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Isolation, fractionation and evaluation of the antiplasmodial properties of *Phyllanthus niruri* resident in its chloroform fraction

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ABSTRACT

Objective: To investigate the antiplasmodial activity of *Phyllanthus niruri* (*P. niruri*) methanol extract (ME) and its fractions in mice. **Methods:** *P. niruri* methanol extract and its chloroform, ethanol and aqueous portions were tested against chloroquine-sensitive *Plasmodium berghei berghei* in early, established and repository models of infection using Knight and Peter´s 4-day suppressive model, Ryley and Peters curative model and Peters prophylactic model respectively. **Results:** Chemosuppression of parasitaemia (37.65%-50.53 %) was elicited by 100-400 mg/kg (b.w.) of ME. At doses of 100 mg/kg b.w., the chloroform fraction (F1) significantly ($P<0.01$) suppressed parasitaemia by 85.29%, while ethanol and aqueous fractions (F2 and F3, respectively) elicited 67.06% and 51.18% chemosuppression. The most active fraction, F1 was selected for further antiplasmodial screening. In established infection, ME reduced parasitaemia (15.81% - 62.96%) while F1 significantly ($P<0.01$) reduced parasitaemia (44.36%-90.48%), with effects comparable to that of chloroquine (96.48%). The prophylactic antiplasmodial activity of ME (92.50% suppression) was also significant ($P<0.01$) and was more effective than pyrimethamine (85.00%). Additionally, cell membrane integrity of non-parasitized erythrocytes incubated with 125-500 mg/mL F1 was maintained. **Conclusions:** These findings indicate the antiplasmodial efficacy of *P. niruri* methanol extract, and the localization of this effect in its chloroform fraction.

1. Introduction

The increasing burden of malaria has contributed significantly to poverty, decreased productivity and slow economic growth in malaria endemic regions[1]. In 2010 alone, an estimated 655 000 cases of malaria-associated deaths were recorded, especially in very young African children[2,3]. This trend is attributed mainly to growing resistance of the *Plasmodium* parasite to drugs like chloroquine and sulphadoxine/pyrimethamine that were previously effective[4] but are now widely ineffective. Parasite resistance to commonly used antimalarials such as chloroquine and sulphadoxine/pyrimethamine has also been found to confer cross resistance to other antimalarial drugs[5]. Excessive and dysregulated inflammatory responses in patients infected with *Plasmodium falciparum* (*P. falciparum*) has also been linked with the development of severe forms of malaria exemplified by cerebral malaria, which accounts for 15%-40% mortality in such patients[6]. Prompt and effective treatment remains the primary option for the management of malaria in tropical areas where the disease is endemic with high transmission rates.

The World Health Organization currently advocates artemisinin derivatives and their combinations for the treatment of malaria[7]. However, poverty in many developing countries, especially in Africa, puts artemisinin-based therapies out of the reach of many. This inaccessibility is still largely responsible for the increasing incidence of morbidity and mortality associated with

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malaria infection. Recent reports[8,9] have also shown that *P. falciparum* is becoming resistant to artemisinin. Therefore, there is the need for continuous search for new antimalarial drugs and the use of plants as a promising strategy is on the increase[10,11]. Novel compounds with potent antiplasmodial activity isolated from natural products offer an attractive source of promising antimalarial agents. This is especially true for plants with long history of use in ethno medical practice, as some of the antimalarials used today were derived from such plants. The most significant examples are quinine, isolated from barks of Cinchona succirubra and Cinchona calisaya[12] and artemisinin, obtained from Artemisia annua[13]. *Phyllanthus niruri* (Euphorbiaceae) (*P. niruri*) is an annual herbaceous plant indigenous to rain forests, tropical and sub tropical areas of the world. Preparations of its dried aerial parts are used to stimulate appetite and to treat malaria in Thailand[14] and the West Indies[15]. It is also a popular antipyretic remedy in South America[16]. An aqueous extract of the aerial parts of the plant is also used to break and expel kidney stones in India, Brazil and other Amazonian regions. Harish and Shivanandappa reported[17] that the alcoholic and aqueous extracts of the leaves and fruit exhibit antioxidant, free radical scavenging and hepatoprotective effects. Antiplasmodial activity of aqueous, methanol and chloroform extracts of the plant has also been demonstrated by Mustofa *et al*[18]. Phytoconstituents present in the *P. niruri* responsible for its pharmacological properties include lignans, alkaloids, flavonoids, benzenoids, coumarins, tannins, diterpenes, triterpenes, sterols, phytallates and lipids[19]. To date, no information was found in literature regarding the components of the organic extracts responsible for its antiplasmodial activity. The significant medicinal activity of *P. niruri*[17-19] also underscores the need to assure quality and consistency of its pharmacological effects by identifying phytochemical markers and fingerprints, a basic requirement for validating and standardizing medicinal plants[20].

