**Phytochemical screening, antiplasmodial and antioxidant activities of *Combretum rhodanthum* extracts**

**Abstract**

**Background:** *Plasmodium falciparum* is responsiblefor over 90% of malaria cases and the vast majority of malaria-related deaths globally. Oxidative stress, an imbalance between free radicals and antioxidants, plays a key role in the malaria pathogenesis, potentially leading to increased severity of the disease. Growing evidence has shown that *Plasmodium* species have become resistant to currently available drugs. Thus, there is a pressing need to search for effective treatments against malaria. Because of drug resistance to antimalarial agents, the World Health Organization recommends the use of medicinal plant extracts as alternative therapy for malaria. *Combretum rhodanthum* is such a plant, which is traditionally used to treat malaria symptoms. **Objective:** This study sought to investigate the antiplasmodial and antioxidant activities of *Combretum rhodanthum* extracts. **Methods:** To this end, the ethanol extraction of *Combretum rhodanthum* was carried out by maceration. The as-prepared extract was partitioned with hexane, ethyl acetate and n-butanol. The n-butanol fraction, which was found to be the most potent antiplasmodial fraction was chromatographed to yield eight sub-fractions. Antiplasmodial activity was performed *in vitro* on chloroquino-sensitive (*Plasmodium falciparum* 3D7; Pf3D7) and multi-resistant (*Plasmodium falciparum* Dd2; PfDd2) strains of *P. falciparum* using the SYBR Green-I assay. Antioxidant activity and phytochemical analysis of the most active extracts were performed using standard methods. The acute toxicity of the active extract was studied according to the OECD (Organisation for Economic Co-operation and Development) protocol, guideline number 423. **Results:** Upon incubation of *C.* *rhodanthum* extracts with *Pf*Dd2 and *Pf*3D7, mean inhibitory concentrations ranged from 10.21 to 24.73 μg/mL and from 18.48 to 39.56 μg/mL, respectively. The n-butanol extract, which was chromatographed yielded eight antiplasmodial sub-extracts, the most potent being subfraction [D] (90% ethyl acetate/10% methanol) with IC50 values of 5.30 and 11.21 μg/mL on *Pf*Dd2 and *Pf*3D7, respectively and selectivity for the multidrug-resistant strain (Resistance index<1). Phytochemical analysis of the ethanol extract showed the presence of tannins, terpenoids, phenolic compounds, and glycosides. Upon DPPH and ABTS assays, extracts and fractions scavenged free radicals with mean radical scavenging concentrations (SC50) lower than 39 μg/mL. Moreover, *C.* *rhodanthum* extracts reduced Fe3+ into Fe2+ with mean reduction concentration (RS50) less than 26.1 μg/mL. Acute toxicity study revealed no signs of toxicity in Wistar rats at 2000 mg/kg. **Conclusion:** This novel contribution demonstrates the antiplasmodial and antioxidant activities of *Combretum rhodanthum* extracts, thus substantiating the ethnomedicinal use of this plant in treating malaria-like symptoms.

**Key words:** Antiplasmodial activity, Antioxidant activity, *Combretum rhodanthum*, Acute toxicity, *Plasmodium falciparum*.

**1. Introduction**

According to the latest report from the World Health Organisation (WHO), as high as 263 million malaria cases occurred in 2023 with 597 000 deaths worldwide (WHO, 2023). Malaria is caused by *Plasmodium* species, and is transmitted through infected female anopheles’ mosquitoes. During the mosquito blood meal, sporozoites are released into the bloodstream and then migrate to the liver, where they infect hepatocytes and develop into exoerythrocytic forms, triggered by thousands of merozoites. This stage of the parasite life cycle in humans lasts for 7-14 days, and is clinically silent. From the hepatocytes, merozoites are released from the hepatocytes into the bloodstream to invade red blood cells (erythrocytes), thus initiating the asexual erythrocytic cycle, that cause the disease symptoms (Haldar et al., 2018; Ross and Fidock, 2019; Hodoameda et al., 2022). Among these species, *P. falciparum* is the most virulent causing higher mortality, especially in sub-Saharan Africa, a region with the highest number of malaria cases in the world (White, 2022).

Although there are a number of currently available antimalarial drugs, resistance by *Plasmodium* species has rendered the majority of these therapies inefficient. For instance, the first case of resistance to artemisinins was described in Cambodia in 2006, and has then spread across many parts of Asia (Dondorp et al., 2009 ; Murray et al., 2012). Nagendrappa et al. (2017) have also reported contraindications for antimalarial drugs, such as primaquine, atovaquone, and doxycycline in pregnant women and children. Thus, there is a pressing need to search for effective and safe treatments against malaria.

 In the last half-century, plant-derived natural compounds have played a major role in the discovery of drugs further developed by pharmaceutical industries (Newman and Cragg, 2012). Furthermore, quinine and artemisinin for example, were isolated from cinchona tree and *Artemisia annua*, respectively. A number of synthetic derivatives were also prepared from these pharmacophores. A number of *Combretum* species, including *Combretum micranthum* and *Combretum molle*, are used traditionally to treat malaria symptoms. Accumulated evidence has shown the *in vitro* antiplasmodial activity of *Combretum micranthum* and *Combretum molle* (de Morais Lima et al., 2012 ; Anato and Ketema, 2018 ; Jean Noel et al., 2020, Namadina et al., 2024). However, the antimalarial activity of *Combretum rhodanthum* has not yet been investigated. Thus, the present study aims to evaluate the antiplasmodial and antioxidant activities of *Combretum rhodanthum.*

**2. Materials and methods**

**2.1. Materials**

**2.1.1. Plant collection and identification**

Vines of*C. rhodantum* were collected on 27th December 2022 at the Bafou village (West Region of Cameroon). The identification of the plant was done by Mr Nana Victor, a Botanist of the National Herbarium of Cameroon (Yaounde, Cameroon), where a specimen (voucher number: 3444/HNC) was deposited. The plant was further taken to the Research Unit of Environmental and Applied Chemistry of the University of Dschang (Dschang, Cameroon). Next, the plant was washed to eliminate waste and other contaminants, then shade dried at room temperature to remove water and moisture. Subsequently, the dried plant was coarsely powdered for further use.

