



Cuscuta reflexa Possess Potent Inhibitory Activity Against Human Malaria Parasite: An In Vitro and In Vivo Study

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Abstract

Drug resistance to practically all antimalarial drugs in use necessitate the development of new chemotherapeutics against malaria. In this aspect, traditionally used plants with folklore reputation are the pillar for drug discovery. *Cuscuta reflexa* being traditionally used in the treatment of malaria in Odisha, India we aimed to experimentally validate its antimalarial potential. Different solvent extracts of *C. reflexa* or column fractions from a promising solvent extract were evaluated for in vitro anti-plasmodial activity against *Plasmodium falciparum* strain Pf3D7. Potent fractions were further evaluated for inhibition of parasite growth against different drug resistant strains. Safety of these fractions was determined by in vitro cyto-toxicity, and therapeutic effectiveness was evaluated by suppression of parasitemia and improvement in survival of experimental mice. Besides, their immunomodulatory effect was investigated in Pf-antigen stimulated RAW cells. GCMS fingerprints of active fractions was determined. Column separation of methanol extract which showed the highest in vitro antiplasmodial activity ($IC_{50} = 14.48 \mu\text{g/ml}$) resulted in eleven fractions, three of which (F2, F3, and F4) had anti-plasmodial IC_{50} ranging from ≤ 10 to $2.2 \mu\text{g/ml}$ against various *P. falciparum* strains with no demonstration of in vitro cytotoxicity. F4 displayed the highest in vivo parasite suppression, and had a mean survival time similar to artesunate (19.3 vs. 20.6 days). These fractions significantly modulated expression of inflammatory cytokines in Pf-antigen stimulated RAW cells. The findings of the study confirm the antimalarial potential of *C. reflexa*. Exploration of phyto-molecules in GCMS fingerprints of active fractions is warranted for possible identification of lead anti-malarial phyto-drugs.

Introduction

Human malaria, one of the deadliest infectious diseases of global health problems is caused by six species of *Plasmodium* (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. Knowlesi* and *P. simium*). Of these, *P. falciparum* malaria is often associated with severe manifestations of the disease, with a case fatality rate of 15–40% despite effective treatment with anti-malarial drugs [1]. Further, long term cognitive impairment is reported in as much as 25% of the survivors from cerebral malaria [2, 3]. This indicates that

parasite clearance alone is insufficient to prevent mortality and cognitive deficits in malaria. During malaria infection, the host defence mechanism activates phagocytosis of parasite infected red blood cells (RBCs) by monocytes, macrophages, and dendritic cells including the production of pro-inflammatory cytokines [4, 5]. Although considered as part of the effective host defence at the initial stage of infection [6, 7], excessive and uncontrolled overproduction or persistent host inflammatory responses [5, 8] are deleterious to the host. It is complicated further by the emergence and spread of drug resistance *P. falciparum* to almost all the currently used antimalarial drugs including last effective artemisinin and its derivatives, particularly in South-East Asia [9]. Besides, delays in parasite clearance against isolates originating from West Africa and other parts of the globe are challenging [10]. Therefore, there is an urgent requirement to identify or develop effective antimalarial drugs having a regulatory effect on inflammatory cytokines production, which may resolve post survival malarial attributed deficits and be a powerful new therapeutic strategy.

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In this aspect, plant derived compounds are the pillar for drug discovery, which constitutes the backbone of primary healthcare for about 70–95% of the world's population and has the broadest range of therapeutic applications in curing various diseases [11]. According to an estimate, about 14–28% of 250,000 number of total plant species around the world are being used as drugs [12], of which 1854 plant species from 196 families and 1012 genera are recorded for treatment of malaria or fever globally [13]. Studies indicate that 25% of modern medicines could have been a reality because of their primary discovery in phyto-genic natural sources based on their use in folklore and traditional medicine [11]. Interestingly, the most pharmacologically active and widely used antimalarials, such as quinine and artemisinin were obtained from plants having a folklore reputation for antimalarial properties, such as *Cinchona calisaya* and *Artemisia annua*, respectively [14]. Of the several medicinal plants used by indigenous people of Odisha, India for malaria treatment, use of *Cuscuta reflexa* Roxb. (Family: Convolvulaceae) is reported only in Mayurbhanj, Odisha [15], and it is not listed amongst 1854 plant species which are recorded for malaria and fever treatment globally. Based on its folklore reputation in the treatment of malaria in the locality and the lack of or limited investigation done on pharmacological and toxicological aspects, the present study was conducted to ascertain the antimalarial properties of *Cuscuta reflexa*, scientifically.

Cuscuta reflexa is an angiosperm parasite, popularly known as Amar bel or Devil's Hair. Besides having mosquito-cidal activity against malaria vectors [16], *C. reflexa* is reported to exhibit antibacterial, anti-helminthic, immunomodulatory, and hepatoprotective activity. Further, it is used in the treatments of various human ailments such as jaundice, diseases of the spleen, diabetes, epilepsy, ulcer and hair loss, etc. [17, 18]. The bioactivity of plant material is dependent on the nature of its constituent compounds and is influenced by the type of solvent used for extraction, their solubility in specific solvent systems and the methods used for extraction. However, information on original extractor solvent (s) used, and method of traditional processing of *Cuscuta reflexa* for effective treatment of malaria (in which the bioactive phyto-constituents are more likely to be found) are lacking since its first report in 2010 [15]. To maximally extract the diverse group of plant metabolites of differential solvent solubility we used sequential-soxhlet extraction involving both polar and non-polar solvents. In order to attest the traditional use of *C. reflexa* and to validate its antimalarial activity scientifically, these solvent extracts were evaluated for their in vitro anti-plasmodial activity against chloroquine sensitive 3D7 strain of *Plasmodium falciparum*. Further, to characterise the more potent fractions having the ability to kill multiple drug resistant strains of *Plasmodium falciparum*, the most active solvent extract

