



Marmesin isolated from *Celtis durandii* Engl. root bioactive fraction inhibits β -hematin formation and contributes to antiplasmodial activity

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ABSTRACT

Ethnopharmacological relevance: Malaria is a leading cause of death in many developing countries, especially in sub-Saharan Africa. Nigeria is endowed with an abundance of medicinal plants, many of which are used to treat malaria. *Celtis durandii* Engl. is one such plant used as a traditional antimalarial remedy in southeast Nigeria. However, its antiplasmodial potential is poorly explored.

Aim of the study: The study aimed at identifying the antiplasmodial components of *C. durandii* root extract through antiplasmodial activity-guided fractionation.

Materials and methods: Dichloromethane/methanol mixture extract (1:1 v/v) of *C. durandii* root was prepared and partitioned against water to obtain the organic phase, which was further separated by column chromatography into nine (C1 – C9) fractions. The antiplasmodial activity was evaluated by *in vitro* screening of the different fractions against drug-sensitive and drug-resistant *Plasmodium falciparum* strains. Further purification of the active column fractions resulted in a potent anti-*Plasmodium* compound that was subsequently investigated for its effect on β -hematin formation. Additionally, the isolated compound was characterized and identified as marmesin using mass spectrometry and nuclear magnetic resonance spectroscopy.

Results: *Celtis durandii* root extract exhibited promising antiplasmodial activity {IC₅₀ (μ g/ml) 5.92, 6.04, and 6.92} against PfW2mef, PfINDO, and Pf3D7 respectively. Pooled fractions with good antiplasmodial activity {IC₅₀ (μ g/ml) Pf3D7: 3.99; PfINDO: 2.24} and selectivity for the parasites (SI: 21) yielded a compound that was fourteen-fold potent in antiplasmodial activity against Pf3D7 (IC₅₀: 0.28 μ g/ml). It also inhibited β -hematin formation with an IC₅₀ = 150 μ M. Further studies using spectral data, literature, and chemical databases identified the purified compound as marmesin.

Conclusion: This work has demonstrated that *Celtis durandii* root extract has good antiplasmodial activity against drug-sensitive and drug-resistant *P. falciparum*. The inhibition of β -hematin formation by marmesin accounts in part for this activity.

1. Introduction

Malaria causes significant morbidity and mortality, especially in sub-Saharan Africa, and has become a global health concern. In 2021, an estimated 247 million malaria cases were recorded globally, with nearly 619, 000 deaths (World Health Organization, 2022). This infectious disease is spread by an infected female *Anopheles* mosquito which

transmits the parasites from its salivary glands during a human blood meal. Blood-stage infection is symptomized by headache, fever, myalgia, and malaise, and these can occur as paroxysms. Young children, pregnant women, and non-immune adults are particularly susceptible to severe, complicated forms of the disease and account for most malaria-related deaths. Recommended drug treatments for malaria vary by country and region, but artemisinin-based combination therapies

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(ACTs) are the mainstay for treating infection. Although ACTs are highly effective antimalarials, there is evidence of growing artemisinin resistance in some parts of Africa and the Greater Mekong sub-region of Asia (Fidock and Rosenthal, 2021; Menard and Dondorp, 2017; Ndwiga et al., 2021; Rosenthal, 2021). The development of the RTS, S malaria vaccine, and more recently R21 offer hope for controlling the disease, but without new, effective treatments, progress toward malaria elimination may be threatened by drug-resistant strains in patients with poor immunity despite vaccination. In the context of malaria, drug resistance has been a constant challenge faced in malaria treatment, as the parasite has shown the ability to evolve and survive drug exposure. Thus in parts of Africa where malaria is highly endemic, resistance to low-cost antimalarials such as chloroquine and sulfadoxine/pyrimethamine has led to the use of ACTs as frontline drugs (Whitty et al., 2008). However, the use of ACTs is hindered by their unaffordable costs for poor populations, and by the growing reports of resistance to them (Haldar et al., 2018; Fidock and Rosenthal, 2021). Therapeutic options for drug-resistant malaria remain very limited and it is crucial to discover and develop new safe and effective alternatives acting at diverse drug targets. In searching for new and affordable antimalarial alternatives, medicinal plants continue to be attractive starting points given their long history of use and the chemical diversity of their secondary metabolites. A recent systematic review that documents the antimalarial properties of medicinal plants used in African traditional medicine provides evidence to support the traditional use of such plants (Tajbakhsh et al., 2021). Indeed in large parts of Africa where malaria is endemic, the practice of using plant preparations to treat symptoms of malaria is quite common (Bekono et al., 2020). *Celtis durandii* Engl. (Ulmaceae) is one such medicinal plant, where the root of the plant is used by traditional healers to treat malaria in Nigeria (Adebayo and Krettli, 2011; Ibe and Nwufu, 2005). It is also used in Cameroon for the treatment of cardiovascular disorders, migraines, and pain (Ntchapda et al., 2010). *C. durandii* is a deciduous tree that can grow to a height of 20 m and often has a spreading crown (Burkill, 1985). It is found commonly dispersed across Africa and its wood serves different agricultural, building, and other economic purposes. Some pharmacological properties associated with *C. durandii* include vasorelaxant and antihypertensive activity (Dimo et al., 2005; Ntchapda et al., 2010). In our previous study on selected plant extracts, an aqueous ethanolic extract of *C. durandii* root was found to have modest antiparasmodial activity (IC₅₀ 54.2 µg/ml) against malaria parasites in culture, with no cytotoxic effects at 500 µg/ml against human cells in culture (Ezenyi et al., 2020). In this study, we have used an antiparasmodial activity-guided screen of different extracts and chromatographic fractions of *C. durandii* root extract against *in vitro* cultured *Plasmodium falciparum* to identify its bioactive constituents. Inhibition of β-hematin formation by one of the active constituents was also explored as a likely mechanism mediating antiparasmodial activity.

2. Materials and methods

2.1. Plant identification and collection

Celtis durandii Engl. roots were collected from Nsukka, Enugu State, Nigeria (6.8429° N, 7.3733° E). The collection was done in February 2020 and the plant was identified by Mr. Anthony Ozioko of the International Centre for Ethnomedicine and Drug Development (Inter CEDD), Nsukka, Enugu State. A voucher specimen was prepared and deposited in InterCEDD herbarium (InterCEDD/16098). The plant name was checked at <http://www.theplantlist.org> on 3rd November 2022.

