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In-vivo antiplasmodial activity of methanol whole plant extracts of *Tapinanthus dodoneifolius* (DC) Danser in mice

Z. Abdullahi*, J. A. Anuka, A. O. Salawu and I. M. Hussaini

Department of Pharmacology and Toxicology, Kaduna State University, Kaduna-Nigeria.

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Aqueous preparations of the whole plant of *Tapinanthus dodoneifolius* (DC) Danser growing on *Parkia biglobosa* tree are used by the nomads in Northern part of Nigeria for managing malaria, and many other ailments example, diabetes, fever, diarrhoea and wounds. To date, there are no efficacy or safety studies carried out to support its ethno-medicinal use in malaria management. This study aims to investigate the pharmacological activity of the plant relevant to the symptomatic treatment of malaria. High-performance liquid chromatography (HPLC) fractionation of methanol extract of *Tapinanthus dodoneifolius* (MCETD) produced six fractions (TDF1 –TDF6). Three concentrations of the MCETD (100, 200 and 400 mg/kg body weight); and TDF3 (25, 50 and 100 mg/kg) were evaluated for anti-plasmodial activity against *Plasmodium berghei* parasite in mice using three models: early (suppressive) infection, established (curative) infection and residual (repository, prophylactic) infection. Normal saline and Chloroquine phosphate were used as negative and positive controls respectively. All three models used showed that both the methanol whole plant extract of *T. dodoneifolius* and TDF3 fraction produced significant ($p< 0.01$) and dose-dependent chemo-suppressive effect when compared with the negative control group. They also produced a reduction in parasite count and a significant ($p< 0.01$) and dose-dependent increase in the survival times of the infected mice as compared to the negative untreated group. The phytochemical analysis revealed the presence of carbohydrate, tannins, flavonoids, anthracene, cardiac glycosides, saponin glycosides, steroid and triterpenes. The oral and intraperitoneal medium lethal doses (LD_{50}) were estimated to be greater than 5000 mg/kg and 3800 mg/kg respectively. The results suggest the presence of pharmacologically active constituents in the extract with anti-plasmodial activity against *Plasmodium berghei* that justifies its use in malaria ethno-medicine.

Key words: *Tapinanthus dodoneifolius, Plasmodium berghei,* anti-plasmodial activity, ethno-medicine, malaria.

INTRODUCTION

Malaria is one of the major public health concerns in more than 90 developing countries of the world today,

*Corresponding author. E-mail: zulaihatua@yahoo.com.

affecting about 40% of the world's population. It is responsible for about 1.5 to 2.7 million deaths annually, with 75% of these deaths occurring in sub-Saharan African children. The World Health Organisation (WHO) estimated that in 2013, 3.3 billion people were at risk of malaria, of which 1.2 billion were at high risk. In high risk areas, more than one million cases occurred per 1000 population (WHO, 2014). Malaria is more serious in children, pregnant women and non-immune persons, or those suffering from sickle cell disease or Acquired Immune Deficiency Syndrome (AIDS), and can be fatal within hours or days, depending on the infecting species involved.

Malaria is endemic throughout Nigeria. The WHO mortality estimate for Nigeria is 729 cases per 100,000. Malaria is a serious worldwide health problem due to the emergence of parasites that are resistant to well established antimalarial drugs (Geib, 2007). Medicinal plants have in the past and in the last few years been the source of some of the most successful anti-malarial agents. The antimalarial potential of compounds derived from plants is proven by examples of quinine from *Cinchona* species and Artemisinin from *Artemisia annua*. Medicinal plants are commonly available in abundance, especially in the tropics. It is, therefore, of interest to screen medicinal plants for an evaluation of possible *in vitro* anti-plasmodial and *in-vivo* antimalarial activity (Adzu et al., 2007; Odugbemi, 2003).

