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Antiplasmodial Potential of the Ethanol Leaf Extract of *Triclisia macrophylla* and Its Fractions

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Authors' contributions

This work was carried out in collaboration between all authors. Author CEU wrote the protocol, and wrote the first draft of the manuscript. Author UPME designed the study and managed the analyses of the study. Author AEE managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Different plant extracts are used in Africa by the population as treatment for malaria. The antiplasmodial effect of the ethanol extract and fractions of *Triclisia macrophylla* was thus evaluated using Swiss albino mice 15-25 g. The mice were challenged with different doses of the extract and its n-hexane and butanol fractions in separate curative and suppressive tests. Results showed that the extracts exhibited significant ($p<0.001$) antiplasmodial activity which was in a dose dependent manner. They also showed 28% of chemosuppression and 23.4% and 20.2% for the n-hexane and butanol fractions and 20.2% for the 1500mg/kg/day dose of the extract. The mean survival time of the animal groups treated with the extracts were also significant ($p<0.001$) relative to the control. The LD_{50} test result confirmed that the plant is relatively safe as the animals that were given a high dose of 5000 mg/kg survived while initial phytochemical screening revealed the presence of saponins, tannins, flavonoids, cardiac glycosides, terpenes and alkaloids. The trado-medicinal use

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of this plant for the treatment of malaria is justified and its potential for further exploration in antimalarial therapy is acknowledged.

Keywords: Extract; antiplasmodial; Triclisia macrophylla; chemosuppression; malaria.

1. INTRODUCTION

Malaria is a mosquito borne disease which affects man and animals. It is caused by a protist of the genus *Plasmodium* and transmitted through the bite of an infected Female Anopheles mosquito. The infected mosquito transmits the parasite through the salivary gland from where it gets to the circulatory system of the host.

The disease is confined to tropical and subtropical regions of the world with African population accounting for the largest burden of the disease [1]. Malaria parasites are able to survive only in the cellular environment and are concentrated in the red blood cells. The affected persons may have feverish attacks, tiredness, headache and a whole range of other symptoms.

The resistance of malaria parasites to drugs in use for their control has become a serious problem especially in areas where malaria is endemic. Despite extensive interventions by the World Health Organization to control and possibly eliminate malaria, the transmission has continued in many countries of the world [2]. The most outstanding reason for this is the escalation of drug resistance parasites. Malaria therefore remains Africa's leading cause of mortality in patients under five years and constitutes the continents disease burden [3]. The search for novel and possibly more effective antimalarial compounds especially from medicinal plants is therefore of primary importance.

The resistance of plasmodium to synthetic drugs e.g chloroquine in endemic regions of the tropics remains the most important reason that has led to the increased use of certain herbs by many locals for malarial control. This has renewed the focus on plants as a source of potential antiplasmodial compounds [4]. This problem has become even more pronounced with the understanding of the gentle strength in plant preparations and the fact that they can be used safely without the known side effects of most drugs [5].

Triclisia macropylla commonly referred to as "Isim Oyood" by the Ibibio people of Akwa Ibom State, Nigeria is a perennial shrub occurring also as a climber and found growing on farmlands and forests. The leaves are hairy and alternate while the fruits which contains only one seed are green when not ripe but yellow when ripe. Generally, species of *Triclisia* are also found in Liberia, Tanzania, Angola, and The Central African Republic. They have been used in treating a wide range of ailments in places where they are found. In Cote d'Ivoire, the root pulp is used to treat joint pains and epileptic attack while the leaf juice is used to ease cough. In the Democratic Republic of Congo, a decoction of the twig bark is used to treat fever and malaria. *Triclisia microphylla* is one of the plants used by the indigenous people of Akwa Ibom State for the treatment of malaria.

2. METHODS

2.1 Plant Specimen Collection

Fresh leaves of *Triclisia microphylla* were collected from a farmland at Ekpene Ukim in Uruan Local Government Area of Akwa Ibom state Nigeria in the month of March. Taxonomic keys for the identification of the plant were provided by Professor (Mrs) Margaret Bassey, A taxanomist in the Department of Botany and Ecological studies, University of Uyo, Uyo Nigeria. A herbarium specimen with voucher No UUH49b was deposited at the herbarium of the Faculty of Pharmacy, University of Uyo.