was column separated into different fractions, each of which were then evaluated for their in vitro anti-plasmodial activity against chloroquine sensitive 3D7, chloroquine resistant RKL-9 and artemisinin resistant R539T strains of *Plasmodium falciparum*. To determine the therapeutic effectiveness and safety of the potent fractions, their selectivity index (SI) was calculated after in vitro evaluation of these fractions for cyto-toxicity against normal cells. Since bioactive fractions/compounds exhibiting anti-plasmodial activity alone is insufficient, the potent fractions were further evaluated for their regulatory effect on inflammatory cytokines production in Pf-antigen stimulated RAW cells. Moreover, the antimalarial effect of potent fractions was evaluated in *P. berghei* infected Swiss-albino mice by examining the suppression of parasitemia and improvement in survival of infected mice in in vivo studies. Finally, the constituent compounds in potent fractions were identified in gas chromatography mass spectrometry (GC–MS) analysis. The findings of the study confirm antimalarial potential of *C. reflexa* in both in vitro and in vivo studies.

Materials and Method

Preparation of Extracts and Fractions

After identification and authentication of *C. reflexa* by World Flora Online, 2022 (<http://www.worldfloraonline.org/taxon/wfo-0001296658>), 30 g of dried powder prepared by pulverization of shed dried whole plant of *C. reflexa* was used in sequential-Soxhlet extraction with different organic solvents based on their polarity [such as petroleum benzene (PB), chloroform (CH), ethyl acetate (EA) and methanol (M)] at 50 °C and for a minimum of 20 cycles with 300 ml of the solvent [19]. The solvents from plant extracts were allowed to evaporate at RT and the percentage yield of extract was determined as [(weight of extract obtained – initial weight)/Initial weight]*100.

TLC Analysis of Bioactive Solvent Extracts

For selection of a suitable solvent system, 10 µl of crude extract in different solvent systems were run in analytical Thin Layer Chromatography (TLC) on a glass plate with silica gel (GF-254, SRL) [20]. The solvent system CH: M: AQ in 4:4:2 ratio showed a distinct chromatogram (Supplementary table) and was used for bulk separation of crude extract through column chromatography.

Column Chromatography

Column chromatography was performed by loading 1 g/ml of crude extract on to the top of the column (Borosil-6101062,

size 300×18 mm) prepared with slurry of silica (60–120 mesh size, SRL) in a selected solvent system. The compounds in the crude extract were separated through careful and continuous slow adding of solvents mixture on the top and collection of about 1 ml of eluents in separate test tubes by turning on the tap below of the column [21]. Each eluent was run in TLC, which was followed by the grouping of similar eluents into fractions. Solvents from the fractions were evaporated using a centrifuge concentrator (concentrator plus, Eppendorf, 5305000568) and percentage yields were calculated.

Drug Preparations

For in vitro assays, 100% DMSO was used to prepare a stock solution of 40 mg/ml for all solvent extracts and 30 mg/ml for different column fractions. Working solution for solvent extract (200 µg/ml) and column fractions (30 µg/ml) were prepared by appropriate dilution with culture media (containing 1% parasitemia and 2% hematocrit) to make the final concentration of DMSO in the working solution to be 0.1% (which is shown to be non-toxic and is commonly used in in vitro cell assays). This was followed by subsequent two-fold serial dilution with culture media to yield a range of final concentrations of drugs (ranging from 200 to 1.56 µg/ml) or fractions (ranging from 30 to 0.23 µg/ml). Intraperitoneal injections of 5% DMSO at a dose of 10 ml/kg for seven consecutive days are proven safe to mice in a recent study [22]. Therefore, for in vivo studies, a drug dose of 20 mg/kg was considered safe and given to test mice by injecting 200 µl of drug solution obtained after tenfold dilution (with PBS, pH 7.4) of a 30 mg/ml stock of fractions prepared in 100% DMSO. Similarly, stock for the standard drug artesunate (Sigma, A3731) was prepared with DMSO and amphotericin-B with incomplete culture media, whereas sterilized distilled water was used to dilute chloroquine (CQ).

Parasite Culture and In Vitro Anti-Plasmodial Assay

In vitro anti-plasmodial activities of various solvent extracts (PB, CH, EA and Methanol), and column fractions of crude methanol extract of *C. reflexa* (which showed highest in vitro anti-plasmodial activity) were assessed with different laboratory strains of *P. falciparum* using SYBR green-I based fluorescence assay following our established protocol [23]. Briefly, cultures of three different strains of *P. falciparum* such as 3D7, RKL-9 and R539T maintained in O positive human erythrocytes and complete RPMI-1640 (Sigma) media [supplemented with 2 g/l sodium bicarbonate (Sigma), 50 mg/l hypoxanthine (Sigma), 10 µg/ml gentamicin (Sigma), and 5 g/l Albumax I (Gibco)] at 37 °C under mixed gas environment (5% O₂, 5% CO₂ and 90% N₂) were 5% sorbitol synchronized separately, prior to use

in the growth inhibition assay. About 24 h post incubation, 100 µl of late ring stage or early trophozoite stage of *P. falciparum* diluted to 1% parasitemia and 2% hematocrit with complete culture media (and packed erythrocytes wherever needed) were incubated with serially diluted concentrations of test samples in triplicate to yield the final concentrations of the solvent extracts (200–1.56 µg/ml), column fractions (30–0.23 µg/ml), and standard antimalarial drugs (chloroquine at 100 nm and artesunate at 50 nm) in a 96-well plate. Untreated wells with parasitized erythrocytes were used as control. Growth inhibition due to plant extracts, fractions and standard drugs were assessed by SYBR-green based fluorescence assay [23] and fluorescence was recorded in a multimode microplate reader (Bio-Rad) at an excitation and emission wavelength of 485 and 530 nm, respectively. Percent growth inhibition was calculated using the following formula: % Growth Inhibition = {(absorbance of control – absorbance of treated)/ (absorbance of control) * 100}.

