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In vivo Antiplasmodial Activity of Ethanolic Leaf Extract of *Tithonia diversifolia* (Hemsl.) A. Gray against *Plasmodium berghei* Nk65 in Infected Swiss Albino Mice

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Authors' contributions

This work was carried out in collaboration between both authors. Author DAO designed the study, wrote the protocol, and wrote the first draft of the manuscript. Authors EOD and DAO managed the literature searches, analyses of the study performed the spectroscopy analysis and author DAO managed the experimental process and author EOD identified the species of plant. Authors EOD and DAO read and approved the final manuscript.

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ABSTRACT

Antiplasmodial activity of the ethanolic leaf extract of *Tithonia diversifolia* was studied in Swiss albino mice infected with *Plasmodium berghei* (NK65). The study was carried out at the Department of Microbiology, Federal University of Technology, Akure, Nigeria between April and December, 2015. Qualitative phytochemical screening was carried out using standard procedure. Thirty experimental mice (average weight of 18-22 g) were divided into six groups (five mice per group). Groups 1, 2 and 3 were infected with *P. berghei* and treated with 0.2 ml of 200, 400 and 600 mg/kg body weight of *T. diversifolia* extract respectively. Group 4 (positive control) was infected with *P. berghei* and treated with 0.2 ml of 5 mg/kg body weight of chloroquine. Group 5 (negative control) was infected with *P. berghei* and treated with 0.2 ml of normal saline and group 6 (normal control) was not infected but administered with 0.2 ml of normal saline. Qualitative phytochemical

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screening revealed the presence of saponin, alkaloid, flavonoid, tannin and cardiac glycoside while quantitative screening revealed the presence of tannins (0.39 mg/100 g), flavonoids (0.87 mg/ 100 g), cardiac glycosides (1.19 mg / 100 g), alkaloids (1.23 mg / 100 g) and saponins (1.37 mg / 100 g). Suppressive test shows that leaf extract exerted significant (P<.05) dose-dependent reduction against the parasite. Chemo-suppression of 60.02%, 63.45% and 92.0% were respectively observed in mice treated with 0.2 ml of 200 mg/kg, 400 mg/kg and 600 mg/kg body weight dosage in groups 1, 2 and 3. Group 4 (positive control) and group 5 (negative control) had 100% and 0.0% chemo-suppression respectively. In conclusion, the ethanolic leaf extract of *T. diversifolia* at 600 mg/kg significantly suppressed the parasitaemia in the infected experimental mice.

Keywords: Antiplasmodial; Tithonia diversifolia; Swiss albino mice; Plasmodium berghei; NK65.

1. INTRODUCTION

Malaria has not only been reported to be the world's most devastating human parasitic infection but it has also been implicated as one of the major public health problems and greatest health challenges faced by the world's population. The causative agent is a protozoan parasite belonging to the genus *Plasmodium* and vectored by the bite of the female *Anopheles* mosquito. The disease has caused much suffering and premature death among pregnant women and children less than five years of age in the poorer region of the tropics and sub tropics [1].

According to WHO [2], the global estimate of malaria cases was within the range of 149-303 million in year 2015. Most of these cases have occurred in WHO African region with 88%, followed by the WHO South-East Asia region with 10% and WHO Eastern Mediterranean region with 2%. Despite the increase in the population growth, malaria cases had drastically reduced by 37% between years 2000 and 2015. In addition, the number of malaria deaths globally fell from 839000 in year 2000 to 438000 in year 2015.

Several years ago, antimalarial drugs such as chloroquine, fansidar, mefloquine and malarone were effectively used to treat malaria. However, development of resistant strains of *Plasmodium*, particularly *P. falciparum* to these drugs has since led to their abandonment in treating malaria in many affected areas. In view of this, scientists are into research for new anti-malarial compounds, either natural or synthetic that are important for the killing of either the parasite or vector. Consequently, urgent research becomes necessary to put into use, the naturally endowed rich biodiversity around us for the benefit of mankind. Hence, studies on medicinal plants could come out with useful information for the synthesis of important active and non-toxic compounds as antimalarials [3].