2.2 Preparation of Ethanol Extract

The leaves were sorted and washed to remove any unwanted particles. They were then reduced to smaller size by slicing with a knife and then air dried for 14 days at room temperature. Dried leaves were then reduced to powder using mortar and pestle. The ethanol extract was obtained by maceration process using 2.5 L of ethanol (99%) and 400 g of the powder. This was done with stirring for 72 hours at room temperature. The filtrate obtained was concentrated and extract obtained was stored in a refrigerator at 4°C prior to use.

2.3 Experimental Animals

Male and female Swiss albino mice (15-25 g) with normal body temperature (35°C -37°C)

obtained from the animal house, Faculty of Pharmacy, University of Uyo, were used for the experiments. They were maintained on standard animal feed pellets ad libitum according to internationally known standards for the guide, care and use of laboratory animals. The principles of laboratory animal care (NIH publication No 85-23, revised 1985) were followed as well as specific national laws where applicable. All experiments were examined and approved by the animal ethical committee of the Faculty of Pharmacy, University of Uyo-Nigeria.

2.4 Phytochemical Screening

The leaf extract was screened for its phytochemical constituents using the methods described by [5a].

2.5 Fractionation of Extract

Ethanol extract (20 g) was dissolved in 100ml of distilled water in a separating funnel. N-hexane (100 mL) was then added to the solution and shaken vigorously for between 5 and 10 seconds. The mixture was allowed to stand in a clamped position for 10 minutes during which adequate separation took place. The n-hexane fraction which was on top was collected. The same process was repeated for the butanol fraction using the left over aqueous fraction and 100 ml of butanol. Both fractions were concentrated in vacuo using temperature less than 40°C.

2.6 Malaria Parasite

The chloroquine sensitive *Plasmodium berghei* (NK 65) was used for the experiment. They were obtained from the Nigerian Institute for Medical Research (NIMR) Lagos and maintained in the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Uyo, Nigeria.

2.7 Parasite Inoculation

The mice used for the experiment were firstly confirmed to be free from any malaria parasites before use. This was done by observing smears made using blood obtained from the tail of the animals under the microscope. Each of the screened animals was then inoculated with 0.2mL of infected blood. They were left for four days to incubate after which blood samples were taken from the tip of their tails to determine the parasitaemia levels before the extract and fractions were administered. After the adminisration, blood samples were also collected from the tip of the tails of the animals at regular intervals of 48 hours and the parasitaemia levels were determined.

2.8 Acute Toxicity Study

Acute toxicity study was done to determine the safety of the extract used in the study. The median lethal dose (LD_{50}) was determined using albino mice which were divided into 6 groups of three mice each. Doses, 500mg/kg, 1000mg/kg, 1500 up to 5000 mg/kg were administered to each group. Toxicity signs and mortality were looked out for while the LD_{50} was calculated according to the method of Lorke, [6]

$$LD_{50}=\sqrt{AB}$$

A=Maximum dose that produced 0% mortality
B=Minimum dose that produced 100% mortality

3. ANTIPLASMODIAL STUDIES

3.1 Suppressive Test

Tests were performed according to the method of Knight and Peters (6a). A total of 42 mice were used for the study. Each mouse was given intraperitoneally, 0.2ml of blood which contained $1.0x10^7$ *P. berghei* parasites. Then they were divided into seven groups of six mice each. Using sterile disposable syringes, the groups 1, 2 and 3 were orally treated with 500, 1000 and 1500 mg/kg/day respectively. The group 4 received 5 mg/kg/day of chloroquine (standard control) while group 5 was given 10 mL/kg/body weight of distilled water (positive control). The groups 6 and 7 were given 1000 mg/kg/day each of hexane and butanol fractions respectively. All extracts and fractions administrations were done orally during four days after which blood samples were obtained from the tip of the tails, smeared, Giemsa stained and observed under the microscope for the level of parasitaemia at 48 hours intervals. The average percentage chemosuppression was thus calculated using the formula.