In Vitro Cytotoxicity Assay Against Monocytes

A 40-mg/ml stock solutions of different column fractions was prepared in 100% DMSO. Column fractions possessing potent in vitro anti-plasmodial activity (such as F2, F3, F4, F5 and F8) were evaluated for cytotoxicity starting with a concentration of 100 µg/ml to 0.78 µg/ml in a two-fold dilution series in RPMI-1640 media supplemented with 10% foetal bovine serum following Ramu et al. [24] and incubated at 37 °C and 5% CO₂. 72 h post incubation, 20 µl of MTT in PBS (5 mg/ml) was added to each well and it was incubated for 4 h at 37 °C in 5% CO₂. Cell viability was checked by solubilisation of purple formazan product in viable cells after addition and gentle mixing of 100 µl of DMSO/well. Wells having amphotericin-B (29 µg/ml) were used as positive control, whereas wells without any column fractions/drugs were considered as control (100% growth). The absorbance was recorded at 570 nm on a microplate reader, and the percentage viability of cells was determined using the formula: % Cell viability = (Absorbance of control – Absorbance of treated/Absorbance of control)*100.

In Vitro Inhibition of Inflammatory Cytokines Production

The inhibitory effects of potent fractions (F2, F3, F4 and F5) on the production of three different inflammatory cytokines (such as TNF-α, IL-10 and IL-12) by *P. falciparum* (Pf) antigen stimulated murine RAW 264.7 macrophage cells were assessed by qRT-PCR. Briefly, a running culture of RAW cells maintained in DMEM media following the previously described method [25] was trypsinized after a confluence of about 70–80% (in about 24 h culture). The cells were then seeded at 10,000

cells/200 μ l/well in 4X12 well plates after appropriate dilution with complete DMEM media and cell counting. Allowing about 1 h of cell adherence, these cells were treated with Pf antigen (at a ratio of 1 RAW:10 trophozoites) alone, and in combination with different fractions (F2, F3, F4 and F5) at a final concentration corresponding to their respective anti-plasmodial IC₅₀ values. While RAW cells un-stimulated with Pf antigen but treated with different fractions were considered as normal control, LPS (10 ng/ml) stimulated cells treated with these fractions were considered as their respective positive controls. About 24 h post-treatment at 37 °C, RNA was isolated from the cell pellets (TRIZOL reagent, Invitrogen) and quantified by Nanodrop. Using random hexamer primers, cDNA prepared from 1 μ g of RNase-free DNase treated total RNA were evaluated for the expression of TNF- α , IL-12, and IL-10 in Power Up SYBR Green PCR master mix (Thermo Fisher, USA) by Real-Time PCR system (Applied Biosystem, CA, USA) following [26]. Melting curves were generated along with the mean C_T values and confirmed the generation of a specific PCR product. Amplification of RNU6AP was used as internal control for normalization. Each experiment was done in triplicates.

In Vivo Studies

Experimental Animal and Parasite Inoculation

The in vivo experiment was performed in accordance with the internationally accepted principles for laboratory animal use and care (NIH publication no 85-23, revised 1985), and standard operation procedures of the Institutional Animal Ethics Committee (IAEC) of Jawaharlal Nehru University (JNU), Delhi (JNU/IBSC/2020/18). The male Swiss-albino mice, aged about 2 weeks and having an average body weight of ~30 g housed at Central Laboratory Animal Resources, Jawaharlal Nehru University, Delhi were maintained under standard conditions of food, temperature (25 °C \pm 3), relative humidity (55 \pm 10%) and illumination (12 h light/dark cycles) throughout the experiment. Donor albino mouse previously infected with chloroquine sensitive lethal strain of *Plasmodium berghei* ANKA and having above 30% parasitemia (3 days post infection) was anaesthetized. About 1 ml of blood was then drawn from this infected mouse through the eye vein using a glass capillary. It was then transferred to a sterile vial containing 200 μ l of citrate-phosphate-dextrose (Sigma, C7165) as anticoagulant. After centrifugation at 2000 rpm for 6 min, parasitemia in packed RBCs was determined. Following appropriate dilution, each mouse was intraperitoneally injected with 200 μ l of suspension in PBS containing 10⁷ infected RBCs.

In Vivo Suppressive Test and Survival Analysis

Infected mice were randomly divided into seven groups of three each, and the schizonticidal activity of fractions or standard drugs was evaluated following the method described by [27] with some modifications. Briefly, about 24 h post infection, five different column fractions (such as F2, F3, F4, F5 and F8) were injected intraperitoneally into five groups of mice separately at a dose of 20 mg/kg and continued for four consecutive days (D₁–D₄). The positive control group was similarly treated with the standard drug artesunate (ART) at a dose of 5 mg/kg, whereas the group of mice injected with 200 μ l of 2% DMSO was used as the infected control group. The ART dose of 5 mg/kg was selected due to the non-reduction of parasitemia level in infected mice after intraperitoneal injection of a fixed daily dose of ART at 1.7 mg/kg (data not shown) based on previous study [28]. Since a single dose of 30 mg/kg of tested fractions did not result in mortality or adverse clinical symptoms within the first 24 h and up to a follow-up of 7 days (data not shown), a test dose of 20 mg/kg (four times the standard drug concentration) was also anticipated to be safe and selected for this study.