2.2. Culture of malaria parasites

Chloroquine (CQ)-sensitive 3D7, CQ-resistant INDO, and multidrug (CQ, pyrimethamine, sulfadoxine, mefloquine) - resistant W2mef *Plasmodium falciparum* strains were maintained in our laboratories and used for *in vitro* antiparasmodial screening. The parasites were cultured

essentially as described by Trager and Jensen (1976). Parasites were grown in O⁺ erythrocytes and complete RPMI-1640 culture medium containing sodium bicarbonate 2 g/L, hypoxanthine 50 mg/L, gentamycin 10 mg/L, and enriched with Albumax I (5 g/L). Cultures were maintained at 37 °C under an atmosphere of 5% CO₂, 5% O₂, and 90% nitrogen.

2.3. General experimental procedures

Column chromatographic separations were carried out using silica gel (Davisil Grade 643, pore size 150 Å, 200–425 mesh, Sigma Aldrich, Germany). Thin-layer chromatography (TLC) analysis was carried out using silica gel on pre-coated aluminum sheets (Merck, Germany). The melting point of the compound was determined on a differential scanning calorimeter (DSC-60A, Shimadzu, Japan), and Nuclear Magnetic Resonance (NMR) experiments were carried out on a Bruker AVIII (400 MHz) spectrophotometer using CDCl₃ as solvent and tetramethylsilane (TMS) as internal standard. The spectra were processed using MestReNova software.

2.4. Extract preparation and column chromatography

C. durandii roots were washed clean under running water, wiped dry, cut to small pieces, and air-dried away from direct sunlight. Pieces of the dried roots (1.0 kg) of the plant were ground to coarse powder using an electric hammer mill. The coarse powder was soaked in a 3.5 L mixture of dichloromethane/methanol (1:1v/v) and stirred for 48 h. The mixture was filtered, and the marc was re-extracted with 1.5 L of the same solvent. The combined filtrate (5L) was concentrated by rotary evaporation and dried over a water bath maintained at 45 °C to give 120 g of the dichloromethane/methanol mixture extract, A. Fifty grams of A was dissolved in distilled water (100 ml) and partitioned thrice in a separatory funnel with 500 mL dichloromethane (DCM) each time. The combined DCM phase (1.5 L) was concentrated to dryness and 35 g of the powder (B) obtained was subjected to column chromatography using wet-packed silica gel in a glass column (70 cm × 8 cm). This was eluted with 100% hexane and increasing step gradients of ethyl acetate in hexane (20–100%), followed by 100% ethyl acetate. The last step of elution used increasing gradients of methanol in ethyl acetate (20–100%). Approximately 25 mL fractions were collected and those with matching TLC profiles were combined to give nine pools that were screened for antiparasmodial activity. The pool (fractions 5–8) with the highest promise was concentrated to dryness (3.07 g). This sample was subjected to Silica gel column (60 cm × 1.5 cm) chromatography by gradient elution with chloroform (100 mL × 5) and increasing concentrations of methanol in chloroform (10–100%) to give 14 fractions. Antiparasmodial activity-guided purification of the pool (fractions 5–8) yielded a pure compound in the form of a pale brown powder (20.1 mg). Fig. 1 shows a pictorial description of the extraction protocol and anti-*Plasmodial* activity-guided isolation steps.

2.5. SYBR green microtiter plated based antiparasmodial assay

In an initial primary screening of A, different concentrations (0.064 µg/mL – 25 µg/mL) were used to obtain its IC₅₀, whereas fractions of B were screened at 10 µg/ml in order to identify potent fractions. Thereafter, the IC₅₀ of active fractions and the isolated compound were determined by preparing a 2.5 mg/mL stock in DMSO. The extract, active fractions, and purified compound were screened against the parasites at final well concentrations in the range of 0.07–10 µg/mL following serial dilutions using RPMI. The final DMSO concentration in culture was 0.4% DMSO, which was confirmed to be non-toxic to the parasite. Parasite culture was synchronized with 5% sorbitol to obtain ring-stage parasites (Lambros and Vanderberg, 1979). The synchronized culture was adjusted to 1% parasitemia (P) and 2% hematocrit (H). The growth inhibition assays (Smilkstein et al., 2004; Erhunse et al., 2023)