Our study is therefore aimed at investigating the antiplasmodial activity of *P. niruri* methanol extract and fractions, to identify the most active fraction using mouse models of infection.

2. Materials and methods

2.1. Plant material

Fresh aerial parts of *P. niruri* were collected in June 2008 from an uncultivated farmland in Orba, Enugu State, Nigeria. The plant material was identified and authenticated by Mr A. Ozioko of the International Centre for Ethno medicines and Drug Development (InterCEDD) Nsukka, Enugu state. A voucher specimen was prepared and deposited in NIPRD herbarium (NIPRD/H/6565). The roots of the plant were cut off and the aerial parts dried at room temperature for two weeks then pulverized to coarse powder using an electric hammer mill.

2.2. Extraction and fractionation

The powdered plant material (900 g) was extracted with 5.85 L methanol (98% v/v) for 48 h with occasional mechanical shaking (4 h/day). At the end of 48 h, the mixture was filtered using ashless filter paper (Whatman, USA), the residue reconstituted in 4 L of methanol (98% v/v) and the extraction process repeated. The filtrates were pooled together and concentrated under reduced pressure using a rotary evaporator to afford 98 g of the crude methanol extract (ME). A 33 g quantity of ME was portioned between water and chloroform (4 × 600 mL) using a separatory funnel. The combined lower chloroform layer was concentrated to dryness under vacuum by rotary evaporation at 40 ℃ and the slurry obtained was designated F1 (9.09% w/w). The upper aqueous layer was concentrated on a water bath and extracted with ethanol (96% v/v). The ethanol extract was concentrated under vacuum and evaporated to dryness to give a brown, sticky fraction, F2 (18.18%). The residue left after extraction with ethanol was evaporated to dryness on a water bath to give a brown powder, F3 (54.55%). The crude extract and its fractions were stored in a refrigerator until required.

2.3. Phytochemical screening

Phytochemical screening of the crude extract and fractions was carried out in accordance with standard test procedures described by Evans[21].

2.4. Animals

Swiss albino mice of either sex weighing 18-25 g were acclimatized for two weeks to laboratory conditions in the animal facility centre of the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja. The mice were housed in plastic cages in a ventilated room at a temperature of (20 ± 5) ℃, fed with standard rodent chow and allowed free access to potable water. All experiments were carried out in accordance with NIH Guide for the Care and Use of Laboratory Animals; NIH publication (No 82-23) revised, 1985[22] and NIPRD's standard operating procedures.

2.5. Rodent parasite

Chloroquine-sensitive rodent parasite (NK 65 strain) was sourced from the National Institute for Medical Research

(NIMR), Lagos, Nigeria. The parasite was maintained by continuous reinfection (intraperitoneal) of healthy mice.

2.6. Parasite inoculation

One week after parasite inoculation, a leishman-stained thin blood film was prepared from tail vein blood of a donor mouse on the surface of a glass slide for the assessment of parasitaemia and its red blood cell count determined with a Neubauer hemocytometer. Using the parasitaemia and red blood cell count of the donor mouse, the withdrawn blood sample was diluted with normal saline such that 0.2 mL contained approximately 10^5 parasitized red blood cells. Each experimental mouse was inoculated intraperitoneally with 0.2 mL of the diluted infected blood.

2.7. Preliminary antiplasmodial assessment of extract and fractions in early infection (4-day test)

The method described by Kalra *et al*[23] was adopted. Briefly, sixty mice were randomized into groups of five mice each, such that mean weight within each group was kept close. On day 0, within 4 h after inoculation of mice with the parasite, treatment of all the groups was initiated orally. Groups 1 and 2 served as controls and received distilled water and chloroquine (5 mL and 5 mg/kg b.w., respectively), while groups 3, 4 and 5 received 100, 200 and 400 mg ME/kg b.w., respectively. Graded doses (25-100 mg/kg b.w.) of the fractions were administered to groups 6-14. Treatment was carried out at the same time each day on days 1, 2 and 3. On day 4, thin films of tail vein blood were prepared and stained with Leishman's stain. The films were examined microscopically and parasitaemia was expressed as the mean number of parasitized erythrocytes counted in 10 fields of approximately 250 erythrocytes per field.

