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Original Article

Rubia cordifolia L. Crude Aerial Part Extracts Show a High Potential as Antimalarials against Chloroquine Resistant *Plasmodium falciparum* Strains *in vitro*

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ABSTRACT

Introduction: *Plasmodium falciparum* human malaria parasite is resistant to most available medicines in Africa, necessitating research for new drugs. *Rubia cordifolia* L. is used in traditional malaria treatment in Kenya despite no supporting scientific data. **Objectives:** To evaluate anti-plasmodial efficacy of extracts from the aerial part of *Rubia cordifolia* against chloroquine sensitive (3D7) and resistant (W2) *Plasmodium falciparum* strains. **Methods:** Hexane, methanol and water were separately used in crude extractions from open shade dried aerial plant parts. Phytochemical composition was determined qualitatively. Anti-plasmodial efficacy of serial dilutions (range; 0.4 to 50 µg/ml) of each extract were evaluated *in vitro* and compared using ANOVA and Tukey's HSD test, at $p<0.05$). **Results:** All the extracts significantly inhibited parasites growth, in a dose dependent manner. At 50 µg/ml, inhibition was (84.07%, 77.94% and 66.08%) for hexane, methanol and water extracts respectively against W2 and (80.06%, 78.36% and 60.92%) against 3D7 strain. Hexane extract showed IC_{50} 0.551 µg/ml and 2.747 micrograms/ml against W2 and 3D7 respectively. Methanol extract, IC_{50} 1.231 and 3.528 µ g/ml respectively, aqueous extract showed moderate activity, IC_{50} 5.348 µg/ml and 17.341 µg/ml against the strains respectively. Chloroquine resistant (W2 strain) was more responsive to the extracts than 3D7 strain. **Conclusion:** Anti-plasmodial activities were in the order, Hexane>methanol>aqueous and were attributed to the phytochemicals present in the extracts. This presents evidence of *R. cordifolia* as anti-malarial drugs' source. Further work is required to identify, purify, and characterize active molecules for development of anti-malaria drugs.

Key words:Antimalarial activity, Crude extracts, *in vitro, Plasmodium falciparum, Rubia cordifolia*.

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INTRODUCTION

Despite control and elimination measures implemented through international and national programs worldwide, malaria remains one of the most dreadful parasitic disease of humans. In tropical and subtropical countries where malaria is endemic it is a leading cause of illness and death. Malaria is caused by one of the four main species of Plasmodium; *P. vivax, P. falciparum, P. malariae, P. ovale.*[1] *Plasmodium knowlesi* previously known to infect non-human primates, is acknowledged as the fifth human malaria parasite.[2-4] Among the five parasites, *P. falciparum,* accounts for 99% of confirmed malaria cases and have developed resistance to conventional malaria medications.[5] The most vulnerable groups are young children below age 5 years, whose immunity is yet to develop and pregnant mothers whose immunity is diminished because of pregnancy. Immune compromised people with underlying conditions such as HIV/AIDS and non-immune travelers to endemic areas are highly susceptible to infection.[6,7] Recently, patients with COVID-19 and *P. falciparum* co-infection were observed to develop clinical symptoms such as confusion and vomiting more frequently than those with single infection and this causes potential clinical and therapeutic implications.[8] It was estimated that a half of the world's population was at risk of malaria in the year 2020, where the number of cases increased to 241 million from 227 million in 2019,[9] (retrieved July 2022). The number of deaths due to malaria increased by 69,000 to an estimated 627,000 deaths globally from 2019. Africa suffered the highest malaria burden and over 96% of all malaria deaths in year 2020.[9]

Medicinal plants have proved to be the best natural sources of various drugs. About 80% of people from developing countries use traditional medicines, involving the use of herbs, crude herbal

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preparations and/or finished herbal products that contain plants parts or substances as active ingredients.[10] In the past decade, a rising trend in the use of complementary and alternative medicine (CAM) or herbal remedies, which is closely related to traditional medicine, was witnessed in the developed countries.[11] In Ethiopia, about 90% of the population use herbal remedies for their primary healthcare, while 70% of the population in developed countries such as Germany and Canada are reported to have tried CAM at least once.[11,12] Traditional medicines have been used to treat malaria for many years while the two main antimalarial compounds, artemisinin and quinine were originally obtained from plants. With the increasing drug resistance, unaffordability, and inaccessibility of conventional antimalarial drugs in poor rural areas, traditional medicines are an important and sustainable source of treatment.[13] In Kenya, majority of the population (above 70%) live in rural areas and are the most affected by malaria and depend on herbal medication.[14,15] However, even with the extensive use, little or no scientific data is available to justify the use of some herbs.[16] It is important that such plants be investigated for better understanding of their properties, safety and efficiency.