Tapinanthus dodoneifolius belongs to the family Loranthaceae. It is a bushy parasitic plant, with stem of up to 1 metre long that grows on a wide range of trees and bushes of the wooded savannah zone. In Nigeria, it is called Etu-lonchi (Nupe), Elozie (Ibo), Kauchi (Hausa) and Afomu Igba (Yoruba). *T. dodoneifolius* plant has been used in folk medicine by the Hausa and the Fulani tribes in northern part of Nigeria (Deeni and Sadiq, 2002). It is used to treat many ailments including stomach ache, diarrhoea, dysentery, diabetes, epilepsy, hepatitis, hypertension, wounds and even cancer. In Nigeria, it is used as an antimicrobial agent in farm animals (Deeni and Sadiq 2002). It has also been reported to be used for inflammation, fever, infection, dizziness, energy loss, irritability, vertigo and headache. The plant was also shown to have a wide spectrum of antimicrobial activity against certain multiple drug resistant bacteria and fungal isolates of farm animals (Deeni and Sadiq, 2002; Cepleanu et al., 1994) revealed larvicidal and molluscidal effects of *T. dodoneifolius*. Closely related African mistletoe specie, *Agelanthus dodoneifolius* Polh and Wiens, was shown to possess antiplasmodial activity (Builders et al., 2012).

MATERIALS AND METHODS

Plant collection and preparation

T. dodoneifolius whole plant was collected from Abuja municipal area. It was identified and authenticated at the National Institute of Pharmaceutical Research and Development (NIPRD), Abuja.A voucher specimen (NIPRD/H /6591) was prepared and deposited at the NIPRD herbarium for future references. The plant material was cleaned, air-dried in the shade and pulverized into coarse powder using mortar and pestle. The powder was stored in a dry air-tight container until it was ready for use in the study.

Plant extraction

400 g of the coarse powdered plant was weighed and macerated in 2.5 L of 70% v/v methanol in water for 72 h with constant shaking using a GFL shaker. The resultant mixture was filtered using muslin cloth, followed by Whatman filter paper (No.1) and freeze-dried using AMSCO/FINN-AQUA GT2 Freeze dryer (Germany). The total yield of a dark green pasty substance was 39.78% w/w of crude stating material. Distilled water was used to dilute the extract before administration.

Experimental animals

Swiss albino adult mice (male and female) of 18 to 22 g body weight obtained from the Animal Facility Centre of NIPRD, Abuja were used in the study. They were kept in clean, dry cages and maintained in well-ventilated animal house maintained under standard conditions of temperature, humidity and 12 h light/darkness cycle. Water and pelleted feed were given *ad libitum* for the duration of the study, except when fasting was necessary in the course of the study. All experiments conformed to the principles for research involving animals as recommended by the Helsinki Declaration and the "National Academy of Sciences guide" (1996), on the care and use of Laboratory animals.

Malaria parasite preparation and inoculation

Chloroquine-sensitive malaria parasite, NK 65, *Plasmodium beghei* used in this study was obtained from the National Institute of Medical Research (NIMR) Lagos, and kept at the Department of Pharmacology and Toxicology National Institute of Pharmaceutical Research and Development, Abuja, Nigeria. The parasites were maintained by continuous re-infestation intraperitoneally in mice every 4 days (Adzu et al., 2007). Prior to the start of the study, one of the infected mice was kept and observed to reproduce signs and symptoms of disease similar to human malarial infection. The inoculum consisted of 1×10^7 of *P. beghei* parasitized erythrocytes per ml. Each mouse used was inoculated intraperitoneally with 0.2 ml infected blood containing 1×10^7 *P. beghei* parasitized red blood cells (Peters et al., 1993).

Phytochemical screening

The methanol whole plant extract of *T. dodoneifolius* was subjected to phytochemical screening to determine the presence of Phytochemicals such as alkaloids, carbohydrate, flavonoids, tannins, saponin, anthraquinones, steroids and triterpenoids (Trease and Evans, 1989).

Acute toxicity study

The modified method of Lorke was used. The study was carried out in two phases. In the first phase, 9 mice fasted overnight were randomly distributed into three groups of three mice each. Doses of 10, 100, 1000 mg extract/kg body weight were administered orally. The procedure was repeated using the intraperitoneal route. The

mice were observed for signs of toxicity and mortality for the first critical four hours and thereafter daily for 7 days. Symptoms of all adverse effects and death were observed and recorded. The second phase was determined by the outcome of the Phase one study. Another set of three groups of three mice were given 1600, 2900 and 5000 mg extract/kg body weight orally. The procedure was repeated using the intraperitoneal route. These mice were also observed for signs of toxicity and pattern of mortality for the first four hours, and thereafter daily for 7 days. The LD_{50} is expressed as: LD_{50} = geometric mean of $\sqrt{A \times B}$, where A = lowest lethal dose and B = higher non-lethal dose (0/1 and 1/1) respectively