**2.1.2. *Plasmodium falciparum* strains**

 Chloroquine-sensitive (3D7) and multidrug-resistant (Dd2) strains of *P. falciparum*, which were supplied by BEI-Resource (Biodefense and Emerging Infections Research Resources Repository) were used to assay the antiplasmodial effects of *C. rhodantum* extracts. These strains are maintained in continuous culture at the Laboratory for Phytobiochemistry and Medicinal Plants Studies at the University of Yaounde 1.

**2.1.3. Mammalian cells**

Murine macrophage Raw cells 264.7 cell line, which was gifted by the Noguchi Memorial Institute for Medical Research (University of Ghana) was used to evaluate the *in vitro* cytotoxicity of active extract and fractions from *C. rhodantum*. The Raw cells were maintained in continuous culture at the Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, University of Yaounde I.

**2.2. Methods**

**2.2.1. Preparation of the extract and fractions**

The maceration of 3.2 Kg of *C. rhodanthum* powder in 16 litres of 96% ethanol for 24 hours afforded the ethanol extract. This process was repeated thrice to allow complete extraction of compounds. After that, the preparation was filtered using a paper (Whatman number 1), and subsequently evaporated under reduced pressure by using a rotary evaporator (Rotavapor BUCHI R-100) at 50°C to yield 220 g of crude extract. Next, the extract was stored at 4°C until future use. The yield of the reaction was ultimately calculated.

The ethanol extract of *C. rhodanthum* was partitioned using different solvents of increasing polarity. In brief, the ethanol extract (210 g) was suspended in 600 mL of distilled water. Next, the preparation was successively partitioned with hexane, ethyl acetate and *n*-butanol to yield after solvent evaporation, hexane, ethyl acetate and *n*-butanol fractions, as well as an aqueous residue (Figure 1), which were stored at 4°C for subsequent use in biological assays.



**Figure 1 :** Protocol of extraction and solvent-solvent partitioning of *Combretum rhodanthum*.

**2.2.2.  Fractionation of *C. rhodanthum*****extract**

After screening *C. rhodanthum* extracts against *Plasmodium falciparum*, the *n*-butanol fraction revealed the lowest IC50 value, and was submitted to flash chromatography using solvent systems of increasing polarity, such as hexane-ethyl acetate, ethyl acetate, and ethyl acetate-methanol for elution. Thus, fractions (500 mL each) were collected, evaporated under reduced pressure and pooled on the basis of their similarity on the thin layer chromatography (TLC) plate to obtain eight (08) sub-fractions (A to H) : [A] (50% hexane-50% ethyl acetate) ; [B] (100% ethyl acetate) ; [C] (95% ethyl acetate-5% methanol) ; [D] (90% ethyl acetate-10% methanol) ; [E] (85% ethyl acetate-15% methanol) ; [F] (80% ethyl acetate-20% methanol) ; [G] (70% ethyl acetate-30% methanol) ; and [H] (50% ethyl acetate-50% methanol) chromatography (Mbaku et al., 2025).

**2.2.4.  *In vitro*antiplasmodial activity**

**a. Preparation of the intermediate plates**

The crude extract and fractions from *C. rhodanthum* were prepared by dissolving 100 mg of each sample in 1 mL of dimethyl sulfoxide (DMSO ; 100%), to obtain a final concentration of 100 mg/ml for each sample. After that, 2 μl of each extract’s solution was introduced into 96 well round bottom microplates, followed by an addition of 198 μL of incomplete RPMI 1640 medium in the first wells to achieve 1 mg/mL concentration, while only 160 μL of incomplete RPMI 1640 were added to the remaining wells. Afterwards, a serial dilution of geometric sequence 5 was performed to yield concentrations ranging from 1 mg/mL to 0.0016 mg/mL for extracts and from 1% to 0.0016% for DMSO. Artemisinin, which was obtained from Sigma-Aldrich, was used as a reference molecule (positive control) and was prepared from a 10 mM stock solution to obtain concentrations varying from 10 μM to 0.016 μM. The as-prepared intermediate plates were stored at -80°C for further use in antiplasmodial assays.