Rubia cordifolia, is a flowering plant of the *Rubiaceae* family, similar to coffee. It is native in forests in India, China, Pakistan, Korea, Japan and Mongolia where it is known as common madder, manjshtha or Indian madder. It is a climbing herb with leaves arranged in a whorl of four with quadrangular petioles and prickly on the angles. Flowers occur in cymes and are green-white in color while fruits are didymous or globose, smooth, shining, and purplish black when ripe.[17] *Rubia cordifolia* extracts from the various parts of the plant are shown to have several pharmacological benefits including acne and wound treatment, anti-diuretic, anti-ulcer, cardiac disorders and various anti-inflammatory conditions.[18,19] *R. cordifolia* is commonly used mostly by the Maasai, Kuria and Kisii communities in Kenya for the treatment of malaria and other diseases.[20] This study aimed to investigate the anti-plasmodial efficacy of extracts from the aerial parts of *Rubia cordifolia* against *P. falciparum* chloroquine sensitive and resistant strains and qualitative phytochemical composition. No previous work had investigated the potential anti-malarial activity of this plant on *P. falciparum* strains. *In vitro* assays were used as they are effective for testing the sensitivity of malaria parasites to anti- malarial drugs and provide complementary data to epidemiological studies of drug-resistant malaria.[21]

MATERIALS AND METHODS

Collection and Processing of Plant Materials

Fresh leaves and stems of *R. cordifolia* L. were collected in Trans Mara West sub-county, Kenya occupied by the Maasai community in April 2015. The plant was identified at the East African herbarium, the National Museums of Kenya (NMK). Leaves were dried under shade for 14 days and then ground into powder using a hammer mill (8 LAB MILL CHRISTY AND MORRIS LIMITED, ENGLAND) at Technical University of Kenya. The powder was packed into clean airtight plastic containers, stored in a dark, cool, and dry place before solvent extraction at the Pharmacognosy Laboratory at Kenyatta University as previously described.[22]

Extraction of Plant Material

One hundred and fifty grams (150g) of powdered dry leaves and stem mixture were soaked in 250 ml of 95% methanol and hexane separately for 48 hr at room temperature with constant shaking to obtain respective extracts.[23,24] Each mixture was filtered (Whatman filter paper No. 1) and filtrate rotor-vapored (BUCHI, SWITZERLAND) to concentrate it to a constant dry weight. To generate an aqueous extract, 150g of the powdered plant material was soaked in 250 ml of deionized water at 60°C for 24 hr. The mixture was filtered and lyophilized at a vacuum pressure of -1 and a temperature of -40°C for two days. The resulting extracts were transferred into separate pre-weighed sample bottles and stored at -20°C until required for assays.[25,26]

Phytochemical Screening of Extracts

Aqueous, methanolic and hexane preparations were subjected to phytochemical analysis using standard methods.[24-27] Betacyanins, quinones, volatile oils, carbohydrates, alkaloids, proteins, resins, phlabotannins, terpenoids, phenols, saponins, fixed oils, flavonoids, tannins and emodals were evaluated qualitatively. Phlobatannin's were assayed by adding 2ml of crude extract of containing 1mg/ml in a test tube followed by 2ml of 1% hydrochloric acid (HCl test) and the mixture heated to boiling. Formation of a red precipitate indicated phlobatannins' presence.[24-27]

The presence of betacyanins was evaluated using 2ml of 1mg/ml of each solvent plant extract mixed with 1ml of 1M sodium hydroxide in clean test tube. The mixture was heated at 100°C for 5 min. Appearance of yellow coloration is an indicator of betacyanins present.[27] Quinones were tested according to the procedure by,[28] where to 1ml of 1mg/ml of plant extract, was added 1ml of concentrated sulphuric acid and allowed to stand for 5 min. Appearance of a red pigmentation indicated the presence of quinones in the extract. Fixed oils and fats were evaluated using Stain/ Spot Test and saponification test.[29] About 100 mg of each extract were separately put a filter paper and pressed against another filter paper. Formation of translucent mark on the filter papers indicated presence of oils and fats.