Fractionation of extract

Methanolic extract of *T. dodoneifolius* (TD) was subjected to column chromatography using the method described by Harborne (Harborne, 1998). This consisted of Octadecylsilyl (ODS) silica gel stationary phase 100 g and 40 g of *T. dodoneifolius* whole plant extract (MCETD). Gradient elution under gravity was performed with 500 ml of each mobile phase mixture in series. The mobile phase consisted of Hexane: Ethyl acetate: Methanol, starting with Hexane (100%) and 10% increments in Ethyl acetate. This was followed by elution with Ethyl acetate (80%) and (20%) increments in Methanol. The final elution was performed with water. A total of 28 fractions were obtained. The elutes, were monitored with thin layer chromatography (TLC) using solvent system Ethyl acetate: Methanol (4:1); and elutes with similar TLC profile were pooled.

***In-vivo* anti-plasmodial study of methanolic extract**

The anti-plasmodial activity of the whole plant extract (100, 200 and 400 mg/kg) were evaluated by determining the suppressive, curative and prophylactic *in-vivo* activity using the methods of Knight and Peters (Peters et al.,1993; Bulus et al., 2003).

Evaluation of activity on early infection (4-day test)

Thirty adult Swiss albino mice were inoculated on the first day (day 0) by intraperitoneal injection with 0.2 ml standard inoculum of *P. berghei* containing 1 x 10^7 infected erythrocytes. The mice were then randomly divided into five groups of 6 mice each four hours after inoculation. Mice in group I were administered 5 ml/kg normal saline, while mice in groups II, III and IV were administered 100, 200 and 400 mg/kg body weight of the extract orally daily for four days (days 0 to 3). Group V mice were given 5 mg/kg body weight Chloroquine orally daily for four days. On the fifth day (day 4) of the study, blood was collected from the tail vein of each mouse and smeared onto a microscope slide to make a film (Saidu et al., 2000). The blood films were stained with 10% Giemsa at pH 7.2 for 10 min and parasitaemia levels determined by counting the number of parasitized erythrocytes out of 200 erythrocytes in random fields of the microscope. The average percentage chemo-suppression was calculated for each dose level by comparing the parasitaemia in infected controls with those of treated mice and multiplying by 100. Average % chemo-suppression = [(A - B)/A] x 100, Where A is the average parasitaemia in the negative control group, and B is the average parasitaemia in the treated group (Okonkon et al., 2006).

Evaluation of activity in established infection (Rane's test)

Thirty adult Swiss albino mice were inoculated with standard inoculum on the first day (day 0) of the study. Seventy two hours later, the mice were randomly divided into five groups of six mice each. Groups I and V mice were administered 5 ml/kg normal saline and 5 mg/kg of Chloroquine respectively. Mice in groups II, III and IV were administered with 100 mg, 200 mg and 400 mg/kg body weight of the extract orally. The administration was for 5 days. Thin blood films were prepared from tail blood of each mouse daily for five days on microscope slide to monitor the parasitaemia level (Peters et al.,1993). The mean survival time (MST) for each group was determined arithmetically by finding the average survival time (days) of the mice (post inoculum) in each group over a period of 28 days (days 0 to 27).

$$\text{MST} = \frac{\text{Number of days survived}}{\text{Total number of days (28)}} \times 100$$

Evaluation of activity in residual (repository) infection

Thirty adult Swiss albino mice were randomly divided into five groups of six mice each. Mice in group I and V were administered normal saline 5 ml/kg and Pyrimethamine 1.2 mg/kg body weight orally respectively. Mice in groups II, III and IV received 100 mg, 200 mg and 400 mg/kg of the extract orally (days 0 to 4). All administration was for five days. On the fifth day (day 4), all the mice were inoculated with 0.2 ml of 1 x 10^7 *P. berghei* NK 65 infected erythrocytes infected with the parasite. Smears were then made from each mouse 72 h after treatment and examined microscopically to monitor the parasitaemia level (Okonkon et al., 2006).

Statistical analysis

All quantitative data were expressed as the mean $\pm$ standard error of mean (SEM). Statistical analysis was carried out using one way analysis of variance (ANOVA), followed by Dunnet's post hoc test. Significant differences between means were assessed at 95% level of significance that is, p-value less than 0.05 ($p < 0.05$) was considered significant.