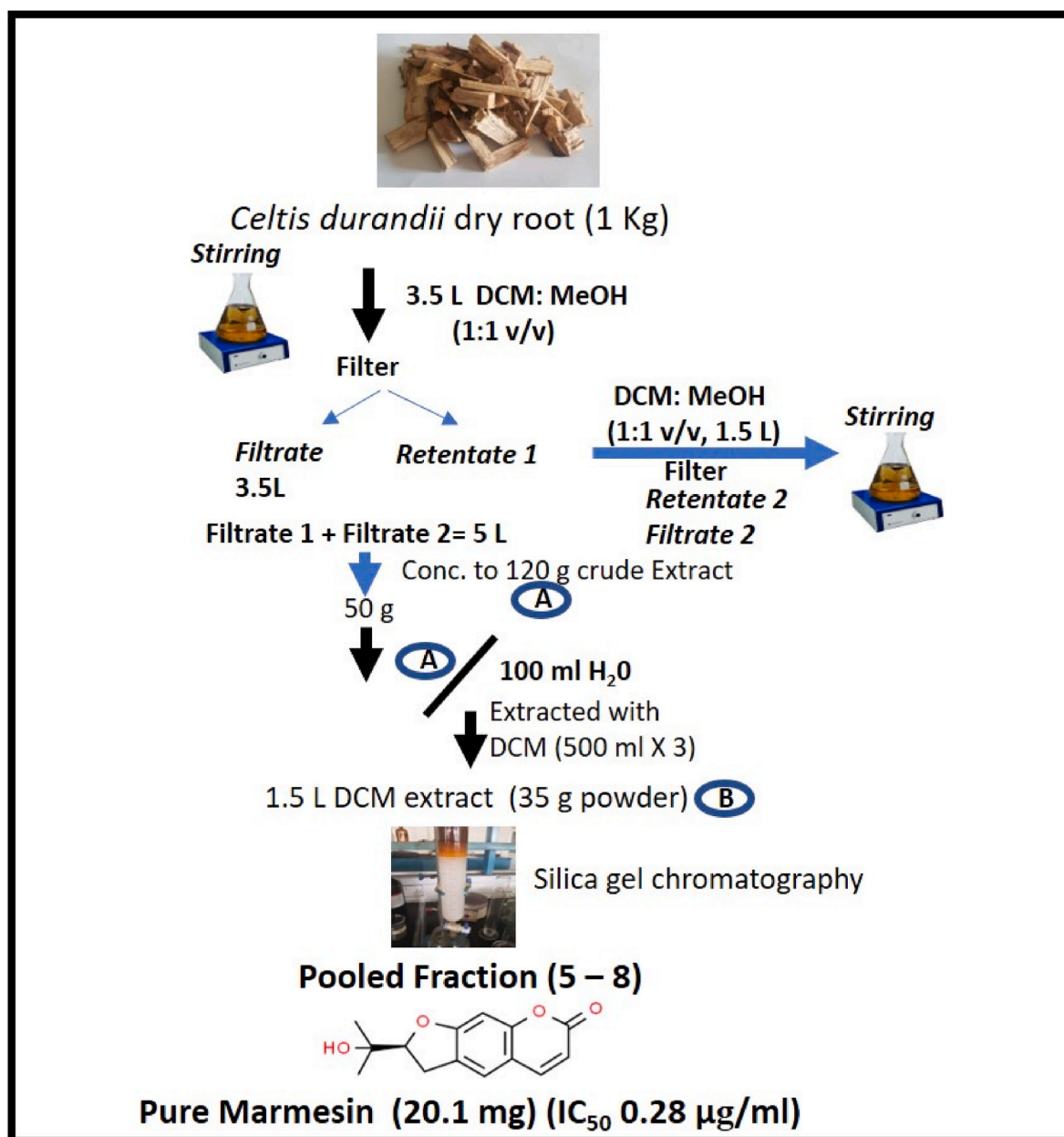


Fig. 1. Extraction and anti-Plasmodial activity guided isolation of potent Marmesin from *Celtis durandii*.

were set up in 96-well microtiter plates with final-well volumes of 100 µL (4 µL of test agent + 96 µL parasite culture at 1% P and 2% H). After 48 h incubation, % parasitemia was determined by adding 100 µL of lysis buffer {20 mM Tris (pH 7.5), 5 mM EDTA, 0.008% (W/V) saponin and 0.08% (V/V) Triton X-100 containing 0.2 µL SYBR green from $\times 10,000$ concentrate per mL of buffer}. This was followed by gentle and thorough mixing to allow for proper lysis of the RBCs after which the plates were incubated at 37 °C in the dark for 1 h and the total fluorescence originating from parasite DNA and SYBR green complexation was determined at excitation and emission wavelengths of 485 nm and 520 nm respectively. Untreated parasite culture (100% growth) and Chloroquine treated culture (0% growth) were used as negative and positive controls in all our experiments. SYBR green counts obtained with positive control were subtracted from counts obtained for all test samples.

2.6. Cytotoxicity test

Human hepatoma (HUH) cells were trypsinized, seeded in a 96-well plate (10^4 cells/100 µL/well), supplemented with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (Gibco), and incubated at 37 °C in an atmosphere of 5% CO₂ for 12 h. Test samples were then added in triplicates followed by 24 h incubation at normal culture conditions. Subsequently, 20 µL of MTT-PBS solution (5 mg/mL) was added to each well and after 3 h incubation, the medium was removed, and 200 µL DMSO/well was added and mixed thoroughly to solubilize the MTT formazan product. The absorbance, an index of cell growth was measured at 570 nm on a multi-well plate reader (Mosmann, 1983) and CC₅₀ values were determined by concentration-response curves. 0.4% DMSO (100% growth) and 10% DMSO (0% growth) were used as negative and positive controls respectively.

2.7. Hemin crystallization assay

This was done according to a recently described method (Herraiz et al., 2019). Briefly, the following were mixed in 5 mL tubes in the order shown below: hemin chloride (0.288 µg) dissolved in DMSO (8 µL, 0.036 mg/mL), chloroquine dissolved in distilled water (0–500 µM) or test compound dissolved in DMSO (0–500 µM), freshly prepared detergent Tween 20 dissolved in water (15 µL, 0.015 mg/mL), and 100 mM acetate buffer (pH 4.8) to attain a final volume of 2 mL. A duplicate set of tubes with the same reagents as above were prepared and before incubation (t_0), the mixture was vortexed and the difference in absorbance at 415 nm and 630 nm (ΔA , $A_{415} - A_{630}$), against assay blank of 100 mM acetate buffer (pH 4.8) was taken. The other set of tubes for incubation was then incubated in dark at 37 °C for 3 h, with constant agitation at 300 rpm using an incubator shaker (Eppendorf, New Brunswick Scientific). The tubes were removed and kept in dark for 1 h at room temperature to allow crystallization/ β -hemin formation, before centrifuging at 10,000 rpm for 10 min. Free hemin remaining in supernatant was determined by calculating the ΔA . Controls without the drug or test sample were also prepared using DMSO in the same way as described for the samples above. The amount of free hemin (%) that correlated with β -hemin inhibition was calculated as:

$$[\Delta A_{(\text{drug or test molecule})} - \Delta A_{(\text{no drug/control})}] / (\Delta A_{(\text{pre-incubation})}) \times 100$$

2.8. GC-MS analysis of DCM phase B of *C. durandii* extract

GC-MS analysis was performed according to a method described previously (Awolola et al., 2019). The extract B (5 mg) was dissolved in

dichloromethane and chromatographed on a silica gel column under isocratic elution with methanol, and the eluate obtained was concentrated to 2 mL. A 1 µL injection was made into the GC-MS machine in a split-less mode, using ultra-high purity Helium as a carrier gas (2 mL/min). Injector temperature (250 °C) and oven temperature (100 °C) were held for 5 min, and then ramped at 8 °C/min, for 7.5 min. The GC-MS transfer line temperature was 240 °C, while the MS operated in electron impact mode was at 70 eV. The ion source and detector temperatures were 200 °C and 300 °C respectively. Data was acquired on an Agilent Mass Hunter software and compounds were identified based on information in the National Institute of Standards and Technology (Gaithersburg, USA) standard reference library (NIST v14).

2.9. Statistical analysis

Each experiment was set up and performed in triplicate. Data are given as mean $\pm$ standard deviation. The 50% inhibitory concentration (IC_{50}) was determined by analysis of concentration–response curves using non-linear regression using GraphPad Prism Version 7.0 (GraphPad Software, USA).

