating additional stabilizing interactions within the binding pocket (Fig. 3D). Our docking studies elucidated key interactions between the aromatic moieties of the compounds and residues within the *Pf*DHODH binding pocket. These interactions, in combination with robust hydrophobic contacts, are crucial for ligand binding and efficacy (Fig. 3E–H). We observed that N-Methyl-1H-indole-3-propanamide (I) forms a similar hydrogen bond with His-185 (at a distance of 2.2 Å) and engages in a 1.9 Å hydrogen bond with Arg-265 via its carbonyl moiety (Fig. 3E). This parallels the interaction profile of the benchmark compound, DSM705, which forms bifurcated hydrogen bonds of distances 2 Å and 2.4 Å with His-185, and a hydrogen bond with Arg-265 (at a distance of 1.8 Å) (Fig. 3H). This concurrence with experimentally observed interactions in the crystal structure of *Pf*DHODH-bound DSM705 reinforces the predictive validity of our docking simulations [21].

Tazolol (II) forms a hydrogen bond with Gly-181 (at a distance of 2.1 Å), an interaction similarly noted in other potent inhibitors of *Pf*DHODH (Fig. 3F) [21]. In contrast, Isopentyl salicylate (III) lacks hydrogen bond interactions but compensates with substantial aromatic and hydrophobic interactions that play a critical role in stabilizing an inhibitor within the binding pocket (Fig. 3G) [21]. Corroborating our findings, mutagenesis studies have revealed that specific point mutations (Gly181Cys, Leu531Phe, Arg265Gly, Cys276Tyr, and Glu182Asp) within *Pf*DHODH significantly impact binding affinity, resulting in diminished inhibitor potency [38]. Overall, although the absence of experimental data hinders direct validation of our docking simulations, the current findings strongly suggest that N-Methyl-1H-indole-3-propanamide (I), Tazolol (II), and Isopentyl salicylate (III) could serve as potential inhibitors of *Pf*DHODH. Consequently, the antimalarial activity observed in *T. tetraptera* extracts may be attributed to these compounds targeting *Pf*DHODH enzymatic activity.

Conclusion

The findings of this study highlight the remarkable antiplasmodial properties exhibited by the ethanolic extract and the

subfractions from the ethanolic extract of *T. tetraptera* fruit. The antiplasmodial activity against both chloroquine-sensitive and resistant strains of *P. falciparum* underscores the potential significance of these extracts in combating malaria. Furthermore, the ethanolic extract demonstrated superior antioxidant capabilities, suggesting a multifaceted therapeutic potential. The identification of seven bioactive compounds through LC-MS analysis opens an exciting avenue for understanding the mechanisms underlying the observed antimalarial activity. Notably, N-Methyl-1H-indole-3-propanamide (I), Tazolol (II), and Isopentyl salicylate (III) have emerged as promising candidates for inhibiting *Pf*DHODH, a validated target for malaria. The results underscore the therapeutic potential of *T. tetraptera* fruit extracts in the realm of malaria. Beyond their antiplasmodial capabilities, the identified compounds, especially N-Methyl-1H-indole-3-propanamide, Tazolol, and Isopentyl salicylate, present a promising foundation for novel antimalarial drug development, with the potential to target specific interactions and offer multifaceted therapeutic benefits.

However, several crucial considerations warrant attention as we move forward. Firstly, the strain specificity of the results necessitates broader investigations across various Plasmodium species and strains to ascertain the generalizability of these outcomes. While the identified bioactive compounds hold promise, their transformation into effective antimalarial drugs mandates rigorous evaluation encompassing safety, optimal dosage determination, and comprehensive pharmacological studies. In light of these findings, it is imperative to emphasize the pressing need for continued research. Further exploration into the *in vivo* effectiveness and safety profiles of these extracts and their bioactive components is essential to establish their therapeutic potential. This study provides a compelling foundation upon which subsequent studies can build, paving the way for a potential breakthrough in the development of novel antimalarial agents.

Ethical considerations

There were no ethical considerations for this study.

CRediT authorship contribution statement

Sherif Hamidu: Conceptualization, Methodology, Software, Data curation, Writing – original draft. **Latif Adams:** Conceptualization, Methodology, Software, Data curation, Writing – original draft, Writing – review & editing. **Patrick Kwabena Oduro:** Conceptualization, Methodology, Software, Data curation, Writing – original draft. **Felix Kwame Zoiku:** Data curation, Investigation. **Abigail Aning:** Data curation, Investigation. **Eunice Ampem-Danso:** Data curation, Investigation. **Janet Abena Ampofo:** Data curation, Investigation. **Michael Afiadenyo:** Data curation, Visualization, Software. **Siobhan Moane:** Validation, Methodology. **Michelle McKeon Bennett:** Validation, Methodology. **Kwadwo Asamoah Kusi:** Writing – review & editing, Supervision, Validation. **Dorcas Obiri -Yeboah:** Writing – review & editing, Supervision, Validation.

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.

Data availability

The datasets used or analyzed during the current study are available from the corresponding author upon request.

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The *Plasmodium* parasites (the chloroquine-sensitive strain (3D7) and the chloroquine-resistant strain (DD2)) were received from the Department of Immunology at the Noguchi Memorial Institute of Medical Research for use in this experiment (NMIMR).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sciaf.2023.e01944.

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