Average percentage chemosuppression:

$$100\left(\frac{A-B}{A}\right)$$

A=Average percentage parasitaemia in the control group
B=Average percentage parasitaemia in the test group

3.2 Curative Test

The animals were treated in the same way as in the suppressive test except that the drugs/extracts were administered once daily for six days. The level of parasitaemia was determined every 48 hours using the blood obtained from the tip of the tail of the mice. The mean survival time for each group was thus calculated within a 28 day period.

$$\% \text{ Parasitaemia} = \frac{No.of\ Parasitized\ RBC}{Total\ No.\ of\ RBC\ counted} x100$$

$$\text{MST} = \frac{Number\ of\ days\ survived}{Total\ number\ of\ days\ (28)} x100$$

3.3 Statistical Analysis

Differences in the mean were expressed using ANOVA and values of P< 0.001 were considered as significant.

4. RESULTS

4.1 Phytochemical Screening

The results of the phytochemical screenings are as shown in Table 1. They reveal the presence of tannins, flavonoids, saponins, alkaloids, cardiac glycosides, terpenes and anthraquinones.

4.2 Acute Toxicity Test

Results obtained showed that there was no mortality recorded on oral administration of the extract even at doses as high as 5000 mg/kg body weight. This confirms the relative safety of the extract.

4.3 Test Animal Responses

Before the parasites were inoculated, the experimental mice were in good condition. They fed normally and had normal body temperature of 35 -37.4°C. They also showed normal movement of whiskers and limbs. Four days post inoculation of the parasites however, the following signs were observed. 1. Increased body temperature 37.9-38.1°C 2.Decline in feeding rate. 3. Raised fur. 3. Shivering and paleness of limbs and tails pointing to an anemic condition. A reduction in the movement of whiskers was also observed.

Table 1. Results of phytochemical screening of the ethanol extract of the leaf of *Triclisia microphylla*

Phyto constituents	Test	Result
Tannins	Ferric Chloride	+
Flavonoids	Magnesium Metal	+
Alkanoids	Dragendorff's	+
Saponins	Frothing	+
	Sodium Bicarbonate	+
Terpenes	Glacial acetic acid	+
Anthraquinones	Free anthraquinones	-
	Combined Anthraquinones	-
Cardiac glycosides	Lieberman	+
	Salkowski	+
	Keller-kiliani	+

4.4 Suppressive Test

Results obtained showed that the ethanol extract produced a dose dependent chemo suppressive effect (Table 2). While the n-hexane and butanol fractions showed 28.80 and 23.42% chemosuppression respectively, the ethanol extract showed 13.79, 18.02, 20.19% suppression when 500, 1000 and 1500mg/kg body weight of the extract doses were administered.

4.5 Curative Test

A daily decrease in the parasitaemia levels of the mice in the groups treated with the ethanol extract and fractions was observed. This was also true for the group treated with chloroquine (Positive control). On the contrary, there was a daily increase in the parasitaemia level of the negative control group not treated with any of the extracts. The results are presented in Table 3 as well as in Figs. 1 and 2.

4.6 Mean Survival Time

Results obtained showed that the mean survival time of the group of mice treated with the extracts and fractions were significantly longer than those not treated (Negative control). Mice treated with the standard drug chloroquine survived beyond the 28 day observation period. Results are presented in Table 4 and Figs. 3 and 4.