The leaf extract of *Tithonia diversifolia* (Hemsl.) A. Gray in this study could be one of such medicinal plants for the treatment of malaria. The plant belongs to the family of genus Astereacea and is commonly referred to as Mexican sunflower, or tree marigold. It is a bushy perennial weed commonly found on the fields, wasteland and road sides of Nigeria [4,5]. Mexican sunflower is a robust plant that can vary significantly in habit. Sometimes an unbranched or sparsely-branched annual, it can also be a short-lived perennial, or the plant can become shrub-like with more or less woody stems. *Tithonia diversifolia* is 2–3 m (6.6–9.8 ft) in height with upright and sometimes ligneous stalks in the form of woody shrubs. In addition, *Tithonia diversifolia* often form part of remedies for farmers as manure for farming and human therapies for treatment of diabetes mellitus, stomach pains, indigestion, sore throat, liver pains and malaria [6].

2. METHODOLOGY

2.1 Collection and Identification of *Tithonia diversifolia* Leaves

Fresh leaves of *Tithonia diversifolia* were collected in April, 2015 from Ikere-Ekiti, along Ise-Ekiti road, Ekiti State, Nigeria. Leaves were identified and authenticated by a plant scientist (Dr. L. Fayehun), in the Department of Crop Soil & Pest Management, School of Agriculture and Agricultural Technology, The Federal University of Technology, Akure.

2.2 Extraction and Phytochemical Screening of Leaves

The extraction process of the plant leaves were carried out using the organic solvent extraction

method by mechanical mixing. The leaves were washed in distilled water and air dried at room temperature for four weeks until they become crispy and then pulverized using mortar and pestle. Five hundred grams (500 g) of the pulverized leaf powder was macerated in 4.5 litre of 75% ethanol for 72 hours and then filtered using Millipore (pore size 0.7 µm) filter paper. The filtrate was concentrated using rotary evaporator, the resulting extract was freeze-dried and then stored in an air tight container.

2.2.1 Qualitative determination of phyto-chemicals

2.2.1.1 Alkaloids

The ethanolic leaf extract was warmed with 2% sulphuric acid for two minutes. It was filtered with Whatman No.1 filter paper. Two drops of Wagner's reagents were added. A reddish-brown precipitate appeared which confirm the presence of alkaloids in the extract [7].

2.2.1.2 Flavonoids

Half a gram (0.5 g) of the extract was heated with 10 ml of ethyl acetate in boiling water for 3 minutes. The mixture was filtered; thereafter the filtrate was shaken with 1 ml of 1% dilute ammonia solution. A yellow colouration which was observed at the ammonia layer was taken to indicate the presence of flavonoid in the extract [7].

2.2.1.3 Tannins

Half a gram (0.5 g) of the extract was boiled with 5 ml of 45% solution of ethanol for 5 minutes. The mixture was cooled and filtered thereafter, 1ml of filtrate was diluted with distilled water and two drops of ferric chloride solution was added. A blue greenish coloured precipitate indicated the presence of tannins [7].

2.2.1.4 Glycosides

Two mililitre (2 ml) of aqueous extract was mixed with 2 ml of glacial acetic acid containing one drop of 5% ferric chloride. The mixture was added to 1 ml of concentrated sulphuric acid. Reddish brown ring colour appeared at the junction of the two liquid layers confirming the presence of glycosides [7].

2.2.1.5 Saponins

Foam test were conducted to detect saponins. About 3 g of the extract was diluted with 20 ml of distilled water and it was shaken in a graduated cylinder for 15 minutes. A layer of foam indicated the presence of saponins [7].

2.2.2 Quantitative determination of phytochemicals

2.2.2.1 Alkaloids

A suspension was prepared by dispersing 5 g of the leaf extract in 10% acetic acid solution in ethanol and kept at 28℃ for 4 hrs which was further filtered through Whatman filter paper No. 42. Thereafter alkaloid was precipitated by concentrating the filtrate to one quarter of its original volume and drops of concentrated ammonium hydroxide were added until precipitation was complete. Finally the precipitate was washed with dilute ammonia solution and dried at 80℃ in the oven, then weighed [8].