**b. Maintenance of *Plasmodium falciparum* through *in vitro* culture**

The *in vitro* culturing of the chloroquine-sensitive (3D7) and multidrug-resistant (Dd2) *P. falciparum* strains was carried out using human erythrocytes by reference with a previously reported protocol (Trager and Jensen, 1976). Cryotubes containing *P. falciparum* strains, which were collected from the -80°C freezer were thawed to 37°C for 2 minutes. The preparation in each cryotube was then relocated into a 50 mL Falcon conical tube, followed by a drop-wise addition of 100 μL of NaCl solution (12%). After incubation of the preparation for 5 minutes, an additional 5 mL of NaCl solution at 1.6% was gradually added to the mixture. The as-prepared solution was centrifuged at 2500 rpm for 5 minutes, followed by a removal of the pellet and washing using a complete RPMI 1640 medium. After that, the remaining pellet was cultured while maintaining in suspension at 4% haematocrit in fresh human erythrocytes obtained from a blood group donor (O+) and complete RPMI 1640 medium. Then, cultures were grown in a humidified atmosphere with 5% CO2 at 37°C. To ensure optimal and continuous growth of *P. falciparum*, the culture medium was replenished daily with a fresh complete medium. To examine the parasitized red blood cells, blood smears were prepared on cleaned slides, stained with a Giemsa staining solution (10%) and subsequently observed under microscope (Huma Scope Classic) at 100X objective with immersion oil. Forty-eight hours before each antiplasmodial experiment, *P. falciparum* cultures containing predominantly the ring stage were synchronised (Lambros and Vanderberg, 1979).

**c. Culture synchronisation**

To assay antiplasmodial effects of *C. rhodanthum* extractsover a complete *P. falciparum* cycle (Lambros and Vanderberg, 1979), parasites were synchronized at ring stage by 5% (w/v) sorbitol cell treatment to eliminate trophozoites and schizonts (advanced stages). In the antiplasmodial assays, *P. falciparum* cultures containing at least 80% of the ring stage parasites were centrifuged at 2500 rpm for 5 min, followed by a removal of the supernatant and replacement by 5 mL of 5% (w/v) sorbitol solution (Yuan et al., 2014). After that, the preparation was homogenized and incubated at 37°C for 10 minutes, then centrifuged again at 2500 rpm for 5 minutes. Next, the as-prepared solution was washed three times with incomplete RPMI 1640 medium to remove sorbitol as much as possible. After synchronisation, the parasites were re-suspended in complete RPMI 1640 medium with 4% haematocrit. After 48 hours of incubation, *C. rhodanthum* extract and its fractions were screened for antiplasmodial activity (Maier and Rug, 2013).

**d. Inhibitory effects of plantextracts against *Plasmodium falciparum***

A previously described protocol (Smilkstein et al., 2004) was used to assay the antiplasmodial effects of *C. rhodanthum* extracts in 96-well flat-bottom microplates (Sigma-Aldrich) using a SYBR green I-based fluorescence assay. In brief, under normal culture conditions, sorbitol-synchronized ring stage parasites (hematocrit: 4%, parasitemia: 2%, 90 µL) were incubated for 72 hours at 37℃ with *C. rhodanthum* extracts and artemisinin, a reference antiplasmodial drug (10 µL). After that, 100 µL of SYBR Green I buffer [6 µL of 10,000 × SYBR Green I (Invitrogen), in addition to 600 µL of Red Blood Cells lysis buffer {Tris (25 mM ; pH 7.5)} and 360 µL of EDTA (7.5 mM) + 19.2 µL of parasites lysis solution {saponin (0.012%, wt/vol)} as well as 28.8 µL of Triton X-100 (0.08% ; vol/vol)}] was added to each well, mixed twice gently with a multichannel pipette and incubated in the dark at 37℃ for 1 hour (Kaushik et al., 2015). Fluorescence was measured using a microtiter plate reader TECAN Infinite M200 (USA) at 485 and 538 nm for excitation and emission wavelengths, respectively. The 50% inhibitory concentration (IC50) was determined by plotting fluorescence counts versus the logarithm of the extracts’ concentration, and by analysis of dose-response curves using GraphPad Prism 5.0. Experiments were performed in triplicate.

**2.2.5.  Effects of *C. rhodanthum* extracts on erythrocytes**

The haemolytic activity of *C. rhodanthum* extracts on healthy erythrocytes was determined according to the method described previously by Sinha et al. (2019). Briefly, 1 mL of stock solution of each plant extract solution was diluted in RPMI 1640 (990 µL RPMI+10 µL of extract) at the concentration of 500 µg/mL. Then, 500 µL of RPMI medium were dispensed into four eppendorf tubes, followed by the addition of 500 µL of each extract. After that, 500 µL of the solution were withdrawn from the preparation, followed by a serial dilution of order 2 was performed to obtain different concentrations of each extract. Next, 500 µL of 4% haematocrit solution (1.6 mL of RBCs+18.4 mL of RPMI medium) was added to all the tubes, and then homogenized to achieve final volume of 1 ml and final concentrations ranging from 250 µg/mL to 15.5125 µg/mL. 1% Triton X (990 µL of RPMI medium+10 µlLTriton X) was used as a positive control, whereas only RPMI 1640 medium was used as a negative control. All the experimental tubes were incubated at 37°C for 3 h under a humidified atmosphere with 5% CO2, followed by centrifugation 5% CO2. Next, 200 µL of each supernatant was transferred to a flat-bottomed 96-well (Sigma-Aldrich) plate, and then the absorbance was measured at 540 nm using a microplate reader (TECAN Infinite M200). The percentages of haemolysis were calculated from the optical density of samples using the following formula :

**Percentage of haemolysis (%) =**$\frac{(OD of extract-OD of negative control)}{OD of positive control}$**x100**

**2.2.6. Antioxidant activity**

**a. DPPH• test** The most promising antiplasmodial extracts were subjected to antioxidant activity using the 1,1-diphenyl-2-picryl hydrazyl (DPPH) method. Briefly, twenty-five (25 μL) of each extract at various concentrations (2000 to 31.25 µg/mL) were added to 75 μL of 0.02% DPPH solution to yield final concentrations between 500 and 3.90625 μg/mL. The as-prepared solutions were stored in the dark at room temperature for 30 min, and then the absorbance was measured at 517 nm using a microplate reader (TECAN Infinite M200 at 517 nm) against the blank (only DPPH reagent in methanol). Ascorbic acid, which was used as a positive control, was also incubated with 0.02 % DPPH solution to yield final concentrations varying from 25 to 0.195 μg/mL. The percentages of inhibition, which were calculated from the measured optical densities, were used to express the mean scavenging concentrations (SC50s) using GraphPad Prism 8.0.1. software.