Saponification test was performed by adding a few drops of 0.5M alcoholic potassium hydroxide to small quantities of each extract separately with a drop of phenolphthalein. The mixture was heated in a water bath for 1-2 hr. Formation of soap indicated the presence of fixed oils and fats. The Foam Test procedure was used

to demonstrate saponins' presence using about 0.5g of the plant extracts separately added into test tubes and 5 ml of water added into each fraction. The mixture was shaken vigorously for 15 min and formation of foam that persisted for about 15 min indicated saponins presence in the extract.[29] To test for flavonoids, 2-3 drops of dilute NaOH was added into a clean test tube containing 1 ml of extract solution. An intense yellow color appeared, which became colorless on adding a few drops of dilute acid indicated the presence of flavonoids. Using ferric Chloride test, a few drops of neutral Ferric chloride ($FeCl_3$) solution was added to 2 ml of each solvent extract solution in distilled water (1mg/ml), a blackish red color forms, indicating the presence of flavonoids.[30]

The presence of phenols and tannins was evaluated using Ferric Chloride, where 2-3 drops of neutral (5%) Ferric chloride solution was slowly added along the wall of a test tube containing 1 ml of the filtrate (1mg/ml). A reddish-green-blue or violet coloration indicated the presence of a phenolic hydroxyl group/ phenols presence.[30,31] Tannins were also analyzed by Ferric chloride test using 2 ml of each solvent extract (50mg/ml) by adding 2-3 drops of 5% ferric chloride ($FeCl_3$). A greenish to black transient color was positive for tannins presence. Volatile oils were detected using 1ml of each extract solution at 1mg/ml in separate tubes, and adding 1ml of 90% ethanol followed by four drops of Iron (III) chloride solution. Appearance of a green color indicated volatile oils were presence.[31]

The evaluation of resins, terpenoids, and cardiac glycosides were carried out as per previous studies.[31-33] Acetone-H_2O Test was used to demonstrate resins presence by adding 2ml acetone to 100mg of extract in a test tube and 1ml of distilled water and shaken thoroughly. Turbidity showed the presence of resins in the extract. Terpenoids assay used Salkowski test where 2ml of chloroform was added to 1ml of 1mg/ml of each solvent extract in separate test tubes. While holding the test tube in a slanting level, 3ml of concentrated sulphuric acid was added slowly down the wall of the test tube resulting in a two layered mixture. An appearance of a red-brown color at the boundary between the layers indicated terpenoids presence.[33]

Cardiac glycosides were tested by adding 3 drops of a strong lead acetate solution to 2 ml of the extract solution, then mixed thoroughly and filtered. The filtrate was mixed by shaking with 5 ml of chloroform and the chloroform layer evaporated in a evaporated. The remaining residue was dissolved by adding 2 ml of glacial acetic acid containing a trace of ferric chloride and then transferred to the surface of a tube containing 2 ml concentrated sulphuric acid. The upper layer and interface of the two layers were observed for bluish-green and reddish-brown coloration respectively indicative of cardiac glycosides presence in the extract.[33] Emodols (anthracenosides aglycones) assay used 200mg of each dried extract added to 5ml of 25% ammonia solution in separate tubes. The appearance of a red color in the solution indicated the presence of emodols.

Evaluation of extracts for anti-plasmodial activity

Each of the three solvents extracts (water, methanol and hexane) were used for preparation of the test drugs. To prepare the test drugs, plant extracts were first dissolved separately in 50µl of Dimethyl Sulfoxide (DMSO) and diluted in the malaria culture medium (RPMI 1640 (GIBCO)) to make 10ml of 1mg/ml stock solution and stored at -20°C till required for use.[22] The stock solutions were filtered with 0.45 µm and 0.22 µm micro-filters (Minisart® CE) before use in assays.

Prior to the assay, the 1mg/ml stock solution was further diluted in malaria parasites culture medium to give 50µg/ml being the highest tested extract concentration. Eight serial 2-fold dilutions with a final concentration range of 50 to 0.4µg/ml were prepared in microtiter plates. Stock solutions of chloroquine and artemether (Sigma Aldrich) at a concentration of 1µg/ml each were similarly prepared using distilled water and 70% ethanol respectively for use as reference drugs.