Giemsa-stained thin smear was prepared from the tail blood of all animals on a daily basis till day 6 (by which the first mouse in the control group died) to determine the parasitemia level. Percentage parasitemia was determined by counting infected erythrocytes/100 uninfected erythrocytes in at least 10 fields of 10% giemsa-stained thin smears. The % suppression of parasitemia was determined as [(average parasitemia of infected control – average parasitemia of infected test group)/ average parasitemia of infected control]*100. The mean survival time of mice in each group was determined over a period of 30 days after the day of infection (D₀).

GC–MS Analysis

Prior to GC–MS analysis, a two-stage chemical derivatization was performed to make them suitable for analysis [29]. Briefly, 1 mg of dried fractions were dissolved in 100 μ l of methoxy amine hydrochloride (20 mg/ml in pyridine) followed by vortex mixing for 2–5 min and subsequent incubation at RT for 1 h to convert aldehyde and ketones into their oximes or alkyloximes. Then, 100 μ l of *N*-methyl-*N*-trimethylsilyltrifluoro(0) acetamide was added to this mixture in 1:1 ratio and incubated at 70 °C for 30 min to make the metabolites in the mixture less polar and more volatile, before samples were analysed in GC–MS (Shimadzu GC MS-QP-2010 plus system) at Advanced Instrumentation Research Facility, JNU, New Delhi. GC–MS analysis was conducted using RTx-5 SilMS columns (30 m \times 0.25 mm id \times 0.25 μ m film thickness) with operating conditions as

described previously [30]. The major and minor compounds present in each of these fractions were determined by comparing the mass spectra with data from National Institute of Standards and Technology, WILEY, Pesticide Library 3rd edition, Drug Library, GC-MS Metabolite Mass Spectral Database, and the Flavour and Fragrance Natural and Synthetic Compounds libraries based on their molecular weight and retention time.

Statistical Analysis

The quantitative values were expressed as mean \pm SD. The comparison between two groups was performed by an unpaired *t*-test with Welch's correction. The concentration of extracts/fractions/drugs at which there was 50% inhibition of parasite growth (IC_{50} value) or 50% reduction in cell viability (i.e., cell cytotoxicity, CC_{50} value) was determined by the dose–response curve. The associated SD for the IC_{50} value was calculated from the estimated standard error of mean (SEM) using the formula, $SEM = SD/\text{square root of } n$ (where *n* is the number of repetitions of the study) [31]. The selectivity index (SI) of each fraction was calculated as the ratio of their respective CC_{50} value/ IC_{50} value. The resistance index (RI) was calculated as a ratio of the IC_{50} values of resistant strains (RKL-9, R539T) / IC_{50} value of sensitive strain (3D7). To improve homogeneity of variances, raw data were log transformed where necessary. In qRT-PCR, the Cycle-threshold (Ct) value corresponds to the PCR cycle at which the amplified product was first detected. Using the expression of RNU6AP as reference, gene-expression levels of TNF- α , IL-10 and IL-12 were expressed as fold change by $2^{-\Delta\Delta CT}$ comparative threshold method. Improvement in animal survival is presented in Kaplan–Meier plot and tested by Log-rank, Mantel-Cox test and Gehan-Breslow-Wilcoxon test. Suppression of parasitaemia in infected mice treated with different fractions were compared by post hoc analysis over a period of 6 days with untreated infected control by Dunnett's multiple comparison test. In all cases, a two-tailed *P* value < 0.05 was considered significant. All statistical analyses were performed in GraphPad Prism (version 8.0.1, San Diego, USA).

Results

In Vitro Anti-Plasmodial Activity of Different Solvent Extracts

Methanol extract having a percentage yield (PY) of 10.6% showed the highest anti-Plasmodial activity against 3D7 (*Plasmodium falciparum* strain sensitive to chloroquine and artemisinin) with an IC_{50} value of $14.48 \pm 1.96 \mu\text{g/ml}$ followed by extracts of chloroform (CH) ($IC_{50} = 31.97$;

PY = 11%), Petroleum benzene (PB) ($IC_{50} = 61.83$; PY = 8%) and ethyl acetate (EA) ($IC_{50} = 115.3$; PY = 9.8%).

Fractionations of Methanol Extract, In Vitro Anti-Plasmodial Activity and Cytotoxic Activity

Column chromatography of methanol extract and subsequent grouping of similar fractions by TLC resulted in a total of 11 fractions, four of which (such as F2, F3, F4, F5) showed anti-plasmodial $IC_{50} < 14.48 \mu\text{g/ml}$. Rest of the fractions exhibited anti-plasmodial $IC_{50} > 30 \mu\text{g/ml}$ (Table 1). These four potent fractions, along with one randomly chosen fraction (F8) among those with $IC_{50} > 30 \mu\text{g/ml}$ were selected to investigate their anti-plasmodial activity against two more strains of *P. falciparum* such as R539T (artemisinin resistance strain) and RKL9 (chloroquine resistance strain). Besides, the cytotoxicity of these fractions was evaluated against THP-1 cell lines. The results of the percentage yield, anti-plasmodial activity and cytotoxicity of these fractions are shown in Table 1. Consistent with the results obtained with 3D7, all these potential fractions exhibited promising anti-Plasmodial IC_{50} ranging from < 10.0 to 2.2 $\mu\text{g/ml}$ against R539T and RKL strains (except F5, which had an $IC_{50} > 30.0 \mu\text{g/ml}$ against R539T). The F8 had an anti-plasmodial $IC_{50} > 30 \mu\text{g/ml}$ in accordance with the results obtained with the 3D7 strain. CQ and ART had anti-plasmodial $IC_{50} < 1 \mu\text{g/ml}$ against all the strains of *P. falciparum*. All the column fractions were non-cytotoxic for THP-1 ($CC_{50} > 100 \mu\text{g/ml}$). The SI value was determined to be above 10 $\mu\text{g/ml}$ for column fractions (F2, F3 and F4) against different *P. falciparum* strains. Of note, the highest SI value was documented for F2 against all tested strains of *P. falciparum*.