**b. ABTS assay**

As previously described by Re et al. (1999), a discoloration method was employed to determine the antioxidant effects of active *C. rhodanthum* extracts through ABTS test. In brief, 25 μL of extract at different concentrations (2000-31.25 µg/mL) were added to 75 μL of the ABTS+ solution (0.175 mM), followed by an incubation for 30 min in the dark at room temperature. After that, the optical densities of the as-prepared solutions were measured at 734 nm using a microplate reader (TECAN Infinite M200). ABTS reagent and ascorbic acid, which were used as negative and positive controls, respectively, were prepared at final concentrations varying from 25 to 0.390 μg/ml. The assays were performed in triplicate in 96 well microplates. The reduction of ABTS causes the discoloration of the blue ABTS solution measured at 734 nm after 30 min of incubation

**c. FRAP assay**

The ferric iron reducing activity of *C. rhodanthum* extracts was measured according to a protocol reported by Moffatt et al. (1994). In short, 25 μL of extract at different concentrations (2000-31.25 µg/mL) were added to 25 μL of a solution of Fe3+ (1.2 mg/mL). Next, the microplates were incubated for 15 mins at room temperature, followed by an addition of 50 μL of orthophenanthroline (0.2%) and an additional incubation for 20 min at room temperature. After that, the absorbance of the as-prepared solution was read at 505 nm against the blank (25 μL of methanol + 25 μL Fe3+ + 50 μL of ortho-phenanthroline) using a microplate reader (TECAN Infinite M200). Ascorbic acid (positive control) was prepared in methanol to achieve final concentrations varying from 25 to 0.390 μg/mL.

**2.2.7. Phytochemical screening**

A qualitative analysis of the ethanol extract was conducted to identify various constituents of *C. rhodanthum*, including triterpenes, steroids, phenolic compounds and flavonoids (Trease and Evans, 1989), alkaloids and tannins (Harborne, 1984), and sugars (Odebiyi and Sofowora, 1979), which could be responsible for the antiplasmodial activity.

**a) Liebermann-Burchard test**

The Liebermann-Burchard test is generally used to identify the presence of triterpenes in samples. In this test, one (1) mg of crude extract was dissolved in chloroform, and then acetic anhydride and concentrated sulphuric acid were added to the preparation. After stirring, the presence of steroids was specified by a greenish-blue colour, whereas triterpenes were indicated by a purplish-red colour (Trease and Evans, 1989).

**b) Molisch assay**

The Molisch test was used to identify the presence of sugars in *C. rhodanthum* extract. To this end, 1 mg of extract was dissolved in ethanol, then 1% ethanol solution of α-naphthol was added, followed by an addition of a few drops of concentrated sulfuric acid. After that, the appearance of a purplish-red ring at the interface of the solution marked the presence of sugars in the preparation (Odebiyi and Sofowora, 1979).

**c) Ferric chloride (FeCl3) test**

The presence of phenolic compounds in *C. rhodanthum* extract was detected using the ferric chloride test. Briefly, 1 mg of extract was dissolved in ethanol, followed by an addition of FeCl3 solution. The development of blue or violet complexes indicated the occurrence of phenolic compounds in *C. rhodanthum* extract (Trease and Evans, 1989).

**d) Shinoda test**

After preparing a solution of ethanol extract of *C. rhodanthum*, a few drops of concentrated hydrochloric acid and magnesium sheets were added. The occurrence of flavonoids was unveiled by the appearance of a purple colour (Trease and Evans, 1989).

**e) Dragendorff test**

In this experiment, 10 mg of the ethanol extract of *Combretum rhodanthum* were added to 1% ethanolic solution of HCl, followed by the addition of the Dragendorff reagent. The generation of an orange precipitate revealed the presence of alkaloids (Harborne, 1984).

**f) Tannins test**

In this experiment, 15 mg of *C. rhodanthum* extract was dissolved in 1 mL of ethanol, then, a solution of ferric chloride was added. After that, the presence of tannins in *C. rhodanthum* extract was unveiled by the appearance of a dark brownish-green or blue-black colour in the preparation (Harborne, 1984).

**2.2.8. Acute toxicity *in vivo***

The acute toxicity study was performed according to the OECD protocol, guideline number 423 (OCDE, 2001). For this experiment, 6 adult female rats were used. These animals were randomly divided into 2 groups of 3 animals each : group 1, which was considered as negative control group, received only distilled water at a single dose of 10 mL/kg ; group 2 was administered with the ethanol extract of *C. rhodanthum* at a single dose of 2000 mg/kg. Prior to the experiment, the animals were subjected to 12 hours fasting. After treatment, the animals were carefully observed for the first 4 hours and daily for 14 days. During this time period, the animals were observed for any signs of immediate toxicity, such as aggressiveness, mobility, skin changes, convulsions, etc. The body weights were also recorded every other day. At the end of the experiment, the animals were sacrificed, and their organs (liver, kidneys, spleen, lungs and heart) were removed, defatted and weighed.