3. Results

3.1. Antiplasmodial activity of *C. durandii* extract

The antiplasmodial activities of chloroquine and *C. durandii* dichloromethane/methanol (1:1 v/v) mixture extract against the chloroquine-sensitive (3D7), and resistant (W2mef) strains of *P. falciparum* are shown in Fig. 2. The IC_{50} values of chloroquine against these two strains (7.43 nM for Pf3D7 and 147.5 nM for Pf W2mef) were

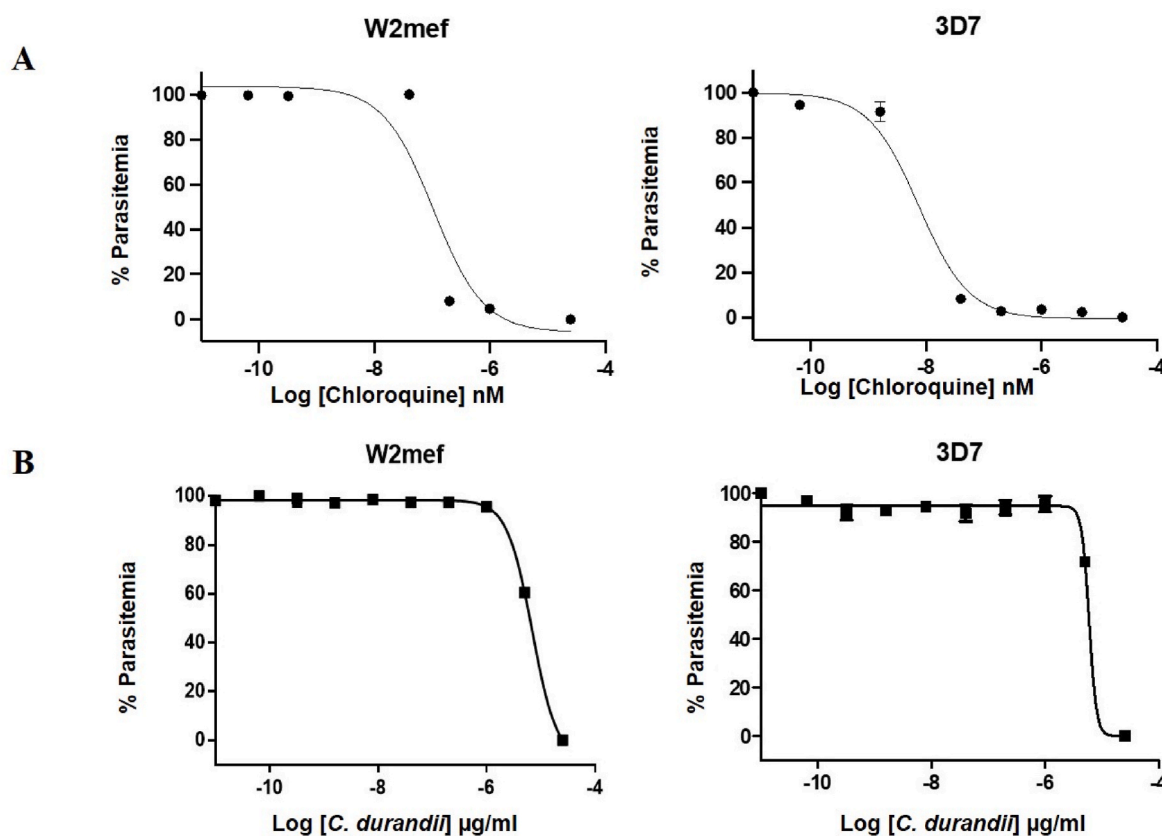


Fig. 2. *In vitro* antiplasmodial activity testing of *C. durandii* extract and standard drug, chloroquine. Panel A shows the growth inhibition profiles for chloroquine against Pf 3D7 and Pf W2mef laboratory strains of *P. falciparum* tested at concentrations in the range from 0.064 nM to 25 nM. Each data point represents the mean $\pm$ SEM (n = 3). The IC_{50} values observed were 3D7: 7.43 nM, W2mef: 147.5 nM. Panel B shows the growth inhibition profiles for *C. durandii* dichloromethane/methanol mixture extract against W2mef (IC_{50} : 5.92 µg/mL) and 3D7 (IC_{50} : 6.92 µg/mL). Each data point represents the mean $\pm$ SEM (n = 3).

within the range reported (Pradines et al., 2011). The IC_{50} values of *C. durandii* extract indicated its higher potency against *Pf*W2mef compared to the *Pf*3D7 strain (Fig. 2B). A resistance index (RI, $IC_{50}PfW2mef/IC_{50}Pf3D7$) of 0.86 obtained for the extract was >20-fold lower than the RI of 19.85 for chloroquine.

3.2. Antiplasmodial activity of *C. durandii* dichloromethane/methanol mixture extract, its chromatographic fractions and isolated compound

The dichloromethane/methanol (1:1 v/v) mixture extract and its fractions were first tested at 10 μ g/mL. Out of the 9 fractions obtained, fraction 8 completely inhibited parasite growth of both strains of the parasite tested, whereas fractions 5–7 showed 0–93% inhibitory effect on growth against *Pf* 3D7 parasites (Fig. 3).

In a concentration-dependent study (0–10 μ g/mL, Fig. 4) fractions 5–7 showed similar and modest activities, while fraction 8 exhibited the most promising antiplasmodial activity (Fig. 4A). The Pool of fractions 5–8 showed comparatively better activity (IC_{50} 3.99 μ g/mL) than fractions 5, 6, or 7 tested individually (IC_{50} 5.94–6.08 μ g/mL) (Fig. 4B) indicating the promise of fraction 8.

The active fractions and parent extract demonstrated resistance indices of ~1 or <1 and high selectivity (SI > 16 for CD and SI = 21 for C5–C8 pool) for the parasites. (Table 1). The isolated compound displayed antiplasmodial IC_{50} of 0.28 μ g/mL against *Pf* 3D7 but its IC_{50} against *Pf* W2mef (83.93 μ g/mL) was significantly higher, resulting in a high RI of 299.75 (Table 1). Interestingly the IC_{50} values for C5–C8 pool from which the potent compound was isolated were 3.99 and 2.24 μ g/mL for CQ-sensitive *Pf* 3D7 and CQ-resistant *Pf* INDO strain respectively, giving a resistance index of 0.56.

3.3. Inhibitory effect of the purified compound on β -hematin formation

Like chloroquine, the purified compound also inhibited heme polymerization (Fig. 5), but with an IC_{50} value that was approximately 5 times higher than that of chloroquine which is expected given the 150-fold difference between the antiplasmodial IC_{50} for purified compound (1.14 μ M) versus the IC_{50} for chloroquine (7.43 nM).