Table 2. Antiplasmodial effect of the leaf extract and fractions of *Triclisia macrophylla* during the 4 day (suppressive) test

Drug/Extract/Fractions	Dose (mg/kg/day)	% parasitaemia	% chemosuppression
Ethanol extract	500	80.79±1.07[a]	13.79
	1000	76.82±0.59[a]	18.02
	1500	74.79±0.55[a]	20.19
n-hexane fraction	1000	66.72±0.83[a]	28.80
Butanol fraction	1000	71.76±0.33[a]	23.42
Chloroquine	5	3.93±0.22[a]	95.80
Distilled water	10 mL	93.71±0.06	

Values are expressed as mean ± SEM, significance relative to control [a] p <0.001, n=6

Table 3. Antiplasmodial effect of the extract and fractions of *Triclisia macrophylla* on established infection with *Plasmodium berghei*

Drug/Extract/Fractions	Dose (mg/kg)	Day 2	Day 4	Day 6
Ethanol extract	500	87.63±2.28[ns]	80.93±0.40[c]	78.45±0.98[c]
	1000	82.56±1.7[a]	73.74±0.68[c]	73.32±0.63c
	1500	79.56±2.54[b]	70.41±0.13[c]	67.15±1.47[c]
n-hexane fraction	1000	72.82±3.28[c]	68.38±2.05[c]	64.83±3.49[c]
Butanol fraction	1000	75.91±3.24[c]	74.79±0.55[c]	69.52±1.15[c]
CQ	5	48.53±2.54[c]	24.17±1.41[c]	15.54±2.72[c]
Distilled water	10 mL	93.57±0.22	96.42±0.56	96.91±0.36

values are expressed as mean ±SEM significance relative to control [a] p <0.05, [b] p <0.01, [c] p <0.001, n=6
[ns] – Not significant, CQ – Chloroquine

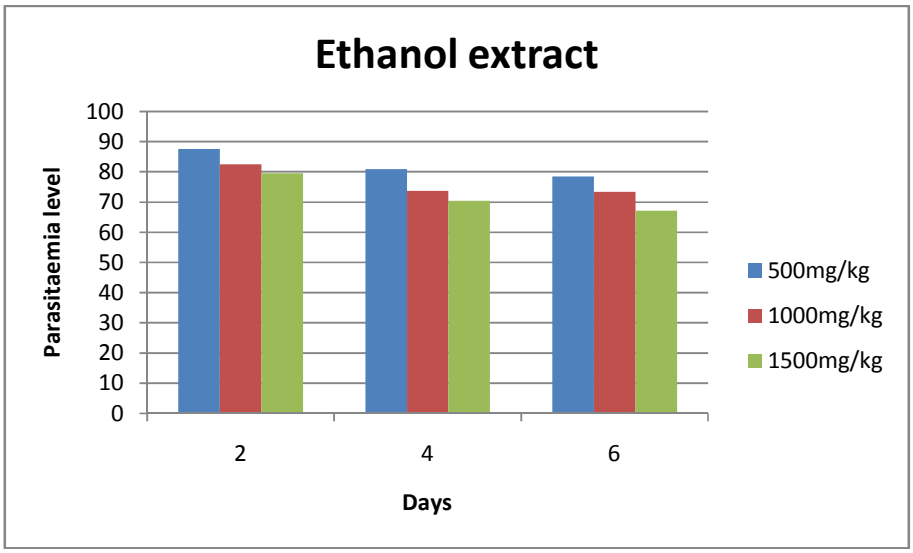

Fig. 1. Parasitaemia levels after administration of different concentrations of Ethanol leaf extract of *Triclisia macrophylla*

5. DISCUSSION

In Africa, indigenous plants have been known to play a very important role in the traditional method of malaria treatment. Interestingly, some of the plants used have shown real antiparasitic activity [7] and many of them are relatively safe [8].

Phytochemical screening results showed that *Triclisia macrophylla* contains tannins, flavonoids, saponins, cardiac glycosides and terpenes. These compounds have been reported to have antiplasmodial effect [9,10,11,12]. Generally, the therapeutic properties of medicinal plants are linked to the phytochemical compounds that they contain.