**2.3. Statistical analyses**

The percentages of inhibition of *Plasmodium falciparum* (% growth inhibition) were calculated using Microsoft® Excel 2020 software. The IC50 values were determined by non-linear regression analysis using Graph Pad Prism 8 software. Results from antioxidant activity were expressed as mean ± standard deviation (mean ± SD). Means were analysed by One-way analysis of variance (ANOVA), then the comparison of means was carried out using the Dunnett’s test. To minimise confounding factors, the test was performed in triplicate. The results of group tests were further compared with those of the positive controls. Values were considered statistically significant at p < 0.05.

**3. Results**

**3.1 Antiplasmodial and haemolytic effects of *Combretum rhodanthum* extracts**

**3.1.1. Antiplasmodial activity**

Upon incubation of *C. rhodanthum* extracts with *P. falciparum*, lower inhibition concentrations were obtained, thus revealing the antiplasmodial activity of these extracts. Against *P. falciparum* Dd2*,* the mean inhibitory concentrations (IC50)ranged from 5.30 to 35.73 μg/ml, whereas the IC50 values obtained against *P. falciparum* 3D7 varied between 11.21 and 105.15 μg/mL. The ethanol extract yielded IC50 values of10.21 and 18.48 μg/mL. Among the fractions derived from the crude ethanol extract, n-butanol revealed more pronounced antiplasmodial activity with IC50 value as low as 17.46 μg/mL against *P. falciparum* Dd2 (Table 1). Subfractions derived from the n-butanol fraction revealed low IC50 values, with fraction [D] and [E] being the most potent subfractions against *P. falciparum* Dd2 and subfractions [A] and [D], the most actives on *P. falciparum* 3D7. According to the classification criteria forIC50 values defined by Kamaraj and coworkers in 2012, extracts with IC50 values ≤ 10are considered to be highly active, whereas 10 **≤** IC50 values ≤ 20 and20 **≤** IC50 values ≤ 40 are considered to be active and moderately active, respectively. Plants extracts with 40 **≤** IC50 values ≤ 70 are considered to be poorly active or inactives.

 Table 1 : Mean Inhibitory Concentration (IC50) of *Combretum rhodanthum* extracts, fractions and subfractions and resistance index (RI) on *Pf*Dd2 and *Pf*3D7

|  |  |  |  |
| --- | --- | --- | --- |
| Extracts | IC50 (μg/mL) sur *Pf*Dd2(Mean ± SD) | IC50 (μg/mL) sur *Pf*3D7(Mean ± SD) | Resistance index(RI) |
| Cr-EtOH | 10.21 ± 4.17 | 18.48 ± 1.58 | 0.55 |
| Cr-AcOEt | 24.73 ± 2.01 | 27.05 ± 0.62 | 0.91 |
| Cr *n*-butanol | 17.46 ± 2.13 | 39.56 ± 2.06 | 0.44 |
| Cr-H2O | 26.91 ± 1.15 | 63.79 ± 5.69 | 0.42 |
| [A] | 22.76 ± 3.42 | 12.32 ± 0.98 | 1.85 |
| [B] | 35.73 ±0.21 | 105.15 ± 3.18 | 0.34 |
| [C] | 19.88 ± 0.63 | 23.74 ± 3.35 | 0.84 |
| [D] | 5.30 ± 1.54 | 11.21 ± 0.06 | 0.47 |
| [E] | 5.89 ± 0.05 | 21.89 ± 0.18 | 0.27 |
| [F] | 9.14 ± 0.19 | 21.52 ± 0.33 | 0.42 |
| [G] | 23.17 ± 6.14 | 43.78 ± 2.47 | 0.53 |
| [H] | 26.45 ± 2.43 | 17.86 ± 3.25 | 1.48 |
| Art (µM) | 0.03 ± 0.01 | 0.02 ± 0.00 | 1.50 |

Cr: *Combretum rhodanthum*, Cr-EtOH: ethanol extract of Cr, Cr-AcOEt: ethyl acetate fraction of Cr, Cr *n*-butanol: *n*-butanol fraction of Cr, Cr-H2O: aqueous residue of Cr, [A] : Cr *n*-butanol hexane/acetate (0.5/0.5) sub-fraction, [B]: Cr *n*-butanol acetate sub-fraction, [C]: Cr *n*-butanol acetate/methanol (0.95/0.05) sub-fraction, [D]: Acetate/methanol (0.9/0.1) subfraction of Cr *n*-butanol, [E]: Ethyl acetate/methanol (0.85/0.15) subfraction of Cr n-butanol, [F]: Ethyl acetate/methanol (0.80/0.2) subfraction of Cr *n*-butanol, [G]: Ethyl acetate/methanol(0.70/0.3) subfraction of Cr *n*-butanol, [H]: acetate/methanol (0.5/0.5) subfraction of Cr *n*-butanol, Art: Artemisinin, SD: standard deviation, μM: micromolar.

According to the classification criteria of IC50s by Kamaraj et al. (2012) *C. rhodanthum* crude extract exhibited moderate anti-plasmodial activity on *Pf*Dd2 and *Pf*3D7, with RI<1 (Table 1). The sub-fraction [D] exhibited the highest antiplasmodial activity on the sensitive (IC50 of 11.21 μg/mL) and resistant strain (IC50 of 5.30 μg/mL) of *P. falciparum*. Extract, fractions and subfractions from *C. rhodanthum* were further assessed for cytotoxicity on red blood cells.