3.4. GC-MS profile of dichloromethane portion of *C. durandii* extract

The volatile components of the dichloromethane extract of *C. durandii* were detected using GC–MS analysis. The chromatogram revealed 35 compounds of which eight were major (SI Table 1, Fig. 6). Based on relative abundances, some identified major components were isopentyl alcohol (9.05 min, 2.5%), phenol (14.56 min, 5.446%), 6-Ethyl-5,6-dihydro-2H-pyran-2-one (15.54 min, 1.96%), 2,6-Dimethylpiperazine (15.68 min, 0.38%), o-Cresol (16.28 min, 2.18%), benzoic acid (18.57 min, 9.69%), catechol (19.02 min, 2.14%), 3-Methyl-2(5H)-furanone (19.34 min, 0.69%), Dihydrocoumarone (19.49 min, 0.89%), 2-Coumaranone (19.97 min, 0.46%), salicyl alcohol (20.42 min, 4.3%), 1,6-anhydro- β -D-glucopyranose (23.94 min, 4.49%), and neophytadiene (30.38 min, 0.83%). Interestingly, Marmesin was not detected in GC-MS analysis. This may have to do with either its small amount to be detectable or its tight binding to the chromatographic resin which may have come in the way of its elution from the column.

3.5. Characterization of isolated compound

The 1H NMR signals in $CDCl_3$ from the compound revealed 2 methyl

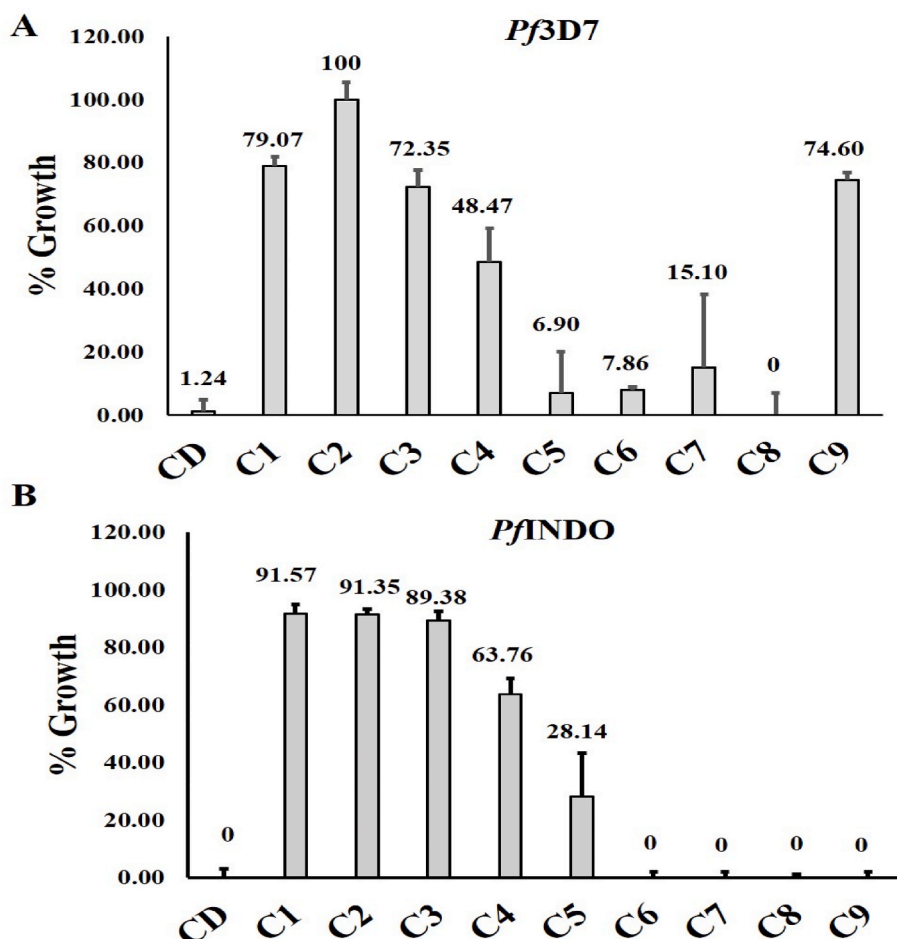


Fig. 3. *In vitro* antiplasmodial activity of *Celtis durandii* dichloromethane/methanol (1:1 v/v) mixture extract (CD) and its chromatographic fractions (C1 – C9) against chloroquine-sensitive (*Pf*3D7) (A), and chloroquine-resistant (*Pf*INDO) (B). Screening was done at 10 μ g/mL. Figures above each bar represent parasite growth inhibition (%) for each sample in comparison to untreated control taken as 100% growth. Each fraction was tested in triplicate, results show the mean $\pm$ standard deviations.