Percentage suppression of parasitaemia was calculated using the following equation[24]:

Mean parasitaemia control − mean parasitaemia treated × 100 / mean parasitaemia control

Based on the outcome of this preliminary test, the chloroform fraction was selected for further antiplasmodial screening.

2.8. Effect of ME and F1 on established infection

The schizonticidal activity of the crude extract and in established infection was evaluated using the method of Ryley and Peters described by Okokon and Nwafor[25]. Forty mice were injected intraperitoneally with dilute infected blood as described above. On day 3, thin blood films of the mice were prepared to determine pre-treatment parasitaemia. The mice were randomized into eight groups of five mice each such that mean parasitaemia of the groups were close. Treatment was then carried out as follows: Group 1 and 2 served as controls and received distilled water and chloroquine (5 mg/kg b.w.) respectively, while groups 6, 7 and 8 were treated with 100, 200 and 400 mg ME/kg b.w. respectively, groups 9-11 were given 25-100 mg F1/kg b.w. Treatment was continued once daily on days 4 to 6. On day 7, blood films were made and the level of parasitaemia assessed. The mice were subsequently monitored for mortality and mean survival time of each group was recorded.

2.9. Assessment of prophylactic antiplasmodial activity of ME and F1

The prophylactic effect of ME and F1 against infection was assessed using the method of Peters, described by Chandel and Bagai[26]. Forty mice were randomized into eight groups (n=5) and treated once daily for three consecutive days. Control groups 1 and 2 received distilled water (5 mL/kg b.w) and pyrimethamine (1.2 mg/kg b.w.) respectively, while the remaining groups were treated with ME (100, 200 and 400 mg/kg b.w.) and F1 (25, 50 and 100 mg/kg b.w.). On the fourth day, the mice were inoculated with *Plasmodium berghei berghei*. After 72 h, a thin blood film of each mouse was made and parasitaemia was assessed.

2.10. Effect of F1 on erythrocyte viability

The effects of F1 on viability of parasitized and non-parasitized red blood cells in vitro were studied using the trypan blue exclusion technique[27]. In brief, blood was withdrawn from the retro orbital plexuses of infected and uninfected mice into EDTA-containing plastic tubes, centrifuged and the packed cells washed thrice in phosphate buffered saline (PBS). A 100 μL volume of a 10% cell suspension was prepared in PBS containing 5% dextrose was mixed with 100 μL of serial dilutions of F1 prepared in dimethylsulfoxide and PBS (1:9). The mixture was incubated at 37 ℃ for 45 min then mixed with an equal volume of 4% trypan blue and allowed to stand for 3 min. The total number of cells was counted and the viable cells counted separately, using a hemocytometer. The test was done in triplicates for each concentration used.

2.11. HPLC fingerprinting of the crude extract and chloroform fraction

Chromatographic fingerprinting of ME and F1 by HPLC was carried out using a Shimadzu LC-20AB Prominence model composed of a programmable auto sampler (SIL-20AC prominence), a Shimadzu SPD-M20A Prominence UV-diode array detector and a binary pump equipped with a degasser (Shimadzu, Kyoto, Japan). The analytical column was a

reversed-phased column Shim-Pack VP-ODS (150 mm × 4.6 mm *id*., 4.6 μm particle size; Shimadzu, Kyoto, Japan). The column temperature was 28 ℃. The mobile phase which gave maximum separation consisted of 0.2% v/v formic acid solution and acetonitrile (80:20) by isocratic elution. Flow rate was 0.6 mL/min. and volume injected was 10 μL. The samples were detected at 254 nm using photodiode array detector. Data analysis was performed with Shimadzu Lab Solutions LCsolution software (version 1.23 SP1).

2.12. Data analysis

Results were expressed as mean ± SEM. Statistical analyses was carried out by one-way ANOVA (GraphPad Prism 3.0), data was further subjected to dunnett's post hoc test and differences between treated groups and control accepted as significant at $P<0.01$ and $P<0.05$.

3. Results

Figure 1A shows the fingerprint of ME with five characteristic peaks of varying relative compositions while a total of fourteen peaks were seen in the fingerprint profile of F1 (Figure 1B). Results in Table 1 show the constituents of *P. niruri* methanol extract and its fractions. Phenols and alkaloids were detected in the crude extract, aqueous, ethanol and chloroform fractions, while terpenes, sterols and anthraquinones were present in the extract, its ethanol and chloroform fractions. Saponins, tannins and flavonoids detected in ME also presented in the aqueous and ethanol fractions. All the fractions except the chloroform fraction were devoid of volatile oils.