2.2.2.2 Tannins

The ethanolic leaf extract of 0.2 g was weighed into a 50 ml sample bottle; 10 ml of 70% aqueous acetone was added and properly covered. The bottle was put in an ice bath shaker and shaken for 2 hours at 30℃. Each solution was then centrifuged and the supernatant was stored in ice bath. Thereafter, 0.2 ml of each solution was pippeted into the test tube and 0.8 ml of distilled water was added. Standard tannic acid solutions were prepared from 0.5 mg/ml of the stock made up to 1 ml with distilled water. Thereafter, 0.5 ml of frolin Ciocaiteau was added to both sample and standard followed by 2.5 ml of 20% Na_2CO_3 .The solution was then vortexed and allowed to incubate for 40 minutes at room temperature. The absorbance of the sample was read at 725 nm against a reagent blank [9].

2.2.2.3 Saponins

Isobutyl alcohol of 100 ml was added to 1 g of leaf extract and stirred for 5 hrs filtered with No 1 Whatman filter paper into 100 ml beaker. 20 ml of 40% saturated solution of magnesium carbonate was added to the filtrates, also 2 ml of 5% ferric chloride solution and 50 ml volume of distilled water was added to 1 ml of colourless solution and kept for 30 min for colour (blood red) development. The absorbances of the samples along with the standard were read at 380 nm [10].

2.2.2.4 Flavonoids

In a test tube, 1 ml of extract was separately mixed with 1 ml of methanol, 0.5 ml of 1.2%

aluminium chloride, and 0.5 ml of 0.12 M potassium acetate and 2.8 ml of distilled water. Mixture was kept at room temperature for 30 minutes; the absorbance of the reaction mixture was measured at 415 nm using a spectrophotometer [11].

2.2.2.5 Cardiac glycosides

Half a gram (0.5 g) of the extract was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was then under layered with 1 ml of concentrated sulphuric acid. A brown colouration which was obtained at the interface indicated of a deoxy-sugar characteristic of cardenolides was measured [11].

2.3 Experimental Mice

Swiss albino mice of both sexes and average weight of 18-22 g were obtained from the Animal House Laboratory of Faculty of Health Sciences, Obafemi Awolowo University, Ile-Ife. Nigeria. The animals were housed in plastic cages with saw dust bedding at room temperature. They were fed with standard diet (Grand cereal) and water ad libitum for 7 days and were allowed to acclimatize prior to the study. Chloroquine sensitive strain of malaria parasite (*Plasmodium berghei* NK 65) in a donor mouse was obtained from the Department of Pharmacognosy, Faculty of Pharmacy Obafemi Awolowo University Ile-Ife, Nigeria. The method described by National Institute of Health was followed in handling of experimental mice throughout the course of the study [12]. The entire experimental management, handling and care were approved by the Research and Ethics Committee of the Federal University of Technology, Akure, Nigeria.

2.4 Preparation of Leaf Extracts Dosages

The dosage of the extract administered to the experimental mice were prepared by dissolving 0.1, 0.2, 0.3, 0.4 and 0.8 mg of the extract in 5 ml of distilled water each in sterile universal bottle based on the weight and total number of mice per group to obtain 200, 400, 600, 800and 1600 mg/kg body weight respectively.

2.5 Acute Toxicity Test

The acute toxicity test was carried out according to modified Lorke's method by Ezeonwumelu [13]. Sixteen mice were randomly divided into four groups (1, 2, 3, and 4) of four mice per group. Group 1 (normal control) was orally administered with 0.2 ml of distilled water, groups 2, 3 and 4 were orally administered with 0.2 ml of 400, 800, 1600 mg/kg body weight of ethanolic leaf extract respectively. They were observed for signs of toxicity and general behaviour such as reduced activities, licking paw, body weakness, convulsion, sleeping, salivation and mortality for 24 hours.

2.6 Preparation of Inoculums

Donor mouse was anaesthetised with chloroform and 0.5 ml of blood containing *P. berghei* infected erythrocytes was withdrawn through the cardiac puncture with syringe, transferred into a screw capped sterile plastic tube and then topped up to 14 ml with normal saline to obtain $1x10^7$ *Plasmodium berghei* infected erythrocytes [14].