Preparation of Parasite culture medium

Malaria parasites culture medium was prepared as described previously,[34,35] by mixing 90.4% v/v RPMI 1640 (GIBCO) with 3.8% HEPES, containing 5% w/v sodium bicarbonate and 1% v/v of 20% w/v D-glucose and 1% of 200mM L-glutamine. The medium was filtered using 0.22µm filters and 25µl of 50mg/ml gentamycin antibiotic added to prevent contamination. The culture was stored at 4°C in aliquots of 45ml in sterile 50ml tubes (CORNING®) until required for use. Prior to use in parasite culturing, each aliquot was supplemented with 5ml (10%) of heat inactivated haemo-serum.

Parasite cultivation

Chloroquine resistant *P. falciparum*, W2 and chloroquine susceptible 3D7 strains were obtained from parasite bank in the Institute of Primate Research, Nairobi, Kenya. Continuous parasite cultures were maintained in RPMI 1640 (GIBCO) media supplemented with human O Rhesus positive erythrocytes and maintained at 2% parasitemia during the assays,[36,37] in sterile tissue culture flasks (CORNING®).

Anti-plasmodial activity test

Anti-plasmodial activity assays were performed in triplicate in sterile flat bottomed 96-well microtiter tissue culture plates (COSTER®). Twenty-five microliters of complete culture media was first put in all wells except row A. Fifty microliters of the extracts at 50µg/ml was added to row A wells in triplicates for each extract. Using a multichannel pipette (Thermo scientific finnpippete® Finland), 8 serial two-fold dilutions of the extracts were done by removing 25µl from row A that was added to row B wells and serially diluting to row H and the last 25µl from row H was discarded. Two hundred µl of parasite cultures at 1% parasitaemia and 1.5% haematocrit majorly at ring stage was

added into each well. Eight extract free wells were included to serve as negative control.[38] Chloroquine and artemether were similarly set alongside the extracts to serve as positive control. The plates covered with lids, were placed in airtight chamber with water dampened paper towel and gassed for three minutes (92% N_2, 5% CO_2, and 3% O_2; BOC, KENYA LTD). Incubation was done at 37°C for 48 hr.[38] All the contents of each well were harvested into sterile labeled 1.5ml Eppendorf tubes and centrifuged at 3500 rpm (Beckman, USA) for 3 sec. The pellet was used to make a thin smear on clean labeled microscope slides and stained (10% Giemsa for 15 min). Parasitemia was determined by counting developed schizonts against a total of 2000 erythrocytes. The untreated control parasite culture was considered as 100% growth and percentage parasitemia as well as percentage growth inhibition for each drug diluted concentration determined as per the formulae.[39]

$$AV\%\ P = \frac{AV\ Ctrl - AV\ Treated}{AV\ Ctrl} \times 100$$

Where AV % P is Average percentage suppression of parasitemia, AV Ctrl is average parasitemia in the control wells and AV treated is the average parasitemia in treated wells.

Percentage parasitemia= (Number of infected red blood cells ÷ Total number of red blood cells) x 100.

% Inhibition = (Mean parasitemia in negative control wells, minus Mean parasitemia in treated wells) ÷ (Mean parasitemia in negative control wells) X 100

IC_{50}, the concentration required to inhibit schizonts' growth by 50% was determined from dose response curves by nonlinear regression analysis using graph pad prism version 5.0. Low 50% inhibitory concentrations (IC_{50} values) indicated that a particular solvent extract was potent against the plasmodium parasite strains.

RESULTS

The weight of extracts obtained from 150g powder of dried plant material were highest with aqueous solvent and decreased with decreasing polarity of solvent (Table 1).

The anti-plasmodial activity of the extracts were indicated by the reduction in the proportion of parasitized erythrocytes in extract treated wells compared to untreated controls at 37°C and 48 hr of incubation of *P. falciparum* W2 and 3D7 strains at the ring or trophozoite stage (Table 2). The mean parasitemia in control wells (Mean, 4.65 ± 0.327) was significantly higher than the parasitemia in extracts treated wells for all treatments, (ANOVA, F= 88.19, $p<0.001$) indicating that all the three *R. cordifolia* crude extracts possessed anti-plasmodial activity.

The percentage mean parasites growth (mean ± standard deviation) in wells treated with varying concentrations of each extract for the plasmodium strains in reference to negative control (assumed to be 100%) are shown in Table 3 while Table 4 summarizes the percentage suppression/ inhibition of parasites growth at various extracts concentrations.