Table 1
Phytochemical composition of *P. niruri* methanol extract and its fractions.

	ME	F1	F2	F3
Carbohydrates	+	ND	ND	+
Terpenes	+	+	+	ND
Sterols	+	+	+	ND
Saponins	+	ND	+	+
Cardiac glycosides	ND	ND	ND	ND
Tannins	+	ND	+	+
Anthraquinones	+	+	+	ND
Balsams	ND	ND	ND	ND
Resins	ND	ND	ND	ND
Alkaloids	+	+	+	+
Phlobatannin	ND	ND	ND	ND
Flavonoids	+	ND	+	+
Phenols	+	+	+	+
Volatile oil	+	+	ND	ND

+: Detected; ND: Not detected, PNME= *P. niruri* methanol extract, F1= chloroform fraction, F2= ethanol fraction, F3= aqueous fraction.

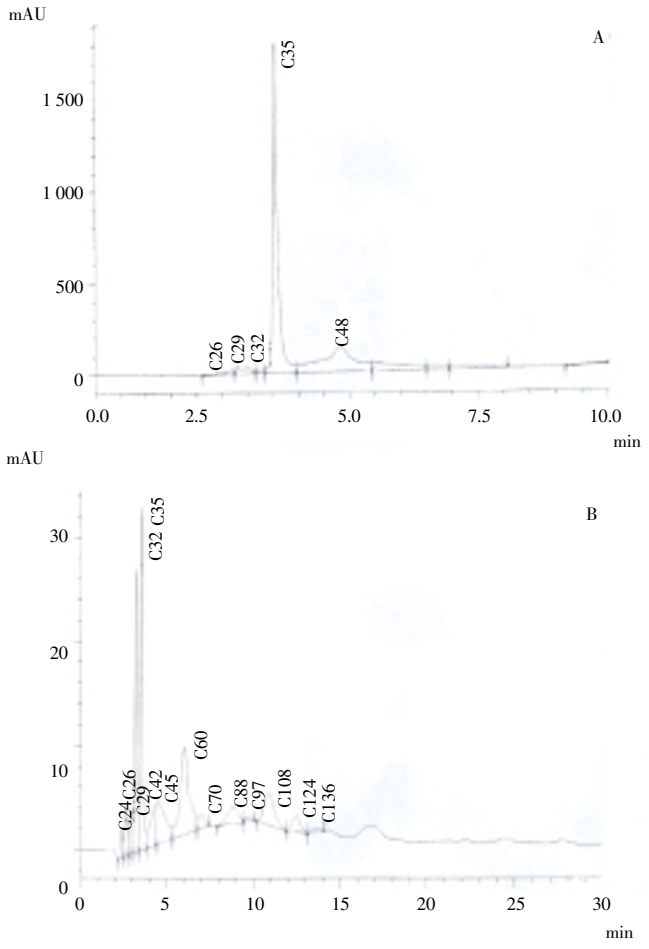

Figure 1. HPLC chromatogram of chloroform fraction of *P. niruri* methanol extract (A) & *P. niruri* methanol extract (B).

Results in Figure 2 show a dose-dependent suppression of parasitaemia by ME and is chloroform, ethanol and aqueous fractions in early infection. At 100-400 mg/kg b.w., ME suppressed parasitaemia by 37.65%-50.53%. The effect of the extract was significant ($P<0.01$) at 400 mg/kg b.w. The aqueous fraction, F3, was the least effective of the three fractions, suppressing parasitaemia by 21.18% - 51.18%, eliciting significant ($P<0.01$) activity only at a dose of 100 mg/kg b.w. The ethanol fraction (F2) had intermediate activity, producing significant ($P<0.01$) suppression of the parasite at 50 (64.71%) and 100 (67.06%) mg/kg b.w. The chloroform fraction possessed remarkable plasmodial suppressive activity. It significantly ($P<0.01$) reduced parasitaemia at 25 (49.41%), 50 (72.94%) and 100 (85.29%) mg/kg b.w. Its effect at 100 mg/kg b.w was comparable to the effect produced by chloroquine (89.41%).

As shown in Table 2, ME significantly ($P<0.01$) reduced parasitaemia in established infection at 200 (34.11%) and 400 (62.96%) mg/kg doses. Mean survival time was observed to increase in PNME-treated mice although this increase was insignificant ($P>0.05$), whereas mean survival time increased significantly ($P<0.05$) in the chloroquine-treated group.