2.7 *In vivo* Antimalarial Activity of the Extracts

Antimalarial activity of the leaf extract was determined using the Peter's 4-day suppressive test method [15]. Thirty mice were randomly distributed into six groups of five mice per group. Groups 1 to 5 were infected intraperitoneally with 0.2 ml of 1 $x10^7$ standard inocula of chloroquine sensitive *P. berghei*. Four hours after post-infection, 0.2 ml of 200, 400 and 600 mg/kg body weight of leaf extract were orally administered to groups 1, 2 and 3 respectively as treatment dose once daily for four consecutive days. Group 4 (positive control) was treated with 0.2 ml of 5 mg/kg body weight of chloroquine, group 5 was given 0.2 ml of normal saline (negative control) and group 6 (normal control) received 0.2 ml of normal saline but was not infected with *P. berghei.*

2.8 Determination of Parasitaemia

The parasitaemia level was determined by the method described by Cheesbrough [16]. On the 5th day of the experiment, 2 drops of blood sample were respectively collected onto grease free microscopic slide from the mice caudal vein. Thick and thin blood smear were made and allowed to air-dry. The air-dried films were fixed with 75% methanol for 2 minutes, they were thereafter stained with 10% Giemsa stain for 15 minutes. The blood smear samples were rinsed with buffered distilled water pH 7.2. The

parasitaemia was determined by counting the part of the film where the white cells and parasites were evenly distributed. Using the oil immersion objective (x100), white blood cells were counted systematically, counting at the same time the number of parasites in ten fields. The counting was done using hard tally counter.

> Number of parasites per microlitre (µL) of blood = (White Blood Cells count (WBC) X Parasites counted against 200WBC / 200)

Percentage suppression (PS) of parasitaemia was calculated using the percentage suppression determination formula [17] stated below:

> PS = (Parasitaemia in negative control - Parasitaemia in study group / Parasitaemia in negative control) X 100

2.9 Measurement of Weight and Temperature

The body weight and temperature of the mice were determined as described by Peters et al. [15]. Body weights of the mice were measured throughout the study on electronic precision balance (KD-KC-2000). Rectal temperatures of mice were obtained using a digital thermometer (BIOSEB-BIO9882). All the control groups and malaria-infected mice were observed visually throughout the experiment for behavioural changes which include diarrhoea, lethargy, reduced activities, sleeplessness and loss of appetite.

2.10 Statistical Analysis

Results obtained from this study were statistically analyzed using one way analysis of variance (ANOVA) and New Duncan's Multiple Range Test using SPSS 20.0. Significant differences between the treatment means were determined at 95% confidence level.

3. RESULTS

The phytochemical screening of ethanolic leaf extract is shown in Table 1. Qualitative screening revealed the presence of saponins, alkaloids, tannins, flavonoids and cardiac glycosides. Quantitative analysis revealed the presence of saponins (1.37 mg / 100 g), alkaloid (1.23 mg/ 100 g), cardiac glycosides (1.19 mg/100g), flavonoids (0.87 mg / 100 g) and tannins (0.39 mg/ 100 g).

Table 1. Phytochemical analysis of ethanolic leaf extract of *Tithonia diversifolia*

Constituents	Quantitative (mg/100 mg)	Qualitative
Saponins	1.37	++
Tannins	0.39	+
Alkaloids	1.23	++
Flavonoids	0.87	++
Cardiac glycosides	1.19	++

The acute toxicity test at the dose of 1600 mg/kg body weight (Table 2) did not reveal any sign of mortality or evidence of toxicity. The treatment did not have significant ($p>.05$) effect on the mice body weight between the groups (Table 3).

Throughout the period of treatment, slight increase in weight was observed in mice of groups 1, 2, 3 which were infected with *P. berghei* and treated with ethanolic leaf extract of *T. diversifolia.* Similar findings occurred in group 4 mice that were infected with *P. berghei* and treated with chloroquine and in group 6 mice that obtained only normal saline for treatment. However, the weight of mice in group 5 which was infected with *P. berghei* but not treated decreased from 20.52 g to 18.75 g from day 2 to day 4 of the post-infection periods (Table 3).