The results revealed a dose dependent inhibition of the parasite growth by all the solvent extracts. All the 8 extract concentrations (range; 0.4 to 50 µg/ml) showed a significant reduction of parasitemia for both parasite strains ($p<0.05$) compared to untreated controls (Table 4). Hexane extracts exhibited the highest inhibition of plasmodium growth at the highest extract concentration against both W2 and 3D7 strains (84.07 and 80.06% respectively). At a concentration of 6.25µg/ml both hexane and methanol extracts showed high (>70%) parasite growth inhibition against both strains, while water extracts showed moderate activity of about 50% inhibition of growth against both strains (Table 4). At the highest concentration, aqueous (water) extract exhibited the lowest schizont growth inhibition against both W2 and 3D7 *P. falciparum* strains (66.08 and 60.92%) respectively. At the highest extract concentration used, schizont growth inhibition was in the order hexane > methanol > water extract for both strains. At the lowest concentration, all extracts exhibited low activity against both strains, (<50% inhibition). Chloroquine resistant (W2) appeared to be overall more responsive to *R. cordifolia* extracts than chloroquine sensitive (3D7) strain.

The mean inhibitory concentration (IC_{50}), inhibiting 50% parasite growth for the extracts and reference drugs against *P. falciparum*, W2 and 3D7 strains are presented in Table 5. Hexane extract recorded the highest anti-plasmodial activity as indicated by its lowest IC_{50} (0.552µg/ml and 2.747µg/ml) against *P. falciparum*, W2 (CQ resistant) and 3D7 (CQ sensitive) respectively, followed by methanol and water extracts. IC_{50} values for water extracts against both strains were significantly higher (t- test, $p=0.02$) (significance level, $p < 0.05$) than IC_{50} values of hexane extract.

Qualitative phytochemical composition of the three solvent extracts of *R. cordifolia* are presented in Table 6. The extracts were shown to contain quinones, terpenoids, flavonoids, (present in all extracts). Hexane and methanol extracts had common phytochemicals except resins and tannins while the aqueous extract contained fewest phytochemicals. Phlobatannins, phenols, saponins and emodals were all absent in all extracts.

DISCUSSION

The present study reports *in vitro* anti-plasmodial activity of hexane, methanol and water crude extracts of *Rubia cordifolia*, a herb used in traditional medicine among the Maasai people, in Narok County, and other communities in Kenya. *R. cordifolia* was chosen based on documented ethnobotanical information.[20-22] The results showed a dose-dependent suppression of *Plasmodium falciparum* parasites growth by all the three solvent extracts. Activity of the extracts was classified as high when percentage parasite inhibition was ≥70%, active for <70% inhibition and >50 and inactive if inhibition <50% at extract concentration

Table 1: Quantity of extract obtained from 150 gram of powdered plant material using the three different extracts.

Plant name	Plant part(s)	Powder Weight (g)	Weight of extracts (g) and solvents used		
R. cordifolia	Stem and leaf	150	Water	Methanol	Hexane
			26.4	8.4	7.29

Table 2: Mean parasitaemia % in extracts treated and control wells for the two strains of *Plasmodium falciparum*.

Treatment	*P. falciparum strains*	
	W2 strain	3D7 strain
	Mean ± S.E	Mean ± S.E
Aqueous	2.49 ± 0.22[a]	2.42 ± 0.09[a]
Hexane	1.84± 0.19[c]	2.05 ± 0.21[a]
Methanol	2.90 ± 0.15[a]	2.79± 0.13[b]
Negative control	4.53± 0.10[b]	4.76 ± 0.12[c]
F-value	51.17	88.19
P-value	0.001	0.001

Mean values in the same column followed by the same letter are not significantly different (Tukeys HSD test, $p \leq 0.05$).

Table 3: Mean Parasites Growth at Varying Extract Concentrations of *R. cordifolia* stem and leaves extracts.