**3.1.2. Effects of *C. rhodanthum* extracts on human red blood cells**

Figure 2 illustrates the haemolysis percentage of red blood cells following treatment with extract, fractions and sub-fractions of *C. rhodanthum*. Ethanol extract and n-butanol fraction showed haemolysis percentages beyond 70% (78.09 and 95.54% for ethanol and butanol extracts, respectively), vs 100% haemolysis for triton X, the positive control. However, subfractions derived from the n butanol fraction did not induce haemolysis (less than 10%) to human erythrocytes (Figure 2). Thus, the process of plant purification has significantly decreased the haemolysis potential of *C. rhodanthum* extracts.



**Figure 2 :** Hemolytic effect of *C. rhodanthum* extracts on erythrocytes.

The values with the same letters (a) and (b) are significantly different from the positive control at *p* ≤0.001 and *p* ≤0.05, respectively ; Cr: *Combretum rhodanthum*, Cr-EtOH: ethanol extract of Cr, Cr-AcOEt: ethyl acetate fraction of Cr, Cr *n*-butanol: *n*-butanol fraction of Cr, Cr-H2O: aqueous residue of Cr, [A]: hexane/ethyl acetate (0.5/0.5) subfraction of Cr *n*-butanol, [B]: Cr *n*-butanol ethyl acetate subfraction, [C]: Cr *n*-butanol ethyl acetate/methanol (0.95/0.05) subfraction, [D]: Cr *n*-butanol ethyl acetate/methanol (0.9/0.1) subfraction, [E]: Cr *n*-butanol ethyl acetate/methanol (0.85/0.15) subfraction, PC: positive control.

**3.1.3. Phytochemical screening**

To identify the chemical constituents responsible for the observed biological activity, ethanol and *n*-butanol extracts were subjected to phytochemical tests using standard protocols. Table 2 depicts the secondary metabolites present in different extracts. According to table 2, the phytochemical screening of ethanol and butanol extracts from *C. rhodanthum* revealed the presence of terpenoids, tannins, phenolic compounds, and glycosides.

Table 2 : Phytochemical screening of ethanol and n-butanol extracts from *C. rhodanthum*

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Extracts** | **Alkaloids** | **Tannins**  | **Terpenoids** | **Steroids** | **Flavonoids** | **Phenolic compounds** | **Glycosides** |
| **Cr-EtOH** | **˗** | **+** | **+** | **˗** | **+/ ˗** | **+** | **+** |
| **Cr *n*-butanol** | **˗** | **+** | **+** | **˗** | **+/ ˗** | **+** | **+** |

Cr: *Combretum rhodanthum*, Cr-EtOH: ethanol extract of Cr, Cr *n*-butanol, +: positive, ˗: negative, +/ ˗: ambiguous.

**3.1.4. Antioxidant activity**

Ethanol extract, n-butanol fraction and subfractions [B], [D], and [E], which revealed significant antiplasmodial activity, were further evaluated for *in vitro* antioxidant activity using DPPH, ABTS and FRAP tests.

**a. ABTS and DPPH assays**

Table 3 shows the radical scavenging concentrations 50 (SC50 ; µg/mL) of *C. rhodanthum* extracts upon DPPH and ABTS assays. Ethanol extract concentrations@39.416 and 23.510 µg/mL were more than enough to respectively scavenge 50% of free radicals of ABTS and DPPH. Meanwhile, *n*-butanol fraction showed SC50 values of 7.942 and 15.965 µg/mL vis-à-vis ABTS and DPPH radicals, respectively. Among the subfractions tested, subfraction [D] was the most potent in scavenging ABTS free radicals (SC50 : 0.884 µg/mL), whereas subfraction [E] showed highest scavenging potential against DPPH free radicals (SC50 : 11.825 µg/mL). Ascorbic acid, which was used as a positive control, revealed SC50 values of 1.645 and 2.835 µg/mL upon ABTS and DPPH assays, respectively.

**Table 3 :** Radical scavenging concentrations (µg/mL) of *C. rhodanthum* extracts upon ABTS and DPPH assays.

|  |  |  |
| --- | --- | --- |
| Extracts | ABTS(Mean SC50 ± SD) | DPPH(Mean SC50 ± SD) |
| Cr-EtOH | 39.416 ± 2.042\*\*\* | 23.510 ± 1.640\*\*\* |
| Cr *n*-butanol | 7.942 ± 2.031\*\* | 15.965 ± 4.617\*\* |
| [B] | 29.56 ± 0.579\*\*\* | 16.315 ± 0.346\*\*\* |
| [D] | 0.884 ± 0.000\* | 13.98 ± 0.629\*\*\* |
| [E] | 5.617 ± 0.008\* | 11.825 ± 2.616\*\* |
|  Ascorbic acid | 1.645 ± 0.725 | 2.835 ± 0.024 |

\*P ≤ 0.05, \*\*P ≤ 0.01, \*\*\*P ≤ 0.001; Values are significantly different, compared to the value of ascorbic acid. Cr: *Combretum rhodanthum*, Cr *n*-butanol: Cr *n*-butanol fraction, Cr-EtOH: Cr ethanol extract, [B]: Cr *n*-butanol ethyl acetate subfraction, [D]: ethyl acetate/methanol (0.9/0.1) subfraction of Cr *n*-butanol, [E]: ethyl acetate/methanol (0.85/0.15) subfraction, SD: standard deviation, SC50: Median scavenging concentration.