RESULTS AND DISCUSSION

This work sought to establish a basis for the ethno-medical use of *T. dodoneifolius* (DC) Danser (*Parkia biglobosa* mistletoe) in the treatment of malaria, and to evaluate the potential of its constituents as possible new antimalarial agent or lead to new antimalarial compound. This is to verify claim and establish a basis for its use in treatment of malaria cases in Northern part of Nigeria by traditional herbalists. This work compared the anti-plasmodial activity of *T. dodoneifolius* with the standard drug, Chloroquine, which has been used for suppressive, curative and prophylactic treatment of malaria.

The preliminary phytochemical screening of methanol extract of *T. dodoneifolius* revealed the presence of carbohydrate, cardiac glycosides, saponin glycoside, free anthraquinones, tannins, flavonoids, unsaturated steroid and triterpenes. The presence of these diverse constituents (secondary metabolites), offer support for the various ways in which this plant is used in traditional medicine, as these compounds are known to exhibit various physiological and biological activities *in-vivo* (Tor-Anyiin and Danisa 2012; Idowu et al., 2010; Fosola and Iyamah 2014; Akinmoladun et al., 2007). Anti-plasmodial

Table 1. Preliminary investigation of anti-plasmodial activity of column fractions of methanol crude extract of *T. dodoneifolius* on *P. berghei* infected mice.

Treatment	Mean parasite count ± SEM	% Chemo suppression
N/S 5 ml/kg	11.60±0.54	-
50 mg/kg F_1	8.67±0.54	25.26**
50 mg/kg F_2	7.00±0.27	39.66**
50 mg/kg F_3	1.80±0.54	84.48***
50 mg/kg F_4	10.00±0.84	13.79*
50 mg/kg F_5	8.01±1.54	31.03**
50 mg/kg F_6	Insufficient quantity	-
CQ 5 mg/kg	0.50±0.02	96.00***

N/S = Normal saline; CQ = Chloroquine; F1 to F6 = Column fractions of methanol whole plant extract of *T. dodoneifolius;* *p < 0.05; **p < 0.01**;** ***p < 0.001**;** One way ANOVA**;** n = 6.

Table 2. The effect of methanol whole plant extract of *T. dodoneifolius in early infection o*n *P. berghei* infected mice.

Treatment	Parasite count	% Suppression
N/S 5 ml/kg	15.00±1.40	-
MCETD 100 mg/kg	9.00±0.58*	40.00
MCETD 200 mg/kg	4.50±0.76**	70.00
MCETD 400 mg/kg	2.20±0.60*	85.00
CQ 5 mg/kg	1.00±0.37***	93.33

TD: *T. dodoneifolius* CQ: Chloroquine N/S: Normal saline, MCETD: Methanol crude extract of *T. dodoneifolius* *p < 0.05**;** ***p < 0.01;***p < 0.001**;** One way ANOVA; n = 6.

effects of natural plant products have been attributed to some of their active phytochemical components. Some of the Phytochemicals, such as tannins, saponins and flavonoids (detected in MCE*TD*) have been reported to have anti-plasmodial activity (Olajide et al., 2000; Sofowora, 2008; Edeoga et al., 2005). Flavonoids, steroids, anthraquinones, alkaloids and terpenes isolated from other plant species have been found to possess anti-plasmodial activity in both *in-vitro* and *in-vivo* studies (Edeoga et al., 2005; Christensen and Kharazmi, 2001; Adebayo and Kretti, 2011; Tona et al., 2001; Karou et al., 2003).

The oral and intraperitoneal LD_{50} of the extract was found to be greater than 5000 mg/kg and 3800 mg/kg body weight respectively. The animals presented with paw and genitalia-licking, salivation and calmness. The LD_{50} after oral administration of 5000 mg/kg body weight of the methanol extract, and intraperitoneal administration of 3800 mg/kg within observation period of 14 days implied that the extract was practically non-toxic, even in the group administered with the highest dose (5000 mg/kg). This indicates that the doses used in the experiment (100, 200 and 400 mg/kg) were safe. Hence higher doses can be given in order to achieve better anti-plasmodial activity. Fractionation of methanol extract produced six fractions (TDF1 to TDF6). However, the yield of fraction six (TDF6) was insignificant and hence was not used in further study. The fraction three (TDF3) gave the highest yield, and when subjected to curative anti-plasmodial tests also produced the highest activity of all the other fractions obtained (Table 1).