Extract concentration (µg/ml)		0.4	0.8	1.6	3.125	6.25	12.5	25	50
P_f Strain	Solvent / extract	Parasite Growth % Mean ±S.D							
3D7	Water	56.51±4.6	53.99±8.5	52.77±9.3	51.52±7.4	49.38±4.8	47.99±3.4	40.63±3.6	39.92±6.3
	MeOH	59.24±3.78	54.71±3.8	51.62±5.7	41.18±4.9	29.26±3.2	27.98±4.6	25.77±2.4	21.64±3.4
	Hxn	61.89±7.7	55.82±5.1	53.11±6.5	41.89±7.6	27.73±5.3	25.84±4.2	24.97±3.4	19.94±3.6
W2	Water	67.94±6.6	65.42±9.1	60.9 ±7.7	53.61±7.7	42.95±5.1	41.68±3.5	37.80±3.8	33.92±6.6
	MeOH	70.48±3.9	60.04±4.5	47.3± 5.9	38.16±9.3	29.85±5.3	25.93±4.9	23.98±3.12	22.06±3.5
	Hxn	52.42±6.8	45.37±5.3	37.68±6.8	32.21±7.9	26.89±5.5	24.95±4.4	20.44±4.7	15.93±3.8

$p \leq 0.05$ Compared to the control, assuming Control= 100% parasite growth; MEOH=Methanol; S.D=standard deviation; Hxn=hexane

Table 4: Percentage Inhibition of Schizonts Growth by the stems and leaves Extracts of *R. cordifolia*.

P_f strain	Solvent Extract	Concentration of Extracts (µg/ml)							
		0.4	0.8	1.6	3.125	6.25	12.5	25	50
3D7	Water	43.49	46.01	47.23	48.52	50.62	52.01	59.37	60.92
	Methanol	40.76	45.29	48.38	58.82	70.74	72.02	74.23	78.36
	Hexane	38.11	44.18	46.89	58.11	72.27	74.16	75.04	80.06
W2	Water	32.06	34.58	39.09	46.39	57.05	58.32	62.20	66.08
	Methanol	29.52	39.96	52.63	61.84	70.15	74.07	76.02	77.94
	Hexane	47.58	54.63	62.32	67.79	73.11	75.05	79.56	84.07

Tukeys HSD test, $p \leq 0.05$ Comparing the control to the treatments, assuming Control= 100% parasite growth; MEOH=Methanol; Hxn=hexane.

Table 5: Mean IC_{50} values for the solvent extracts and reference drugs against *P. falciparum*, W2 and 3D7 strains.

Solvent extract/ Drug	IC_{50} values µg/ml	
	W2 strain	3D7 strain
Aqueous	5.348	17.340
Hexane	0.552	2.747
Methanol	1.231	3.528
CQ	0.129	0.030
ATM	0.065	0.051

Positive control drugs: CQ-Chloroquine; ATM-artemether.

Table 6: Phytochemicals demonstrated in the different solvent extracts of *R. cordifolia* Aerial Parts.

Test	Aqueous extract	Hexane extract	Methanol extract
Betacyanins	+	-	-
Quinones	+	+	+
Volatile oils	-	+	+
Alkaloids	-	+	+
Resins	-	+	-
Phlobatannins	-	-	-
Terpenoids	+	+	+
Phenols	-	-	-
Saponins	-	-	-
Fixed oils	-	+	+
Flavonoids	+	+	+
Tannins	+	-	+
Cardiac glycosides	-	+	+
Emodols	-	-	-

KEY: + indicates presence of bioactive compound. - Absence of bioactive compound. Phytochemicals shown in bold were present in all the three solvent extracts.

of 6µg/ml.[40] The 6µg/ml concentration was emphasized with the reasoning that drugs showing more than 50% inhibition of parasitemia at a concentration of 6µg/ml normally have an IC_{50} <6µg/ml.[40] Based on this, hexane and methanol extracts were classified as highly active against both strains (inhibition ≥70%) at concentration of 6.25µg/ml and 50 µg/ml. Water extract recorded moderate activity (inhibition <60%, and IC_{50} of 5.348µg/ml and 17.34µg/ml against *P. falciparum* 3D7 and W2 respectively. Previous results,[22] recorded a high activity, IC_{50}=1.5µg/ml and 2.5µg/ml for methanol and water leaf extracts of *R. cordifolia* respectively against *Plasmodium knowlesi.* This result also corroborated with those of a related plant, *Morinda lucida* Benth (family: Rubiaceae) which recorded 63.4% and 65.0% inhibition of *P. falciparum* by ethanol extract and by dichloromethane (DCM) or methylene chloride (CH_2Cl_2) extracts.[40,41] At a concentration of 6 µg/ml, crude extract from *Morinda lucida* Benth stem bark using dichloromethane (DCM): Methanol (MeOH) in a ratio of 1:1 (*v/v*), completely suppressed the growth of *P. falciparum* 3D7 strains.[40,41] The results indicate that the many phytochemical compounds demonstrated in the crude extracts may exert varying anti-plasmodial effects and might be acting in synergy and therefore supports the traditional use of *Rubia cordifolia* just as *M. lucida* for malaria treatment. The anti- plasmodial activity observed in the present study was hexane extract>methanol extract >water extract based on IC_{50} values. This variation in activity could be due to presence of active lipophilic constituents which do not extract into the more polar water solvent, as non-polar extracts were more active than polar ones.[40-41] Previous studies using a range of solvents of varying polarity from n-hexane through ethanol to water resulted in higher extract yields with the highly polar solvent but of lower phenolic and flavonoid content as compared to non-polar ones.[42] Different amounts of active phytochemicals extracted by the different polarity solvents could also contribute to the variation in the anti-plasmodial efficacy of the solvent extracts.