In Vitro Inhibition of Inflammatory Cytokines Production

De-regulated inflammatory responses are the hallmark of severe malaria. To test whether these bioactive fractions affect inflammatory cytokine production, normal RAW cells and those stimulated with Pf antigen were treated with F2, F3, F4 and F5 at a concentration corresponding to their respective anti-plasmodial IC_{50} values. As shown in Fig. 1, Pf stimulated RAW cells produced several fold higher pro-inflammatory cytokines (TNF- α , IL-12) similar to LPS (10 ng/ml) treated positive control compared to unstimulated control (Fig. 1a and b), whereas the level of IL-10 between Pf stimulated RAW cells and unstimulated control was comparable (Fig. 1c). Interestingly, treatment with studied fractions significantly reduced the expression of TNF- α and IL-12 in both unstimulated and Pf stimulated RAW cells. However, in Pf stimulated RAW cells treated with F4, still the TNF- α level was two-fold high (1A), and level of IL-12

Table 1 In vitro anti-plasmodial activity and cytotoxicity of different column fractions from methanol extract of *C. reflexa*

Fraction	PY	IC ₅₀ (µg/ml)		CC ₅₀ (µg/ml)		Selectivity Index			Resistance index			
		3D7	RKL9	R539T	THP-1/3D7	THP-1/R539T	THP-1/RKL9	R539T/3D7	RKL-9/3D7			
1	2.4	> 30	-	-	-	-	-	-	-	-	-	-
2	3.0	5.104 ± 0.56	2.19 ± 0.21	6.834 ± 0.43	> 100	> 19.59	> 14.63	> 45.6	1.338	0.429		
3	7.5	10.093 ± 0.94	2.418 ± 0.046	8.879 ± 0.62	> 100	-10	> 11.26	> 41.35	0.879	0.239		
4	3.0	5.67 ± 0.65	2.7 ± 0.36	6.96 ± 0.23	> 100	> 17.63	> 14.35	> 37.03	1.227	0.476		
5	3.1	13.26 ± 0.88	2.814 ± 0.38	> 30	> 100	> 7.54	> 3.33	> 35.53	> 2.26	0.212		
6	2.2	> 30	-	-	-	-	-	-	-	-	-	-
7	2.3	> 30	-	-	-	-	-	-	-	-	-	-
8	2.8	> 30	> 30	> 30	> 100	> 3.33	> 3.33	> 3.33				
9	5.4	> 30	-	-	-	-	-	-	-	-	-	-
10	3.1	> 30	-	-	-	-	-	-	-	-	-	-
11	5.2	> 30	-	-	-	-	-	-	-	-	-	-
*ART/CQ	-	0.0053	0.0038	0.021	-	-	-	-	3.962	0.716		

Four potent fractions with anti-plasmodial IC₅₀ against 3D7 < 14.48 µg/ml and one randomly chosen fraction amongst those with IC₅₀ > 30 µg/ml were investigated for their anti-plasmodial activity against two more strains of *P. falciparum* (RKL9 and R539T). The SD for IC₅₀ value was calculated from SEM. Cytotoxicity was assessed by MTT assay against THP-1 cell line. PY: percentage yield, ART: artemisinin, CQ: Chloroquine, *ART and CQ are used separately as positive control, wherever applicable. Specifically, ART was used as positive control against CQ resistance (RKL9) and CQ sensitive strain (3D7), whereas CQ was used as positive control against ART resistant strain (R539T). Selectivity index is calculated as CC₅₀ / IC₅₀. The value “> than X” represents the IC₅₀ or CC₅₀ value is above the highest concentration used (X) in the study in their respective columns

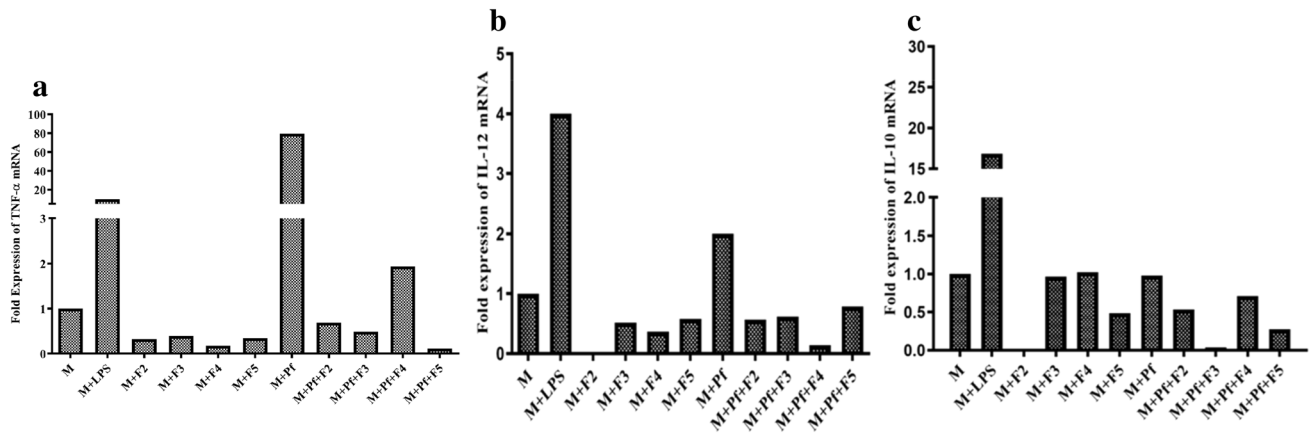


Fig. 1 Effect of different fractions of methanol extracts on TNF- α (a), IL-12 (b) and IL-10 (c) production in RAW cells. RAW cells stimulated with Pf-antigen produced several fold higher pro-inflammatory cytokines (TNF- α , IL-12) similar to LPS treated positive control. Although treatment with studied fractions significantly reduced their expression, F4 treated RAW cells still had significant level of TNF- α (a). M: Untreated RAW cells, LPS: Lipo-polysaccharides treated