**b. FRAP test**

*C. rhodanthum* extracts were also screened for antioxidant activity using the FRAP test. The ethanol extract showed RS50 value of 26.1 µg/mL, whereas n-butanol revealed RS50 value of 20.5 µg/mL, vs ascorbic acid (RC50 : 4.1 µg/mL). Among the sub-fractions tested, subfraction E was the most active (RC50 : 7.1 µg/mL) in reducing Fe3+ into Fe2+ (Table 4).

**Table 4:** Median reduction concentrations (µg/mL) of *C. rhodanthum* extracts upon FRAP assay.

|  |  |
| --- | --- |
| Extracts | FRAP(Mean RS50 ± SD in µg/mL) |
| Cr-EtOH | 26.1 ± 4.9\*\* |
| Cr *n*-butanol | 20.5 ± 3.2\*\*\* |
| [B] | 15.9 ± 1.6\*\* |
| [D] | 14.3 ± 4.8\*\* |
| [E] | 7.1 ± 2.1\* |
| Ascorbic acid | 4.1 ± 0.7 |

\*P ≤ 0.05, \*\*P ≤ 0.01, \*\*\*P ≤ 0.001; Values are significantly different, compared to the value of ascorbic acid. Cr: *Combretum rhodanthum,* Cr *n*-butanol: Cr *n*-butanol fraction, Cr-EtOH: Cr ethanol extract, [B]: Cr *n*-butanol acetate subfraction, [D]: Cr n-butanol acetate/methanol (0.9: 0.1) Cr n-butanol subfraction, [E]: ethyl acetate/methanol (0.85:0.15) subfraction, PC: positive control, SD: standard deviation, RC50: Median reducing concentration.

**3.1.5. Acute toxicity**

**a. Clinical manifestations**

A single oral dose of the ethanol extract *C. rhodanthum* (2000 mg/kg) was administered to Wistar rats, followed by a 14 days’ observation period. Table 5 summarizes the clinical manifestations observed in rats administered with a single dose of *C. rhodanthum* ethanol extract. No death was observed in treated animals after 14 days of observation, and the no observed adverse effect level (NOAEL) for *C. rhodanthum* ethanol extract was considered to be more than 2000 mg/kg.

**Table 5:** Clinical signs of toxicity in rats administered with 2000 mg/kg of *C.* *rhodanthum* extract.

|  |  |  |
| --- | --- | --- |
| **Parameters** | **Negative control** | **Extract@****2000 mg/kg** |
| **Time**  |  **0.5 Hours** | **4 Hours** | **Day 14** | **0.5 Hours** | **4 Hours** | **Day 14** |
| **Number of deaths** | 0 | 0 | 0 | 0 | 0 | 0 |
| **Chills** | - | - | - | - | - | - |
| **Aggressiveness** | - | - | - | - | - | - |
| **Mobility** | + | + | + | + | + | + |
| **Aspect of feces** | N | N | N | N | N | N |
| **Horripilation** | - | - | - | - | - | - |
| **Sensitivity to touch** | - | - | - | - | - | - |
| **Sensitivity** **to** **noise** | - | - | - | - | - | - |

N= normal, + = Present, - = Absent

**b.** **Changes in body weights of rats**

Figure 3 illustrates the evolution of body weights of rats administered with a single oral dose (2000 mg/kg) of *C.* *rhodanthum* extract and observed during 14 days’ post-treatment. An increasing trend of the curves is observed from Day 0 to Day 14. This observation signifies that the animals have gained weights throughout the experimental period.



**Figure 3 :** Changes in the body weights of the animals treated with a single oral dose of *C.* *rhodanthum* ethanol extract and observed for 14 days.

**c. Relative organ weights**

The relative weights of the liver, kidneys, spleen, heart, lungs and brain were measured to identify any signs of toxicity (hypertrophy or hypotrophy) (Figure 4). The results show that there was no significant difference between experimental and control groups of animals in terms of liver, kidneys, spleen, heart, lungs and brain body weights.

 

**Figure 4 :** Relative organ weights of animals administered with a single oral dose of *C.* *rhodanthum* ethanol extract and observed for 14 days.

**4. Discussion**

The increasing resistance to current antimalarial drugs necessitates further research campaigns for the development of efficient treatments for malaria. In the recently published malaria report, the World Health Organization recommends the use of medicinal plant extracts as a Traditional Medicine Strategy to overcome malaria drug resistance (WHO, 2024). An example of such plants include *Combretum rhodanthum*,which has long been used by local practitioners of healing to treat malaria, without proper scientific validation. Therefore, the scientific validation of *C. rhodanthum* in the treatment of malaria conditions is worthy.Thus, this study evaluates the antiplasmodial and antioxidant activities of *C. rhodanthum* extracts. Upon incubation of the ethanol extract of *C. rhodanthum* with *P. falciparum* Dd2 and *P. falciparum* 3D7, as high as 10.21 and 18.48 μg/mL concentrations were respectively sufficient to inhibit 50% of the parasites in culture. Further partitioning of the ethanol extract yielded ethylacetate and n-butanol fractions, which exhibited low IC50 values against both parasites (IC50 range: 17.46-63.79 μg/mL) with low resistance indices for ethanol and n-butanol extracts. The *n*-butanol fraction, which was subsequently chromatographed, yielded eight antiplasmodial sub-fractions [A-H], with subfraction [D] being the most potent antiplasmodial subfraction. Against *P. falciparum* Dd2, IC50s of the subfractions ranged 5.30 to 26.91 μg/mL, whereas values of IC50 varied from 11.21 to 105.5 μg/mL when tested on *P. falciparum* 3D7. The phytochemical analysis of ethanol and n-butanol extracts revealed the presence of terpenoids, phenolic compounds, tannins and glycosides, which might be responsible for the observed antiplasmodial activity. Accumulated evidence has shown the antiplasmodial activity of phenolic compounds against *P. falciparum*. Indeed, previous reports indicate the antiplasmodial potential of phenolic compounds (Azebaze et al., 2015 ; Yun et al., 2016), steroids (Safar et al., 2022), and tannins (Dell'agli et al., 2010). Plants containing high phenolic compounds such as *Moringa olifeira* has also been shown to exhibit antiplasmodial activity (Olaniran et al., 2019; Hassan et al., 2021).