Chloroquine resistant *P. falciparum*, W2 appeared more responsive to *R. cordifolia* extracts than chloroquine sensitive *P. falciparum,* 3D7. For instance, at the highest concentration, hexane extract was observed to have inhibition 84.07%, IC_{50}=0.552µg/ml and 80.06%, IC_{50} 2.747µg/ml against W2 and 3D7 respectively. This suggested possible absence of cross- resistance between the active phytochemicals present in the extracts with chloroquine, most likely because of dissimilarities in the manner of action of their molecules. This finding suggests that active principles from this plant extracts could be potential alternatives of reversing chloroquine resistance. This study therefore corroborates well with,[43] who reported moderate activity, IC_{50} value of 5.6µg/ml and high activity (IC_{50} of 4.4µg/ml) against chloroquine sensitive *P. falciparum*, D6 and chloroquine resistant W2 respectively for water-methanol extract from *Harrisonia abyssinica* Oliv. Comparing the extracts with Chloroquine and artemether positive controls, Chloroquine had IC_{50} values 0.030 µg/ml and 0.129 µg/ml respectively against chloroquine sensitive and resistant strains of *P. falciparum*. On the other hand artemether had IC_{50} values of 0.051 µg/ml and 0.065µg/ml respectively against the CQ sensitive and resistant *P. falciparum* strains.

Previous studies have reported high anti-plasmodial activity for various crude extracts with low IC_{50} values. Other studies,[44] reported IC_{50} of 1.03µg/ml for *Baccharis salcifolia* on *P. falciparum* D6 while,[45] obtained IC_{50} of 0.38 µg/ml for *Cissampelos mucronata* against KI strain. In another study, a significant dose dependent inhibition of plasmodial growth by n-hexane extract from *Pleurotus ostreatus*, edible mushroom on *P. falciparum* 3D7 was reported.[46] *Artemisia annua* and *Azadirachta indica* from which artemisinin and gedunin were isolated with IC_{50} in nanomolar concentrations had IC_{50} of 3.9 and 10 respectively.[47] The present study is the first reporting activity of *R. cordifolia* on *P. falciparum* strains. The low IC_{50} values recorded for *R. cordifolia* herein are indicators of high efficacy and validate the use of the plant in herbal medicine practice. However, recommendations by WHO,[5] are that any potential herbal medicines must be effective, safe, and of good quality before they can be applied for public use.

Phytochemical analysis revealed the presence of betacyanin, quinones, terpenoids, flavonoids and tannins in water extracts and absence of volatile oils, alkaloids, resins, phlabotannins, phenols, saponins, fixed oils, cardiac glycocides, and emodals. Methanolic extract contained quinones, volatile oils, alkaloids, resins, phlabotannins, fixed oils, flavonoids and cardiac glycosides. On the other hand, hexane extract contained quinones, volatile oils, alkaloids, terpenoids, fixed oils, flavonoids, tannins and cardiac glycosides. Different solvents have diverse capacities for different phytochemical compounds.[48] The current study correlated with a study by,[49] which reported the presence of alkaloids, cardiac glycosides, tannins, flavonoids. phenols and steroids in the same plant species from a different geographical region. Many phytochemicals identified in this study including alkaloids, saponin, tannins, phenol, and anthraquinone have been demonstrated in medicinal plants used to treat malaria, such as *Lecaniodiscus cupanioides* while alkaloids have been implicated in anti-malarial activity of many plants.[50] Different classes of alkaloids contained in the extract of Nigella sativa (black seed) are believed to block protein synthesis in *Plasmodium falciparum.*[51] Triterpenoid and steroid saponins have been found to be detrimental to many infectious protozoans including *Plasmodium falciparum.*[52] The findings of this study suggest that the identified phytochemical components may be responsible for the anti-plasmodial activity recorded and that *R. cordifolia* is proving to be a valuable reservoir of active phytochemicals of anti-malarial value.