at 10 ng/ml; F2, F3, F4 and F5 are different fractions of methanol extract treated at their respective IC₅₀ values. Pf: *P. falciparum* antigen. Gene-expression levels are expressed as fold change by 2^{- $\Delta\Delta$ CT} comparative threshold method using the expression of RNU6AP as reference. The results are representative of two independent experiments (n=2) performed in triplicates

was the least (1B) compared to unstimulated control. On the contrary, while F3 treated RAW cells alone had comparable IL-10 level with that of untreated or Pf stimulated RAW cells; in Pf stimulated RAW cells treated with F3, the level of IL-10 reduced significantly (1C).

In Vivo Anti-Plasmodial Activity of Different Bioactive Fractions

Results of the in vivo suppression of parasitemia by different fractions at a dose of 20 mg/kg, and their effect on the mean survival time (MST) of *P. berghei* ANKA infected mice are summarized in Table 2 and Fig. 2. The

results showed a significant reduction in parasitemia levels by all the tested fractions (above 60%) compared to the control, with the highest suppression demonstrated for F4 (85.93), followed by F3 (80.32) in a 4-day suppressive test. Interestingly, Dunnett’s multiple comparison tests revealed these suppressions were maintained throughout the study period of 6 days (Fig. 2a). Notably, mice treated with F4 had significantly higher MST of 19.3 ± 9.6 days (Log-rank P value < 0.0246 and Gehan-Breslow-Wilcoxon test P value < 0.0339), followed by F3 (MST of 17.0 ± 7.0 days), compared to the control group (MST of 6.67 ± 1.1). The F2 showed an MST of 11.0 ± 3.4. However, these observations were still less as compared to ART-treated mice

Table 2 In vivo suppression of parasitemia and improvement in survival of Swiss-albino mice infected with *P.berghei* by different fractions of methanol extract

Groups	Parasitemia	Suppression (%)	MST	P values	
				Log-rank test	Gehan-Breslow-Wilcoxon test
C	23.36 ± 1.6	...	6.67 ± 1.1	–	–
F2	5.26 ± 1.2	77.49	11.0 ± 3.4	< 0.05	< 0.05
F3	4.6 ± 5.8	80.32	17.0 ± 7.0	< 0.05	< 0.05
F4	3.2 ± 4.4	85.93	19.3 ± 9.6	< 0.05	< 0.05
F5	9.1 ± 1.8	60.95	13.0 ± 8.6	0.103	0.093
F8	5.1 ± 3.7	77.98	10.6 ± 3.2	0.115	0.112
ART	1.9 ± 0.4	91.56	20.6 ± 9.5	0.01	0.014

C: Control group, F2-F5 and F8 are different fractions of methanol extract, ART: artesunate, MST: mean survival time, Parasitemia and MST are expressed in mean ± SD, P value < 0.05 are considered significant. Each group comprised of three male mice. Fractions were injected intraperitoneally at a dose of 20 mg/kg, artesunate at a dose of 5 mg/kg, whereas 200 μ l of 2% DMSO were injected into mice in infected control group

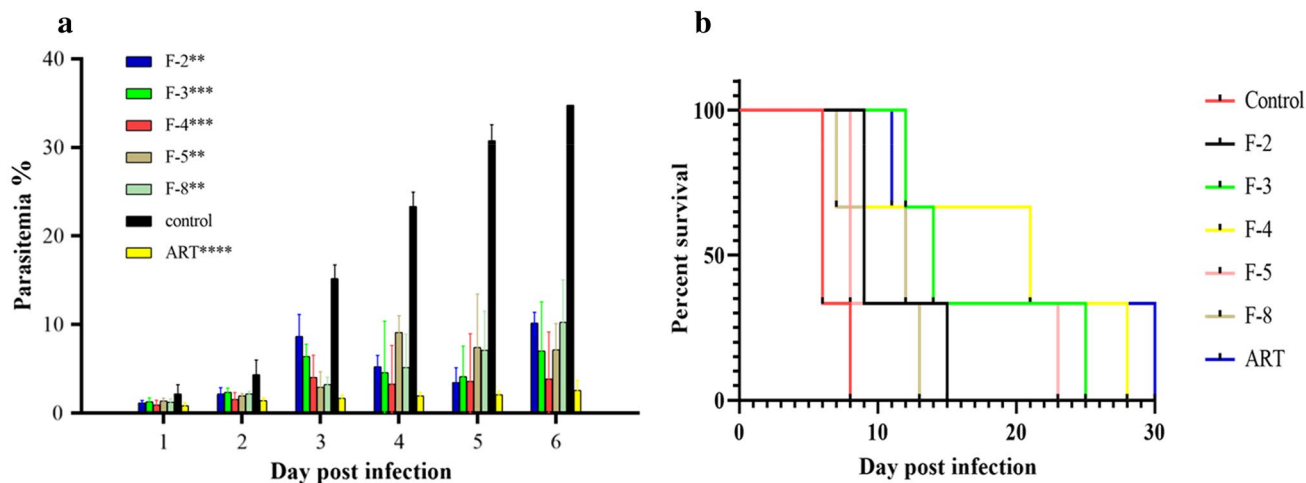


Fig. 2 Effect of different column fractions of methanol extract on in vivo anti-plasmodial activity (**a**) and survival of Swiss-albino mice (**b**) infected with *P. berghei*. F4 treated mice ($n=3$) exhibited highest suppression of parasitemia throughout the study period of 6 days (**a**) compared to untreated infected control as revealed by Dunnett's multiple comparison test. In addition, treatment with F4 improved

the survival of infected mice in 30 days follow-up study (**b**) similar to ART-treated mice as shown in Kaplan–Meier survival curve. Different fractions (F2–F5 and F8) were treated at 20 mg/kg. ART: artesunate was treated at 5 mg/kg. Dunnett's multiple comparison test P value < 0.05 is represented by *, P value < 0.01 by **, P value < 0.001 by *** and P value < 0.0001 by ****

(91.56% reduction in parasitemia, MST of 20.6 ± 9.5 days) (Fig. 2b).