The results of *in-vivo* anti-plasmodial study showed that the methanol whole plant extract of TD possess significant and dose-dependent suppressive effect against early infection; curative effect against established infection and some prophylactic effect against residual infection in *P. berghei* infected mice. The extract also increased the mean survival time period of treated mice. In the early (suppressive) anti-plasmodial study, the methanol whole plant extract of *T. dodoneifolius* had a significant ($p< 0.05$) and dose-dependent parasite suppressive activity (40, 70 and 85%) at doses of 100, 200 and 400 mg/kg doses respectively) compared to the control group (Table 2). The chloroquine treated group showed the highest level of chemo-suppression (93.33%) when compared with all the extract doses used ($p< 0.001$). The TDF3 fraction showed a low to moderate level of suppressive activity ranging from 43.33, 49.33

Table 3. The effect of TDF3 fraction in early infection on *P. berghei* infected mice.

Treatment	Parasitaemia	% Chemo suppression	Mean survival time (days)
N/S 5 ml/kg	15.0±0.18	-	7.00
TDF3 25 mg/kg	8.5±0.72*	43.33	22.00
TDF3 50 mg/kg	7.6±0.16*	49.33	26.00
TDF3 100 mg/kg	5.6±0.16**	62.67	28.00
CQ 5 mg/kg	4.1±0.33***	72.67	29.0

N/S = Normal saline; CQ = Chloroquine; TDF3 = *T. dodoneifolius* column active fraction F3, *p < 0.05; **p < 0.01; ***p < 0.001; One way ANOVA; n = 6.

Table 4. The effect of methanol whole plant extract of TD in established infection in *P. berghei* infected mice.

Treatment	Mean parasite count	% Suppression	Mean survival time (days)
N/S 5 ml/kg	49.00±1.70	-	9.30±0.47
MCETD 100 mg/kg	4.80±0.53*	90.20	24.5 ±1.63*
MCETD 200 mg/kg	1.40±0.40**	97.14	28.00±0.00**
MCETD 400 mg/kg	0.60±0.40**	98.77	28.0 ±0.00**
CQ 5 mg/kg	0.20±0.20**	99.59	28.23±1.32**

TD: *T. dodoneifolius.* CQ: Chloroquine phosphate. N/S: Normal saline, MCETD: Methanol crude extract of *T. dodoneifolius* *p < 0.01**;** **p < 0.001; One way ANOVA, n = 6.

Table 5. The effect of TDF3 in established infection on *P. berghei* infected mice.

Treatment	Parasitaemia	% Chemo suppression	Mean survival time (days)
N/S 5 ml/kg	12.0±0.38	-	7.00
TDF3 25 mg/kg	2.50±0.28*	79.17	26.00
TDF3 50 mg/kg	2.20±0.11*	81.67	28.00
TDF3 100 mg/kg	1.80±0.21*	85.00	28.00
CQ 5 mg/kg	0.50±0.02**	96.00	29.00

N/S = Normal saline; TDF3 = *T. dodoneifolius* column active fraction F3, *P < 0.01; **p < 0.001; One way ANOVA; n = 6.

and 62.67% chemo suppression for the 25, 50 and 100 mg/kg doses respectively (Table 3). Agents with suppressive activity against *P. berghei* were known for antimalarial activity (Adebayo and Kretti, 2011; Tona et al., 2001; Karou et al., 2003).

In the established (curative) anti-plasmodial study, the methanol whole plant extract of TD at doses of 100, 200 and 400 mg/kg body weight significantly ($p < 0.01$) and dose-dependently reduced level of parasitaemia (90.2, 97.14 and 98.77% respectively) compared to the control group. The standard drug chloroquine produced the highest reduction in parasitaemia level (99.59%) when compared with all the extract doses used in the study, $p< 0.001$ (Table 4). The mean survival times for the extract doses (100, 200 and 400 mg/kg) were 24.5, 28 and 28 days respectively. Chloroquine treated group had a mean survival of 28 days, while the infected untreated group had a 9 days survival time (Table 4). The TDF3 fraction showed a high curative activity (79.1, 81.67 and 85.00% chemo suppression at doses of 25, 50 and 100 mg/kg respectively) as compared with control untreated group (Table 5). This suggests that both the extract and fraction F3 of TD possess significant blood schizontocidal activity against *P. berghei* parasite, as seen in the high mean survival times of the treated mice (Tables 4 and 5).