CONCLUSION

This study established that *R. cordifolia* crude extracts contain several bioactive compounds of medicinal importance. The significant plasmodium growth inhibition observed provides evidence supporting *R. cordifoila* use for future anti-malaria drugs development. Hexane and methanol extract showed high anti-plasmodial activity while aqueous extracts showed moderate activity.

RECOMMENDATION

The study provides a scientific support for *R. cordifolia* extracts as antimalarials. The bioactive phytochemicals in the plant extracts should be isolated, purified, characterized and separately investigated to obtain effective, quality and safe chemotherapeutic agents as an alternative treatment for both chloroquine sensitive and resistant *Plasmodium falciparum* strains.

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CONFLICT OF INTEREST

The authors declare that they have no conflicting or competing interests.

AUTHORS' CONTRIBUTIONS

J. Magara- developed the concept, did the experiments, generated and analyzed the data, literature review, prepared the first draft of this manuscript, definition of intellectual content, guarantor. L. Kamau- reviewed the concept, experimental design, literature

search, data collection, analysis and statistics, detailed review of the manuscript, updating and editing, definition of intellectual content, guarantor. G. Nyambati- reviewed the concept, experimental design, literature, data analysis, manuscript review. H. Ozwara- provided the laboratory facilities and materials, reviewed the concept, experimental design, supervised the day-to-day experiments of the project. definition of intellectual content, guarantor. All authors contributed to the final version of the manuscript.

ABBREVIATIONS

ATM: Artemether; **BOC:** British Oxygen Company; **CAM:** Complementally alternative medicine: **CO_2:** Carbon dioxide; **CQ:** Chloroquine; **DMSO:** Dimethylsulphoxide; **D6:** Chloquine sensitive strain of *Plasmodium falciparum*; **g:** grams; **HCL:** Hydrochloric acid; **HEPES:** 2-hydroxyethlpiperazine-N-2ethanesulfonic acid; **IC_{50}**: Drug concentration killing 50% of test organisms *in vitro*; **IPR:** Institute of Primate Research; **KEMRI:** Kenya Medical Research Institute; **K39:** Chloroquine sensitive *P. falciparum* strain; **MeOH :** Methanol; **Ml:** Milliliter; **M:** Molar concentration; **Mg:** milligrams; **Mg:** Microgram; **N_2**: Nitrogen; **NaOH:** Sodium Hydroxide; **NMK:** National Museums of Kenya; **O_2:** Oxygen; **P. f:** *Plasmodium falciparum*; **RBCs:** Red Blood Cells; **Rpm:** Rounds Per Minute; **RPMI**: Roswell Park Memorial Institute Medium; **WHO:** World Health Organization; **W2:** Chloroquine resistant *Plasmodium falciparum* strain; **3D7:** Chloroquine sensitive *Plasmodium falciparum* strain; **ºC:** Degree Celsius.

SUMMARY

The anti-plasmodial activity of stems and leaves of *R. cordifolia* collected in Kenya and extracted separately using Hexane, methanol and aqueous was evaluated *in vitro* against Chloroquine susceptible (3D7) and resistant strains (W2) of *Plasmodium falciparum*. A dose dependent inhibition of parasite growth was shown by all the extracts using concentrations range; 0.4 to 50 µg/ml, (ANOVA, $p<0.05$), compared to untreated controls. Hexane and methanol extracts recorded high anti-plasmodial activity while aqueous was moderate based on parasite growth inhibition and IC_{50} values. Hexane extract gave the lowest IC_{50} (0.552µg/ml against W2 (CQ resistant) and 2.747µg/ml against 3D7 (CQ sensitive) showing high anti-plasmodial activity. The ant-plasmodial effects were attributed to various bioactive compounds present in the plant extracts. The results support the current use of *R. cordifolia* as traditional antimalarials. The bioactive phytochemicals should be isolated, purified, characterized, and separately investigated to obtain effective, safe and quality chemotherapeutic agents as an alternative treatment for malaria.

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