GC–MS Analysis of Potent Anti-Plasmodial Fractions of *C. reflexa*

GC–MS analysis revealed 34 constituent compounds in F2, 80 in F3 and 121 in F4 (S1). Of these, compound 1-(4-isopropylphenyl)-2-methylpropylacetate was found to be common in both F2 and F4, whereas about 37 compounds were observed in both F3 and F4 (S1). There were four major constituent compounds in F2 (such as myristicin, elimicin, Bis(2-ethylhexyl)phthalate and 2H-Pyran-2-dodecanoic acid, 6-(17,19-dimethylheneicosyl)), three in F3 (such as D-(+)-Talose- pentakis(trimethylsilyl)ether-methoxyime, 9,12-octadecadienoic acid(z,z)-trimethylsilyl ester and oelsaeure-trimethylsilyl ester) and four in F4 (such as benzoic acid, 3,4,5-tris(trimethylsiloxy)-trimethylsilyl ester, bis-o-trimethylsilyl-palmitinic acid-glycerin-(2)-monoester including the first two compounds of F3) which had their percentage share $\geq 5\%$ in respective fractions. Similarly, there were five compounds in F2, 22 in F3 and 20 in F4 having percentage share between $1 \leq 5\%$ in respective fractions. Other components were found in less than 1% quantity (S1 and SF1–SF3).

Discussion

With an aim to scientifically confirm the potential of *C. reflexa* for malaria treatment, when different solvent extracts of *C. reflexa* were evaluated for their in vitro

anti-plasmodial activity against *Pf3D7*, methanol extract showed promising anti-plasmodial activity, having an IC_{50} of $14.48 \mu\text{g/ml}$. According to a recent classification [32], plant extract possessing $IC_{50} < 5 \mu\text{g/ml}$ is described to have strong activity, whereas those with $5 \leq IC_{50} < 15 \mu\text{g/ml}$ is described to have good or promising activity. Interestingly, three out of eleven column fractions (such as F2, F3 and F4) of methanol extract exhibited excellent anti-plasmodial activity ($IC_{50} < 10$) against three different tested strains of *P. falciparum* such as 3D7, RKL-9 and R539T with no demonstration of in vitro cytotoxicity up to a concentration of $100 \mu\text{g/ml}$. This indicates a selective index higher than tenfold, which is considered specific against infected erythrocytes but non-toxic to the host [32]. These findings attest to the traditional use of *C. reflexa* for malaria control and highlight the importance of potent column fractions for their containment of possible lead antimalarial phyto-drug. Although in vitro study revealed F2 (high SI value) to be a more effective anti-plasmodial fraction, the same could not be replicated by in vivo studies. Instead, the F4 displayed the highest in vivo anti-plasmodial activity with an 85.93% reduction in parasitemia in a 4-day suppressive test. Further, *P. berghei*-infected mice treated with F4 had comparable MST with that of artesunate-treated mice (19.3 days vs. 20.6 days). This was followed by F3 and F2-treated mice. A similar trend was established through Dunnett's multiple comparison tests over the study period of 6 days. These observed discrepancies of studied fractions (F2, F3 and F4) in in vitro and in vivo studies though could not be explained by the available data at present; all had significant anti-malarial effects and could

be the source of active phyto-molecules for anti-malaria therapeutics.

It is an established fact that hyper-parasitemia is associated with severe malaria in both human studies and experimental mice [33]. However, some mild malaria patients are documented to be tolerant to high-level parasitemia. On the contrary, malaria patients with low-level parasitemia are succumbed to severity in certain cases [33, 34]. These findings suggest parasitemia alone is insufficient in disease progression to severity in malaria. This is evidenced by the observation of a considerably high case fatality rate despite effective antimalarial treatment for severe malaria patients [1]. Therefore, a combination of parasite density and the host immune response to infection is crucial. While pro-inflammatory cytokines (TNF- α , IL-12, IFN- γ etc.) are proven beneficial during the acute phase of malaria infection, an increased level of anti-inflammatory cytokines (TGF- β and IL-10) is shown to limit the progression of mild malaria to a life-threatening severe complication [6, 7, 35], whereas deregulated expression leading to an imbalance between pro-inflammatory and anti-inflammatory cytokines or an extreme level of either, is associated with heightened severity and mortality [5, 8]. Unfortunately, we could not collect blood samples from control and treated mice for gene expression analysis of relevant cytokines and thus the effect of studied fractions on in vivo production of inflammatory cytokines could not be analysed. Alternatively, to explore the effect on inflammatory cytokines production in vitro, when un-stimulated or Pf antigen stimulated RAW cells treated with these active column fractions were examined for pro-inflammatory (TNF- α , IL-12) and anti-inflammatory (IL-10) cytokines production (Fig. 1), a significant reduction in the expression level of these cytokines were observed. This suggests that, besides having direct anti-plasmodial activity, the studied fractions might also affect inflammatory cytokines production in infected mice thereby modulating their survival time. However, in Pf antigen stimulated RAW cells treated with F4, the expression of TNF- α was still significantly high compared to untreated control (Fig. 1a). Although we could not investigate the change in expression of cytokines profile in in vivo studies, our observation of improved survival in F4 treated *P. berghei* infected mice, and previous findings of malaria protective association of early pro-inflammatory cytokine burst [6, 7, 35–37] advocate possible protective maintenance of TNF- α in F4 treated infected mice, adequate enough to suppress malaria infection. On the contrary, despite possessing high in vitro anti-plasmodial activity (Table 1), the impact of F2 in drastically reducing the expression of these early phase malaria protective cytokines might have compromised its overall effectiveness on malaria associated immunopathology and subsequent outcomes. However, more promising antiplasmodial activity with high SI index results being demonstrated for F2 in