In the residual (prophylactic) anti-plasmodial study, the methanol whole plant extract of *T. dodoneifolius* at doses of 100, 200 and 400 mg/kg, significantly ($p< 0.05$) and dose-dependently reduced the level of parasitaemia compared to the control group (Table 6). Pyrimethamine produced the highest prophylactic parasite reduction level ($p < 0.001$) when compared to all the extract doses used in the study. The MCETD produced a dose-dependent chemo-suppressive effect (61.5 and 76.5%) at doses of 200 and 400 mg/kg respectively. However, the 100 mg/kg extract treated group had a much lower prophylactic activity (20%) as compared to the standard drug Pyrimethamine that produced a 91.5% prophylactic effect, $p< 0.001$ (Table 6). The TDF3 fraction had a low level of prophylactic activity at lower dose, but the higher

Table 6. The effect of methanol whole plant extract of *TD* in residual (prophylactic) infection on *P. berghei* infected mice.

Treatment	Parasite count	% Suppression
N/S 5 ml/kg	20.00±0.42	-
MCETD 100 mg/kg	16.00±0.67*	20.00
MCETD 200 mg/kg	7.70±0.71**	61.50
MCETD 400 mg/kg	4.70±1.00***	76.50
Pyrimethamine 1.2 mg/kg	1.70±0.67***	91.50

N/S = Normal saline; MCETD = Methanol crude extract of *T. dodoneifolius*, *p < 0.05**;** **p < 0.01**;** ***p < 0.001; One way ANOVA; n = 6.

Table 7. The effect of TDF3 in residual infection on *P. berghei* infected mice.

Treatment	Parasitaemia	% Chemo suppression	Mean survival time (days)
N/S 5 ml/kg	10.0±0.14	-	9.00
TDF3 25 mg/kg	6.40±0.14*	36.00	18.00
TDF3 50 mg/kg	3.80±0.18**	62.00	24.00
TDF3 100 mg/kg	1.90±0.35**	81.00	27.00
Pyrimeth 1.2mg/kg	1.10±0.32***	89.00	29.0

N/S = Normal saline; TDF3 = *T. dodoneifolius* column active fraction F3, *p < 0.05; **p < 0.01; ***p < 0.001; One way ANOVA; n = 6.

dose levels showed moderate to high prophylactic activity of 62 and 81% for 50 and 100 mg/kg doses (Table 7).

This may be attributed to a short duration of action of the extract, perhaps limited by rapid metabolism. It might also be due to the *in-vivo* model used which lacks the insect vector, and inoculation was done in such a manner and dose that results in rapid infection of the red blood cells without the parasite going through the liver stages (Edeoga et al., 2005). Death for *P. berghei* infected untreated mice were first observed on day 8 post-infection, and most of the animals died by day 10. Parasitaemia in the control untreated group was evident on the second day, and the level increased throughout the period of the study, while those of the extract treated groups were lower but not completely eliminated. The extract treated groups had a longer survival period which was significantly higher than the maximum survival period of the untreated group ($p < 0.01$). The fact that all extract and TDF3 treated mice groups had a prolonged survival times when compared with infected untreated mice showed that the whole plant extract possesses intrinsic anti-plasmodial activity.

Some plant extract are known to have anti-plasmodial activity either by causing elevation of RBC oxidation (Etkin, 1997) or by inhibiting protein synthesis (Kirby et al., 1993). It is not known in this study whether the anti-plasmodial activity of the extract was due to a specific anti-plasmodial action or to general cytotoxicity. Therefore, further bioactivity guided isolation of the constituent needs to be carried out on the extract.

Conclusion

The observed anti-plasmodial activity of the methanol whole plant extract of *T. dodoneifolius* and the column active fraction F3 might be due to the presence of pharmacologically active constituents like flavonoids, triterpenes, steroids and Anthraquinones. The study, therefore provide scientific support for the ethno-medicinal claim of the use of the plant in the management of malaria

Conflicts of interest

The authors declare that they have no conflicts of interest.

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