Table 4 shows the effect of ethanolic leaf extract of *T. diversifolia* on the temperature of the mice. From day 1 to 4, temperature of mice in groups 1, 2, 3, 4 and 6 slightly increased, while temperature of mice in group 5 decreased. However, on day 4, the treatment produced significant decrease ($P<.05$) of 33.00 ± 0.55℃ in group 5, while the temperature of the mice in groups 1, 2, 3, 4 and group 6 were not significantly ($P<.05$) affected.

Table 2. Acute toxicity test of ethanolic leaf extract of *Tithonia diversifolia*

Toxicity sign	Group 1	Group 2	Group 3	Group 4
Mortality	0	0	0	0
Behavioural sign of toxicity	Nil	Nil	Nil	Nil

Group 1: 0.2 ml of distilled water
Group 2: 400 mg/kg body weight of ethanolic leaf extract of Tithonia diversifolia
Group 3: 800 mg/kg body weight of ethanolic leaf extract of Tithonia diversifolia
Group 4: 1600 mg/kg body weight of ethanolic leaf extract of Tithonia diversifolia

Table 3. Effect of ethanolic leaf extract of *Tithonia diversifolia* on body weight (g) of mice

Weight/ Day	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Initial weight	19.71±0.55[a]	19.49±0.44[a]	19.28±0.56[a]	19.64±0.27[a]	19.86±0.52[a]	19.54±0.46[a]
1	20.69±0.55[a]	20.54±0.45[a]	20.54±0.45[a]	20.76±0.43[a]	20.76±0.53[a]	20.50±0.45[a]
2	20.79±0.46[a]	20.68±0.41[a]	20.70±0.41[a]	20.92±0.36[a]	20.52±0.20[a]	20.76±0.40[a]
3	20.83±0.47[a]	20.72±0.39[a]	20.72±0.40[a]	20.98±0.39[a]	19.90±0.20[a]	20.86±0.44[a]
4	20.97±0.43[a]	20.78±0.38[a]	20.76±0.39[a]	21.02±0.37[a]	18.75±0.21[b]	20.90±0.43[a]
P value	0.41	0.20	0.14	0.08	0.01	0.20

Means with different letters in the same column are significantly (P< .05) different.

Group 1: P. berghei + 200 mg/kg body weight of ethanolic leaf extract; Group 2: P. berghei + 400 mg/kg body weight of ethanolic leaf extract; Group 3: P. berghei + 600 mg/kg body weight of ethanolic leaf extract; Group 4: P. berghei + 5 mg/kg body weight of chloroquine; Group 5: P. berghei + 0.2 ml of normal saline; Group 6: 0.2 ml of normal saline

Table 4. Effect of ethanolic leaf extract of *Tithonia diversifolia* on temperature (℃) of mice

Temperature/ Day	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
0	34.02±0.40[a]	34.08±0.45[a]	34.14±0.39[a]	34.06±0.40[a]	34.18±0.27[a]	34.04±0.38[a]
1	34.12±0.44[a]	34.18±0.48[a]	34.38±0.31[a]	34.12±0.43[a]	34.26±0.24[a]	34.14±0.37[a]
2	34.52±0.09[a]	35.54±0.38[a]	34.62±0.62[a]	34.67±0.46[a]	34.50±0.15[a]	33.69±0.09[a]
3	34.78±0.21[a]	34.74±0.58[a]	34.50±0.30[a]	34.57±0.33[a]	33.80±0.33[ab]	34.80±0.28[a]
4	34.92±0.21[a]	34.97±0.57[a]	34.90±0.38[a]	34.86±0.38[a]	33.00±0.55[b]	34.80±0.28[a]
P value	0.14	0.64	0.77	0.57	0.04	0.16

Means with different letters in the same column are significantly (P<.05) different.