in vitro study compared to F3 and F4, possible containment of important anti-plasmodial phyto-molecules in F2 cannot be ruled out.

To the best of our knowledge, this is the first study that confirms experimentally the potential of *C. reflexa* for malaria treatment. Interestingly, acute oral doses of methanol extract of *C. reflexa* are shown to be non-toxic against Swiss-albino mice [38, 39], though not documented in malaria infected mice yet. Phytochemical characterization of active column fractions through GC–MS analysis revealed the presence of several compounds in F2 alone (such as Terpinen-4-ol, myristicin, hexadecanoic acid-methylester- pentacosane and methyl isoeugenol), which are either reported to occur in the bioactive fractions of different plant extracts or shown to exhibit promising anti-malarial activity in previous studies [40–46]. Besides, the 1-(4-isopropylphenyl)-2-methylpropylacetate observed in both F2 and F4 also been found in active fractions of *Syzygiumcumini* methanolic extract [47] indicating its likeliness to be a drug-candidate for malaria. Further, out of 37 compounds found in both F3 and F4 (S2), eight compounds (such as 9,12-octadecadienoicacid(z,z)-trimethylsilyl ester, Trimethylsilyl ethers derivatives (TMS) of stearic acid, azelaic acid 2 TMS derivatives, TMS derivatives of Butane-dioic-acid, Phenyl-phosphonic-acid, Malic acid, meso-Erythritol and Xylitol) are reported to exhibit anti-malarial activity (see S2 for details). Of these, the compound 9,12-octadecadienoicacid(z,z)-,trimethylsilyl ester was observed as above 5% in the entire compound collection in F3 (7.0%) and F4 (5.35%). Interestingly, it is also documented as a major peak in bioactive methanol extract of *Achyranthes aspera*, a plant commonly used in the treatment of chronic malaria [48] However, their target sites or mechanism of action are mostly unknown. Since, both F3 and F4 harbour several anti-malarial compounds in common, the reported anti-malarial activity and immune-pathological effects leading to a significant reduction in parasitemia and improved survival of malaria infected mice by F4, followed by F3 is not surprising and could be due in part to their bioactive constituent components. However, whether the compound (s) exhibiting potent in vivo anti-plasmodial activity are the same or different from those having protective immune-pathological effects remains to be determined. Therefore, anti-plasmodial activity and toxicological analysis of all the major constituents in each of these fractions (S1) require determination. In silico analysis of all major phyto-compounds against important and validated drug targets of *P. falciparum* in virtual screening may help in identifying the potential drug-like candidates in these fractions. However, discovering a single-active and safe antimalarial drug from plant sources is often very difficult due to their synergistic association with diverse compounds in the extract. Interestingly, certain plant extracts are proven to be

more effective than their individual constituent compounds against malaria [49]. This has led to the use of active extract in monotherapy or in combination therapy with standard antimalarial drug [49, 50]. However, identification of single-active phyto-compound would help in synthesizing the more active structural analogs as observed in the case of most active antimalarial drugs such as CQ and artemisinin. Although Soxhlet-extraction is not the traditional practice of preparing herbal medicine, our approach of sequential Soxhlet-extraction with different solvents of increasing polarity extends the prospect of isolating maximum and diverse phyto-constituents in different solvent fractions. Moreover, Soxhlet-extraction being conducted at 50 °C, future exploration of bioactive fractions might lead to isolation of temperature stable interesting anti-malarial compound. Although the explanations relating to the observed discrepancies in active fractions in in vivo and in vitro studies are only speculative, direct and concrete evidence is required to be generated through detailed analysis of major constituent compounds in in vitro and well-designed in vivo studies with additional toxicological analyses.

Conclusions

In conclusion, the results obtained in this study through multiple approaches establish the anti-malarial effect of *C. reflexa* and authenticate its traditional use in the treatment of malaria. The methanol extract is more promising, especially the column fractions F4 along with two other fractions such as F2 and F3. Besides having anti-plasmodial activity against multiple strains of *P. falciparum*, these fractions also exhibited protective immunomodulatory activity with no cytotoxicity. There were several compounds found to be common in F3 and F4. Although F2, F3 and F4 contain several promising anti-malarial compounds, their mechanisms of action are unknown. Further, a large number of compounds in these fractions are yet to be explored for containment of possible lead antimalarial phyto-drug. Therefore, all major phyto-compounds in these fractions should be analysed in silico against validated drug targets of *P. falciparum* in virtual screening to identify the potential drug-like candidates. Information on important hit compounds from this screening would be helpful in synthesising druggable molecules, which can be explored further for lead anti-malarial drug development after appropriate study design.

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Data Availability All data are available with corresponding authors.

Code Availability Not applicable.

Declarations

Conflict of interest All authors have no conflict of interest.

Ethical Approval Approved by IAEC of Jawaharlal Nehru University (JNU), Delhi (JNU/IBSC/2020/18).

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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