Table 2
Effect of *P. niruri* methanol extract and its chloroform fraction on established infection in mice.

Treatment	Dose	Parasitaemia Day 3	Day 6	Mean survival time (days)	% suppression
Distilled water	–	4.31±0.62	6.83±0.54	16.60±0.40	–
PNME	100	4.27±0.36	5.75±0.54	17.00±0.68	15.81
	200	5.40±0.51	4.50±0.56**	17.60±1.91	34.11
	400	3.10±0.84	2.53±0.45**	19.40±1.83	62.96
F1	25	5.40±0.67	3.80±0.76**	18.60±0.68	44.36
	50	4.20±0.83	0.75±0.25**	18.20±0.74	89.01
	100	4.25±0.51	0.65±0.26**	22.80±1.59**	90.48
Chloroquine	5	4.55±0.46	0.24±0.09**	>30**	96.48

PNME = *Phyllanthus niruri* methanol extract, F1= Chloroform fraction, **significantly different from control at P <0.01 (n=5).

Comparatively, F1 dose-dependently and significantly (P<0.01) reduced parasitaemia by 44.36%-90.48%. In particular, a dose of 100 mg/kg b.w. significantly (P<0.01) increased mean survival time and favourably compared with chloroquine in this respect, unlike the crude extract.

In Figure 3, it was shown that ME and F1 also exhibited good prophylactic activity in repository infection. ME significantly (P<0.01) reduced parasitaemia at all the doses used, causing 67.5%-92.5% decrease in parasitaemia. It is noteworthy that the effect of the extract at 200 mg/kg b.w. was comparable to the effect of the standard reference drug, pyrimethamine. A dose of 400 mg/kg b.w. ME was more effective than pyrimethamine (1.2 mg /kg b.w.), reducing parasitaemia by 92.5% and 85.0% respectively. Significant prophylactic activity of F1 was also evident at 50 and 100 mg/kg b.w., yielding 55% and 60% (P<0.01) reduction of parasitaemia respectively.

The anti-hemolytic test carried out showed overall non-cytotoxicity of F1 to non-infected erythrocytes (Figure 4). However, cell membrane integrity of parasitized erythrocytes incubated with 125-500 mg/mL F1 improved in a concentration dependent manner, as concentrations of 250 and 500 mg/mL increased cell viability by 22.61% and 30.26%, respectively.

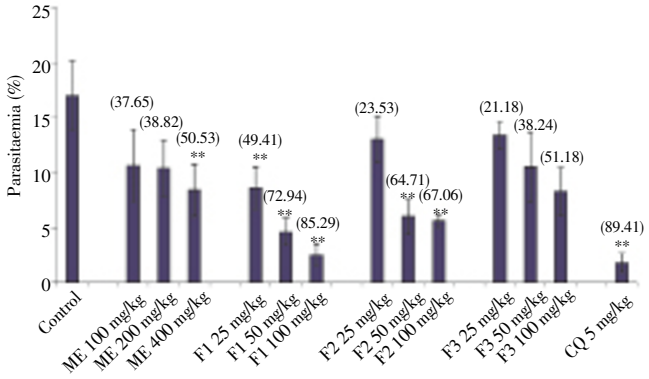

Figure 2. Antiplasmodial activity of *P. niruri* extract and its solvent fractions in early infection.
**significantly different from control at P<0.01 (n=5), values in parenthesis represent percentage suppression relative to control.

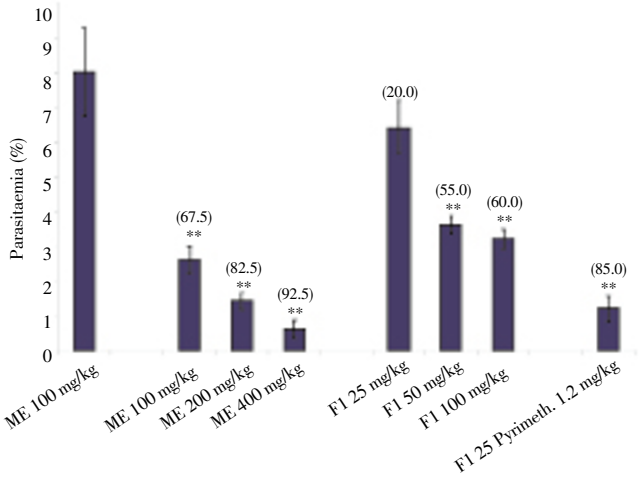

Figure 3. Prophylactic antiplasmodial activity of ME and F1
**significantly different from control at P < 0.01 (n=5).
Values in parenthesis represent percentage suppression relative to control.