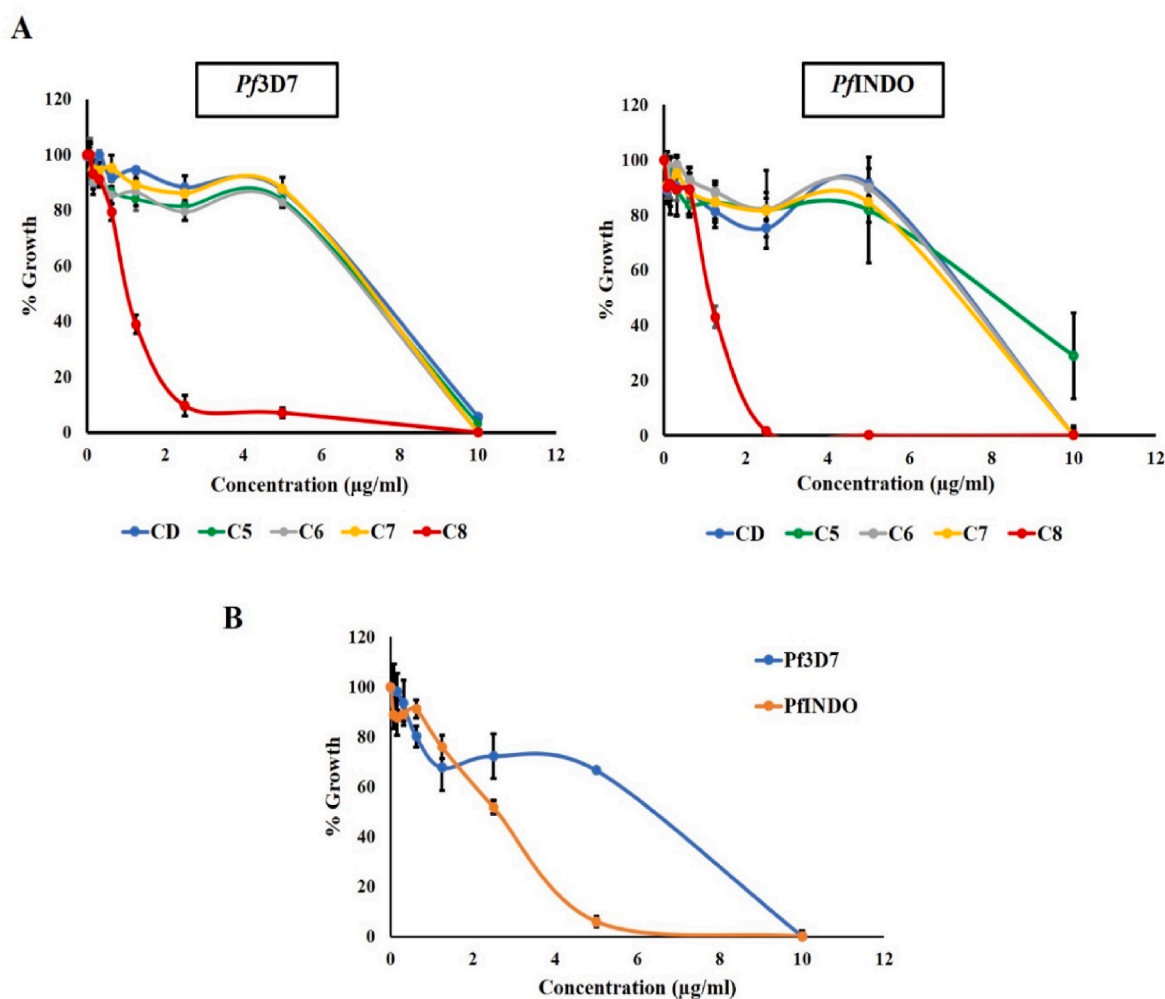


Fig. 4. Growth inhibition curves against the indicated strains of *P. falciparum* caused by *Celtis durandii* dichloromethane extract (CD) and fractions C5–C8 individually (A) and the combined pool of fractions C5–C8 (B).

Table 1

Antiplasmodial activity (IC_{50}), Resistance index (RI), mammalian cell cytotoxicity (CC_{50}), and Selectivity Index (SI) of *Celtis durandii* dichloromethane/methanol mixture extract and potent fractions.

Sample ID	IC_{50} Pf3D7 (μg/ml)	IC_{50} PfW2mef (μg/ml)	IC_{50} PfINDO (μg/ml)	Resistance Index (PfW2mef/Pf3D7)	Resistance Index (PfINDO/Pf3D7)	CC_{50} HUH (μg/ml)	Selectivity index (CC_{50}/IC_{50} Pf3D7)
CD	6.92 ± 0.37	5.92 ± 0.20	6.31 ± 1.00	0.86	0.91	>100	>14.45
C5	5.94 ± 2.43	nd	4.66 ± 1.59	–	0.79	nd	–
C6	5.95 ± 2.15	nd	6.21 ± 0.59	–	1.04	nd	–
C7	6.08 ± 0.39	nd	5.92 ± 0.48	–	0.97	nd	–
C8	1.04 ± 0.05	nd	1.14 ± 0.08	–	1.10	nd	–
C5–C8	3.99 ± 1.17	nd	2.24 ± 0.27	–	0.56	83.80 ± 1.65	21
Marmesin (μM, 95% CI)	1.14 (0.65–1.83)	340.82 (178.63–845.85)	nd	310	–	nd	–
Chloroquine (nM, 95% CI)	7.43 (5.22–10.51)	147.5 (51.88–211.1)	nd	19.85	–	–	–
Mefloquine (nM, 95% CI)	54.13 (1.83–5.05)	29.72 (38.63–112.6)	nd	0.55	–	–	–
Halofantrine (nM, 95% CI)	2.95 (35.15–82.87)	44.46 (3.386–10.31)	nd	15.07	–	–	–
Artesunate (nM, 95% CI)	6.00 (3.63–9.55)	16.79 (10.73–29.48)	nd	2.80	–	–	–

nd = not determined; CD = *Celtis durandii* dichloromethane/methanol mixture extract; C5–C8 = combined fractions.

singlets, a pair of methylene protons and four aromatic protons, while the DEPT ^{13}C NMR revealed 14 signals due to carbons attributable to a furanocoumarin (SI Figs. 1 and 2). Complete NMR chemical shift assignment, 2D NMR correlations (SI Figs. 3–6) and comparison with

literature (Joo et al., 2004; Perel'son et al., 1970; Znati et al., 2014; Awolola et al., 2019) and chemical databases confirmed the compound to be (2S)-2-(2-hydroxypropan-2-yl)-2,3-dihydrofuro [3,2-g] chromen-7-one, or marmesin (Fig. 7). The DSC thermogram of the

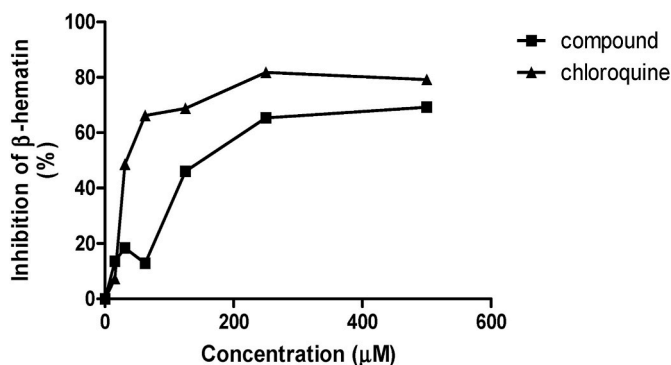


Fig. 5. Inhibition of β -hematin formation by isolated compound Marmesin (IC_{50} : 150 μ M), and Chloroquine (IC_{50} : 36 μ M).

isolated compound showed a melting point of 186.5 $^{\circ}$ C (SI Fig. 7).