Hemozoin, a by-product of hemoglobin metabolism in *Plasmodium* parasites, produces heme and iron, which can activate artemisinin to produce free radicals. The activated artemisinin indiscriminately binds on primary metabolites, such as proteins, or lipids in a number of key biochemical pathways to impede the physiological functions of *Plasmodium* to cause its death (Zheng et al., 2024). In fact, through its endoperoxide ring, artemisinin interacts with heme [Fe(II)] released during parasite hemoglobin digestion to cause a homolytic cleavage of the peroxide (O–O) bridge of trioxanes generating stable cytotoxic species (carbocations) (Zheng et al., 2024). These reactive species cause membrane damage, inhibition of protein and nucleic acid synthesis and interaction with cytochrome oxidase and the glutamine transport system in parasites (Espino-Sanchez et al., 2023 ; Pawłowska et al., 2023 ; Mukherjee et al., 2024).

Several reports have demonstrated the antimalarial activity of terpenoids and steroids via inhibition of key enzymes that are involved in the metabolic pathways of the apicoplast of *Plasmodium falciparum* (Jordão et al., 2011 ; Okada et al., 2022 ; Okada and Sigala, 2023). Since *C. rhodanthum* extracts contain terpenoids and steroids, flavonoids, and tannins, their antimalarial activity might have resulted from at least one of the abovementioned mechanisms. The pathogenesis falciparum malaria triggers oxidative stress, thus causing an overproduction of reactive oxygen species (ROS) to the detriment of natural antioxidant defenses (Gomes et al., 2022). Growing evidence has unveiled the potential benefits of antioxidant therapy in malaria infection (Arrey Tarkang et al., 2013 ; Gomes et al., 2022). Reis et al. (2010) demonstrated that treatment by antioxidants can prevent the development of cerebral malaria. Antioxidants promote an effective immune response vis-à-vis pathogenic microbes by mitigating cellular damage (Gomes et al., 2022). Thus, the need for a dual therapy that defeats the pathogen and neutralizes the oxidative stress generated during infection is paramount.

*Combretum rhodanthum* extracts exhibited antioxidant activity upon DPPH and FRAP assays. This antioxidant indication of *Combretum rhodanthum* extracts might have contributed to the plant’s ability to inhibit the growth of *P. falciparum.* Moreover, the phytochemical analysis of *Combretum rhodanthum* extracts revealed the presence of phenolic compounds, terpenoids, steroids, tannins, etc., which are reported for their antioxidant potential. ROS scavenging effect and inhibition of ROS production and inhibition of free radical-generating enzymes are among the mechanisms of action of antioxidant compounds (Hassanpour and Doroudi, 2023 ; Tumilaar et al., 2024).

 All in all, this novel contribution unveils the antiplasmodial activity of *C. rhodanthum* extracts, which is supported by their phytochemical composition and their antioxidant potential. These findings validate the ethnomedicinal use of *Combretum rhodanthum* in the treatment of malaria.

**5. Conclusions**

*Combretum rhodanthum* organs are used traditionally to overcome parasitic diseases, such as malaria. Therefore, the scientific validation of *C. rhodanthum* in the treatment of malaria is valuable. In this study, the antiplasmodial activity of *C. rhodanthum* extracts was evaluated against chloroquine sensitive and resistant strains of *P. falciparum* using phenotypic screenings. As a result, the ethanol extract and fractions from *C. rhodanthum* inhibited the growth of *P. falciparum* as evidenced by the low IC50 values obtained. The phytochemical screening of the ethanol and butanol extracts revealed the presence of phenolic compounds, tannins, terpenoids and steroids, etc. Furthermore, *C. rhodanthum* extracts demonstrated antioxidant activity following DPPH and FRAP assays, thus revealing that these extracts might reverse the oxidative stress caused by malaria pathogenesis under *in vivo* conditions. Acute toxicity study of *C. rhodanthum* extracts in Wistar rats at 2000 mg/kg revealed non toxicity and the no adverse effect level (NOAEL) was considered to be more than 2000 mg/kg. *Combretum rhodanthum* exhibited significant antiplasmodial and antioxidant properties, thus validating its traditional use in treating malaria and its symptoms. However, the mechanistic basis of the antiplasmodial action, pharmacokinetics, and *in vivo* assays are warranted to support the safe use of *C. rhodanthum* in folk medicine.

**Data Availability Statement:** Data is available from the corresponding authors upon reasonable request.

**Disclaimer (Artificial intelligence):**

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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