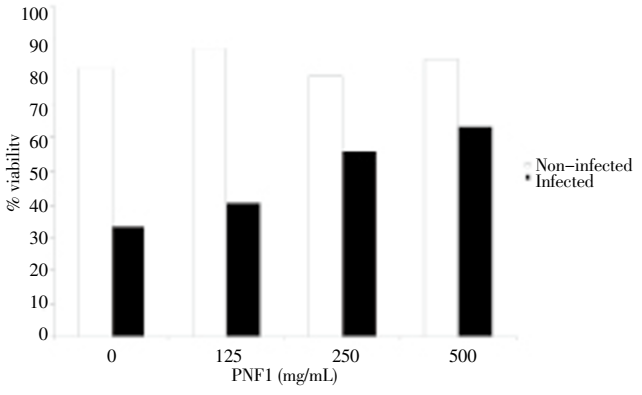

Figure 4. Effect of chloroform fraction of *P. niruri* methanol extract on erythrocyte viability *in vitro*.

4. Discussion

The herbal raw material is prone to a lot of variation due to several factors, the important ones being the identity of the plants and seasonal variation (which has a bearing on the time of collection), the ecotypic, genotypic and chemotypic

variations, drying and storage conditions and the presence of xenobiotics. The National Center for Complementary and Alternative Medicine and the WHO stress the importance of the qualitative and quantitative methods for characterizing the samples, quantification of the biomarkers and/ or chemical markers and the fingerprint profiles. It is a challenging task to develop suitable standards for herbal drugs. The advancements in modern methods of analysis and the development of their application have made it possible to solve many of these problems. Extremely valuable are techniques like high-performance thin-layer chromatography (HPTLC), gas chromatography (GC), mass spectrometry (MS) LC-MS, GC-MS and HPLC which has been applied in this study. The method validation of fingerprint analysis was performed based on the relative retention time and the relative peak area.

Based on these retention times, all the peaks seen in ME except peak 48 were common to F1, although their relative compositions differed. These chromatograms would serve as useful fingerprints, for the identification of *P. niruri* methanol extract and its chloroform fraction.

The secondary metabolites detected in the methanol extract used in our study show consistency with previous reports on phytochemical composition of *P. niruri*[19]. In the present study, the methanol extract of this extract exhibited potent activity. Our findings are supported by Mustofa *et al*[18], who reported that the methanol extract of *P. niruri* showed good *in vivo* and *in vitro* antiplasmodial activity against three strains of *Plasmodium*. The low level biological activity observed with the aqueous and ethanol extracts show that the bioactive components are not sufficiently present in these polar solvents unlike the highly non polar chloroform portion (F1). The observed difference may be attributed to the nature of the solvent. Similar observation has been reported by different investigators[28-30]. These observations indicate that the antiplasmodial components in ME are highly non-polar compounds, mainly resident in F1. This was also evident from its activity in established infection, which can be attributed to the localization of antiplasmodial active compounds in this fraction, relative to the crude extract. However, ME may be considered to possess better prophylactic antiplasmodial activity compared to its chloroform fraction F1, or pyrimethamine.

It has been suggested that, membranolytic effect by substances on erythrocytes could not be precluded in predicting mechanisms of parasiticidal activity. This may not be involved in the antiplasmodial effects of ME and F1, as it did not cause disruption of erythrocytic membranes. Their effects may not however be unrelated to the antioxidant effect of *P. niruri* alcoholic extract as suggested by Harish and Shivanandappa[17] in a separate report. The results of phytochemical screening of F1 indicate a positive correlation between the antiplasmodial activity of F1 and the localization of terpenoid substances, which have been shown to confer some plants with antiplasmodial properties[31-37]. Some terpenoids previously isolated from the plant include limonene, p-cymene and lupeol[38] and these compounds may account for its antiplasmodial activity. These secondary metabolites have been implicated in endoperoxidation, leading to their parasiticidal effects on Plasmodium[25,39].

The results of this study show that the chloroform fraction of *P. niruri* methanol extract possesses significant antimalarial activity *in vivo* and HPLC served as a useful analytical tool for its identification. Further work to isolate, purify and structurally elucidate the antiplasmodial compound(s) resident in this fraction is on going in our laboratories and will form a separate report.

Conflict of interest statement

The authors declare no conflict of interest.

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