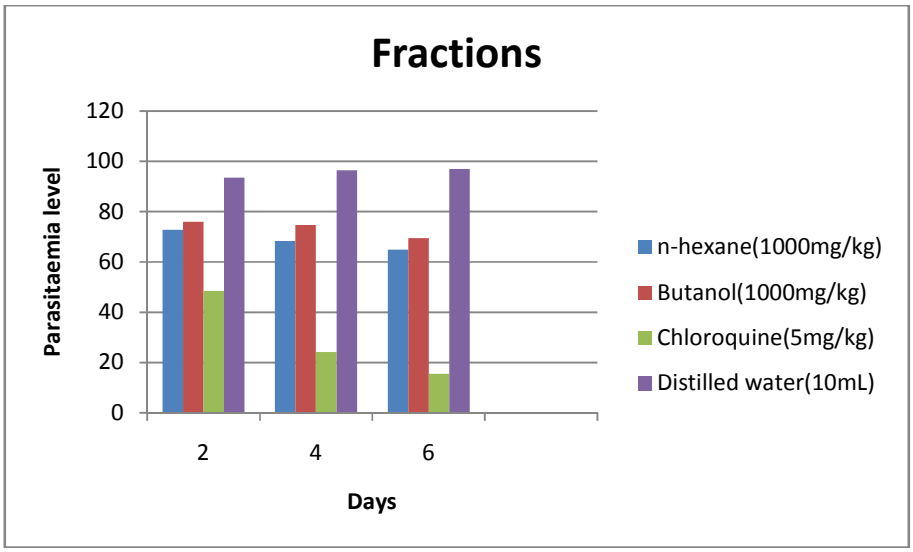

Fig. 2. Parasitaemia levels after administration of butanol and hexane fraction of ethanol leaf extract of *Triclisia macrophylla* using chloroquine as positive control

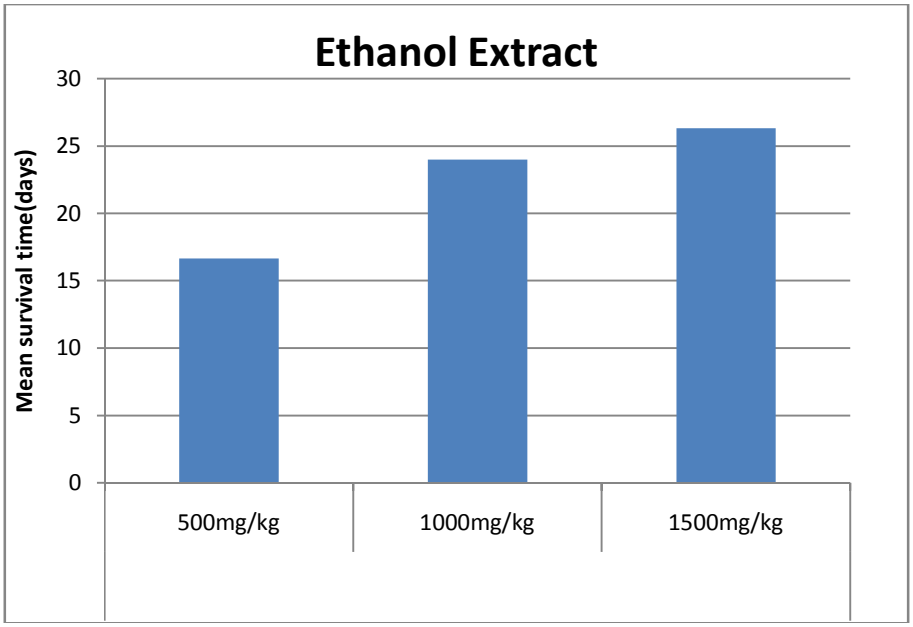

Fig. 3. Mean survival time in days after administration of different concentrations ethanol leaf extract of *Triclisia macrophylla*

The lethal dose test showed that the test animals survived after the administration of very high dose of 5000 mg/kg/day confirming the relative safety of the plant. The greatest problem with the use of plant extracts especially in ethno-medicine has been that of toxicity. Plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well-being [13]. Literature is awash with compounds that have been isolated from a variety of medicinal plants [14]. The problem remains that a lot of them are too toxic to be used on humans. The lethal dose result makes this plant very unique and interesting as most other plant extracts are known to be toxic at even far lower doses.