Group 1: P. berghei + 200 mg/kg body weight of ethanolic leaf extract; Group 2: P. berghei + 400 mg/kg body weight of ethanolic leaf extract; Group 3: P. berghei + 600 mg/kg body weight of ethanolic leaf extract; Group 4: P. berghei + 5 mg/kg body weight of chloroquine; Group 5: P. berghei + 0.2 ml of normal saline; Group 6: 0.2 ml of normal saline

Table 5 shows the antiplasmodial activity of ethanolic leaf extract of *T. diversifolia* in Swiss albino mice. Ethanolic leaf extract of *T. diversifolia* had significant effect (*P*<.05) on the average parasitaemia level in mice. Highest average parasitaemia level was recorded in the negative control (group 5), which was significantly higher (*P*<.05) than groups 1, 2, 3, 4 and 6. On the other hand, the percentage chemo-suppression of parasitaemia was 60.02%, 63.45% and 92% at dosage rate of 200 mg/kg, 400 mg/kg and 600 mg/kg body weight in groups 1, 2 and 3 respectively. Chloroquine at 5 mg/kg body weight produced 100% chemo-suppression, while the lowest effective dose for the experiment was 200 mg/kg body weight.

4. DISCUSSION

This study on the ethanolic leaf extract of *T. diversifolia* in mice infected with *Plasmodium berghei,* revealed that the leaf extract contains some phytochemicals. Quantitative and qualitative phytochemical screening of the extract which revealed the presence of bioactive compounds such as saponins, tannins, alkaloids, flavonoids and glycosides is in agreement with findings of Goffin et al. [18] and Nafiu et al. [19] who worked on the *In-vitro* antiplasmodial activity of *T. diversifolia* and on ethnobotany, chemistry and phytochemical analysis and antimalarial activities of *Tithonia* (Asteraceae) respectively.

Table 5. Antiplasmodial activity of ethanolic leaf extract of *Tithonia diversifolia* in Swiss albino mice

Group	Average parasitaemia	Chemo-suppression (%)
1	1489.37±720.98[b]	60.02
2	1318.25±662.13[bc]	63.45
3	136.75±126.94[c]	92.96
4	0.00±00[c]	100
5	3607.00±391.17[a]	0
6	0.00±0.00[c]	100
P value	0.01	-

Means with different letters in the same column are significantly (P<.05) different.

Group 1: 200 mg/kg body weight + P. berghei; Group 2: 400 mg/kg body weight + P. berghei; Group 3: 600 mg/kg body weight + P. berghei; Group 4: 5 mg/kg body weight of Choloroquine + P. berghei (positive control); Group 5: P. berghei + 0.2 ml of normal saline (negative control); Group 6: 0.2 ml of normal saline only (normal control)

The result of the acute toxicity test of 0.2 ml (1600 mg/kg body weight) in this study which did

not show any observable behavioural signs of toxicity and mortality suggest that the ethanolic leaf extract of the *T. diversifolia* may not be toxic to the experimental mice and could therefore be considered to be probably safe. This result agrees with the observation of Ezeonwumelu et al. [13], who reported no toxicity or mortality using 10,000 mg/kg body weight aqueous extract of *T. diversifolia* in rats.

The decrease in the daily body weight of mice in group 5 that was infected and untreated (negative control) is expected. This observation may probably be attributed to the occurrence of anorexia (loss of appetite) that is usually associated with malaria infection and the effect of disturbed metabolic function coupled with decreased food intake. This is in conformity with the findings of Basir et al. [20].

The increase in temperature observed in the group of mice treated with the leaf extract is in agreement with Ojurongbe [21] who probably attributed it to the presence of saponin acting as an adjuvant which could be responsible for enhancing the immune response of the experimental mice. The decrease in temperature observed in the negative control group could probably be due to hypothermia. This observation agrees with the report of Basir et al. [20] who observed the development of severe hypothermia in post *Plasmodium* infection in mice and attributed it to general debilitating effect of malaria on the infected mice.

The antiplasmodial activity of leaf extract of *Tithonia diversifolia* observed in this study is dose dependent which corroborates the findings of Elufioye and Agbedahunsi [14] who observed 74% parasite clearing with methanolic extract of *T. diversifolia* and be attributed it to the presence of phytoconstituents such as alkaloids and flavonoids in the studied extract.

5. CONCLUSION

The result obtained from this study has revealed that the ethanolic leaf extract of *T. diversifolia* at 600 mg/kg dosage could be safe for the treatment of early malaria infection and can therefore be considered as a future antiplasmodial herbal candidate.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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