4. Discussion

The antiplasmodial activity of *C. durandii* extract reported in this study aligns with its reported traditional use as a remedy against malaria (Adebayo and Krettli, 2011; Ibe and Nwifo, 2005). The extract showed promising activity against both CQ-sensitive *Pf*3D7 (IC_{50} 6.92 μ g/ml), CQ-resistant *Pf*INDO (IC_{50} 6.31 μ g/ml) and mefloquine-resistant *Pf*W2mef (IC_{50} 5.92 μ g/ml) parasites (Fig. 2B; Table 1), indicating that it contains phytoconstituent(s) that do not display cross-resistance with CQ and mefloquine. Reports supporting the antiplasmodial effect of this extract in the literature include Ezenyi et al. (2020) and Laryea and Sheringham Borquaye (2021). Besides earlier studies (Lacroix et al., 2011; Al-Musayeb et al., 2012) reporting antiplasmodial effects produced by coumarin-rich extracts of *Celtis africana* burm.f. further support our findings. *Celtis tessmannii* Rendle stem bark and root extracts also reportedly displayed good and selective antiplasmodial activity against CQ-sensitive and resistant parasites (Kagho et al., 2020). These collectively highlight the genus *Celtis* as a potential source of new, bioactive antiplasmodial compounds.

In our study, *C. durandii* active fractions also had low resistance indices similar to the crude extract, but the compound isolated from the pooled fraction had significantly lower activity against the drug-resistant *Pf* W2mef parasite compared to drug-sensitive *Pf*3D7 giving

rise to a resistance index of >300 (Table 1). Plant extracts are complex mixtures of specialized secondary metabolites in different amounts, and these influence the overall activity of the extract. The activity of these metabolites is influenced usually via synergy, additivity, and sometimes antagonistic effects too (Caesar and Cech, 2019). For instance, the antimalarial herb, *Artemisia annua* is well documented for its active constituent, artemisinin, but the plant contains also other secondary metabolites that demonstrate antiplasmodial efficacy and bioavailability-enhancing ability (Willcox et al., 2004). Bioactivity-directed separation of crude extracts helps to identify active fractions and compounds, but this does not always result in compounds with higher activity (Nothias et al., 2018). This is one of the limitations of focusing on the isolation of single bioactive compounds by fractionation, and approaching drug discovery in this manner may be reductionist and result in the loss of other compounds in an extract that contribute to the activity. Our findings suggest that other hitherto unknown active principles exist in the extracts and fractions of *C. durandii* which may directly or indirectly contribute to the antimalarial efficacy of marmesin.

The furanocoumarin compound, marmesin isolated from *C. durandii* displayed excellent activity (IC_{50} 0.28 μ g/ml) against CQ-sensitive *P. falciparum*. Naturally-occurring furanocoumarins consist of a furan ring fused to a coumarin moiety and are found in fruits and vegetables including celery, grapefruit, lemon, parsley, and parsnip (Alizadeh et al., 2020). Furanocoumarins exemplified by isomerperatorin, isopimpinellin, oxypeucedanin hydrate, and oxypeucedanin methanolate have been reported to exhibit antiplasmodial properties against chloroquine and multidrug-resistant *Plasmodium falciparum* strains *in vitro*, with low resistance indices (Wangchuk et al., 2014). Marmesin is a furanocoumarin that has been isolated from different medicinal plants,

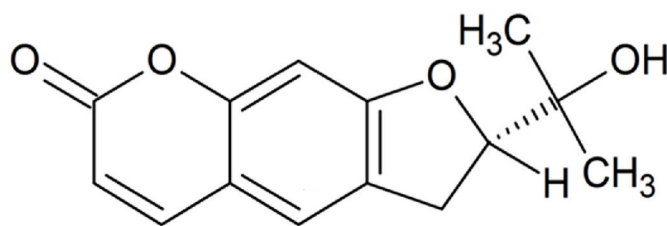


Fig. 7. Marmesin ($C_{14}H_{14}O_4$) isolated from antiplasmodial-active fractions of *C. durandii* root extract.

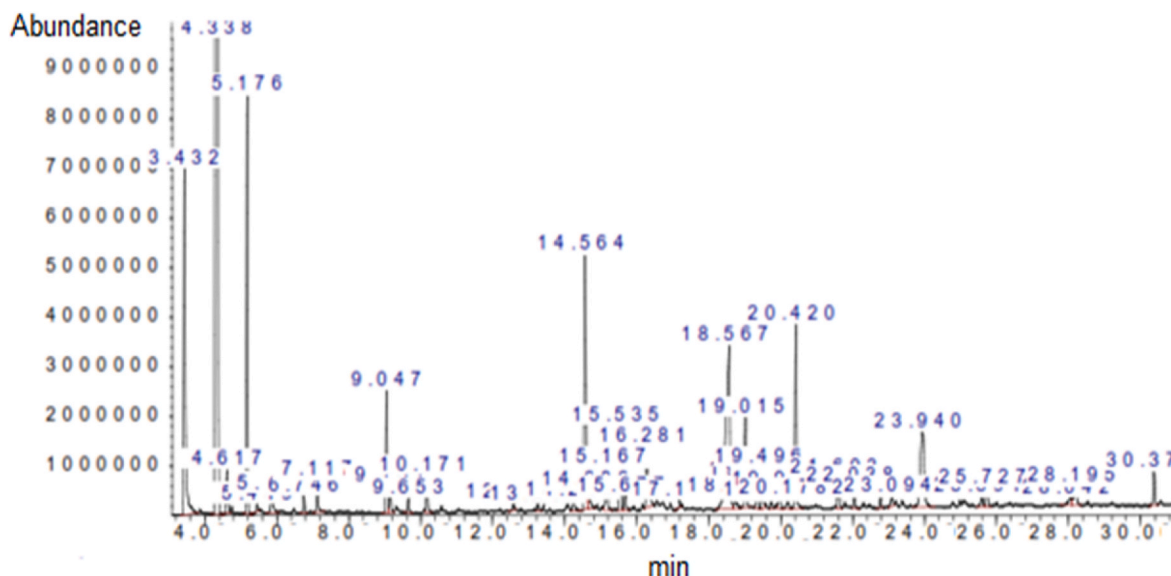


Fig. 6. GC-MS chromatogram of dichloromethane fraction of *C. durandii* extract.

including *Azadirachta indica* that is used as a traditional malaria remedy in Nigeria (Uwaifo, 1984). It also exhibited antiproliferative activity against different human cancer cell lines and was reported to have antimycobacterial and mosquito larvicidal effects (Dong et al., 2018; Kalimuthu et al., 2013; Kim et al., 2017; Suja et al., 2017; Wang et al., 2022). It has been reported to be non-cytotoxic to Human liver hepatoma (HepG2) cells (Jain et al., 2012).