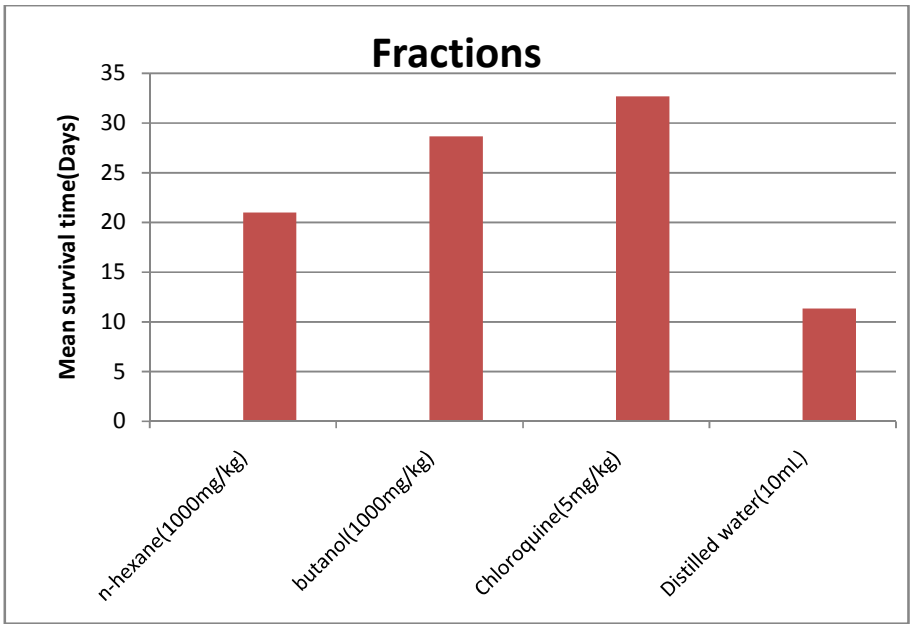

Fig. 4. Mean survival time after administration of butanol and hexane fractions of Ethanol leaf extract of *Triclisia macrophylla* using chloroquine as positive control.

Table 4. Mean survival time (MST)

Drug/Extract/ Fraction	Dose (mg/kg)	MST
Ethanol extract	500	16.67±0.21[a]
	1000	24.00±0.73[a]
	1500	26.33±0.76[a]
Butanol fraction	1000	21.00±0.36[a]
n-hexane fraction	1000	28.67±0.76[a]
Chloroquine	5	32.67±0.56[a]
Distilled water	10 mL	11.33±0.21

Values are expressed as mean ± SEM, significance relative to control [a] p <0.001, n=6

The ethanol extract of *Triclisia macrophylla* exhibited a dose dependent antiplasmodial activity following its oral administration to the mice in groups. The percentage chemo suppression achieved with 500, 1000 and 1500 mg/kg/day doses of the ethanol leaf extracts were 13.79, 18.02 and 20.19 respectively (Table 2). The n-hexane and butanol fractions however gave a higher chemo suppression percentage of 28.80 and 23.42. The fact that these are fractions which are purer than the crude extract may be the explanation. With the fractions, the metabolites which exert the antiplasmodial effect are pooled together in the n- hexane fraction because of their polarity likeness and are more likely to present a better picture of activity than the crude plant extract.

When the antiplasmodial effects of the extracts were evaluated on established infection, results obtained confirmed the trend obtained during the suppression test experiment (Tables 3). The mean survival time (MST) results (Table 4) further confirms the antiplasmodial activity with the number of days of survival comparing favourably with the standard drug–chloroquine.

6. CONCLUSION

Results obtained in this study confirm the antiplasmodial potentials of the plant *Triclisia macrophylla*. This explains the reason for its ethno botanical use as a remedy for malaria by the Ibibio people of Akwa Ibom state-Nigeria. Results obtained also confirm the safety of the plant extracts which makes it an interesting candidate for further antiplasmodial studies.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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