In the current study, marmesin exhibited excellent antiplasmodial activity against CQ-sensitive *Pf*3D7 parasite (IC₅₀: 0.28 µg/ml) but showed a high antiplasmodial resistance index (RI: 310) between CQ-sensitive and multidrug-resistant *Pf*W2mef. However, the resistance index of the pool of fractions C5–C8 from which marmesin was isolated displayed an RI of 0.56 against the CQ-resistant *Pf* INDO strain. The *Pf*W2mef strain is associated with the amplification of *pfmdr1*, leading to mefloquine resistance (Nzila and Mwai, 2009). An inverse relationship exists between *pfmdr1* copy number and susceptibility to chloroquine vs mefloquine. Thus, parasites regained mefloquine susceptibility while resistance to chloroquine increased with the de-amplification of the *pfmdr1* gene (Barnes et al., 1992). The reduced susceptibility of *Pf*W2mef to marmesin is reminiscent of the decreased activity of mefloquine, corresponding with an increase in *pfmdr1* copy number (Nzila and Mwai, 2009). Although chloroquine (CQ) resistance is multifactorial, the resistance transporter *P. falciparum* CQ-resistant transporter (*pfcr1*) is the key protein mediating resistance to CQ with K76T mutation occurring in almost all CQ-resistant field isolates (Summers et al., 2012). CQ acts by inhibiting heme polymerization in the parasite's digestive vacuole (DV). Mutation in *pfcr1* causes CQ to be transported out of the parasite's DV, hence reducing its concentration at the site of action. The chloroquine resistance transporter in W2mef; the parent strain from which Dd2 was cloned carries the K76T mutation (Summers et al., 2012). Thus, marmesin may be a substrate of the mutant *pfcr1*. In essence, our observation suggests that like mefloquine and quinine which were recently reported to target *P. falciparum* purine nucleoside phosphorylase (*PfPNP*) in the parasite's cytosol (Dziekan et al., 2019), marmesin may either have a cytosolic target or may be a substrate for the mutant form of *pfcr1* which mediates CQ resistance. CQ resistance is known to be partially reversed by chemosensitizers like verapamil (Masseno et al., 2009). Therefore, the near-similar activity of the *C. durandii* extract against CQ-sensitive and CQ-resistant malaria parasite strains could be due to the presence of chemosensitizer molecules of the kind represented by verapamil in the extract. By implication, the antiplasmodial property of *C. durandii* is not wholly attributable to marmesin alone but may be due to the synergistic or additive action of the extract's phytoconstituents. It is also noteworthy that the type of effect (synergistic, additive, or antagonistic) produced by constituents of an extract can change, depending on their relative concentrations and the parasite strain used (Suberu et al., 2013).

Marmesin suppressed β-hematin formation and this may correlate with the ability of the compound to interfere with the parasite's heme detoxification pathways, thereby causing toxic free heme to accumulate within malaria parasites. The antimalarial mechanism of action of quinolines exemplified by chloroquine is based on the inhibition of heme detoxification in the parasite's acidic food vacuole where host hemoglobin is digested to amino acids (Herraz et al., 2019). The free heme produced as a by-product during this process is converted to an inert, insoluble pigment called hemozoin or the malaria pigment. Through their action, quinoline drugs cause a lethal build-up of free heme in the parasite, which increases oxidative stress and kills the parasite (Combrinck et al., 2013). Inhibition of heme detoxification is important for parasite death and is still relevant as a validated drug target for antimalarial leads. The *in vitro* β-hematin inhibition assay adopted in this study measures the ability of a compound to inhibit the conversion of free hemin to β-hematin, as observed with quinoline antimalarials (Herraz et al., 2019). Quinoline-resistant parasites possess resistance phenotypes that causes the drug to be effluxed from the parasitic food vacuole. Despite the enhanced fitness this phenomenon

confers on resistant parasites, it has been reported that a high concentration of effluxed drug in surrounding areas outside the food vacuole causes activation of other targetable metabolic pathways in the parasite (Untaroiu et al., 2019). An example of other essential metabolic pathways is the non-mevalonate pathway for isoprenoids metabolism. Computational studies predicted that in the presence of the drug (CQ), CQ-resistant parasites become more susceptible to non-mevalonate pathway inhibitors (Untaroiu et al., 2019). Thus, such metabolically vulnerable parasites can be eliminated by partner drugs which exploit this vulnerability by targeting alternative metabolic pathways. Although marmesin was shown to possess β-hematin inhibition activity (Fig. 5) and strong activity against CQ-sensitive parasites, its activity against drug-resistant parasites could potentially be enhanced by the presence of other non-heme targeting metabolites such as those that are present in the parent fraction and its extract. However, this needs experimental validation using *in vitro* cultured parasites.

Secondary metabolite profiling based on GC–MS is useful for identifying constituents from plants. Benzoic acid and salicyl alcohol also known as saligenin detected in *C. durandii* dichloromethane fraction (Fig. 6) have previously been identified in *Salix* spp. and *Populus laurifolia* (Babst et al., 2010; Greenaway et al., 1992; Zenk, 1967). Furan ring-containing compounds like 3-Methyl-2(5H)-furanone, Dihydrocoumarone, and 2-Coumaranone may be related to the presence of marmesin in *C. durandii* either as precursors or by-products of metabolite biosynthesis. These volatile constituents may serve as biological markers and will therefore be useful in the identification of *C. durandii* extract.

5. Conclusion

Celtis durandii Engl. root extract, fractions and marmesin isolated from the root extract, have antiplasmodial activity, and marmesin inhibited β-hematin formation. This is the first report on the isolation of marmesin from *C. durandii* and its antiplasmodial activity is also reported here for the first time. These findings need to be strengthened through further studies using *in vitro* and *in vivo* models of infection.

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CRediT authorship contribution statement

Ifema C. Ezenyi: Conceptualization, Methodology, Investigation, Funding acquisition, Writing – original draft. **Jersley D. Chirawurah:** Investigation, Resources, Writing – original draft. **Nekpen Erhunse:** Investigation, Writing – original draft. **Prakhar Agrawal:** Investigation. **Dinkar Sahal:** Writing – review & editing, Supervision. **John O. Igoli:** Investigation, Writing – review & editing.

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2023.116804>.

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