

Antimalarial Assessment of *Indigofera tinctoria* Methanol Leaf Extract in Parasitized Albino Rats

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ABSTRACT

This study was undertaken to investigate the antimalarial activity of *Indigofera tinctoria* in *Plasmodium berghei*-infected rats. Phytochemical investigation was conducted using standard method to determine the presence of the bioactive compounds. The *in vitro* anti-malarial assay of *Indigofera tinctoria* was carried out in triplicates in 96 wells microliter plate. The *in-vivo* anti-malarial effect was assessed with group serving as the normal control, group two was left untreated, group three was treated with the standard drug wile group four, five and six were treated with 100mg/kg, 200mg/kg and 400mg/kg respectively. *Indigofera tinctoria* revealed the presence of saponins, tannins, flavonoids, alkaloids, cardiac glycosides, steroids and quinines. For in vitro studies, the drug treated group had the lowest parasite count with a percentage protection of 93.65% while the percentage protection of the group that received the highest dose of the extract had a percentage protection of 79.50%. At Day 3, the untreated group still had its parasite count significantly higher than that of the other groups, while the lowest percentage count was recorded in the drug treated group. For the in vivo studies, the parasite count of the group that received the highest dose of the extract was not significantly different from that of the group treated with the standard drug. The percentage inhibition of the drug control at Day 3 was 93.82 while that of the group that received the highest dose of the extract was 80.06%. The parasite count before treatment (Day 0) for the normal control was significantly different ($P < 0.05$) from that of the other groups. At Day 1, the parasite count for the induced control was significantly ($P < 0.05$) higher than that of the other groups. There was no significant difference ($P > 0.05$) between the groups treated with the extract. At Day 6, the percentage inhibition for the drug control was 87.58%, while that of the extract treated groups were 39.98%, 57.86% and 76.48% respectively. At Day 9, there was not significantly difference between the normal control, the drug control and the group treated with the highest group of the extract. The potent antimalarial activity observed could be attributed to the presence of the secondary metabolites in *Indigofera tinctoria* leaves extracts.

KEYWORDS: *Indigofera tinctoria*, Antimalarial, Parasite Count, *Plasmodium berghei*

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INTRODUCTION

In Africa, an estimated 300-500 million cases of malaria occur each year resulting in approximately one million deaths. In many parts of sub-Saharan Africa, it is still the largest contributor to the burden of disease and premature death^[1], constituting the highest percentage (91%) of the 881,000 people who die of malaria every year, while children under 5 years of age make up 85%^[2]. To be precise, more than half of all estimated malaria cases occur in just five

African countries: Nigeria, Democratic Republic of Congo, Ethiopia, United Republic of Tanzania and Kenya^[3]. In fact among death due to malaria occurring in Africa more than 90% are in under-five children that results in brain damage. Children suffer mostly from malaria and in absolute terms malaria kills 3000 children below 5 years old daily, constitutes 25% of child mortality in Africa and 25-30% in Nigeria^[4]. In young children, malaria can progress from a mild to severe case within 24 hours after the onset of

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symptoms. Prompt diagnosis and timely malaria treatment within 24 hours after onset of first symptoms can reduce illness progression to severe stages and, therefore, decrease mortality^[1].

The degree of morbidity and mortality due to malaria infection is highest in young children especially between 6 months and five years. This is because the acquired protective immunity in this group is usually insufficient to protect against severe disease, mostly in areas of high stable transmission^[5]. Malaria, as a killer disease, accounts for 60% of outpatient visits and 30% of hospitalizations among children under five years of age in Nigeria^[6]. It is estimated that about 50% of the population in Nigeria experience at least one episode yearly while the under-five children have up to 2-4 attacks of malaria annually^[7]. Increased resistance of the parasite to the existing antimalarial drugs militates against the proper treatment of this infection. Antimalarial drug resistance accounts for the failure to control malaria in many areas of the tropical world and the consequent increasing global mortality.

There is a growing resistance to conventional anti-malarial drugs and the associated resurgence in infection rates and malaria-related morbidity and mortality, in sub-Saharan Africa and particularly in Nigeria^[8]. The use of conventional drugs has often led to resistance by the parasite. Plant based treatment has been used from time immemorial. *Indigofera tinctoria* is used traditionally to treat many conditions^[2]. One of the most promising prospects in the search for new antimalarial drugs is the large repository of medicinal plants used in the treatment of malaria in traditional societies^[9]. As such, it is imperative to investigate the antimalarial potential of *Indigofera tinctoria* and validate its ethno medicinal usage in the treatment of malaria.

MATERIAL AND METHODS

Plant Collection and Identification.

The leaves of *Indigofera tinctoria* were collected from Aliero, Aliero Local Government Area of Kebbi State, Nigeria and was authenticated by a botanist in the Herbarium Unit, Plant Science and Biotechnology Department, Kebbi State University of Science and Technology, Aliero and given a voucher number (45A).

Preparation of Sample

The leaves of *Indigofera tinctoria* were dried at room temperature for two weeks. Four hundred and twenty (420) grams of coarse plant material was macerated with 1400ml of methanol and kept in an air-tight aspirator for seventy two hours. The mixture was then filtered using sterile muslin cloth and filter paper. The filtrate was evaporated using a rotary evaporator (Kern KB 2000-2N) at 45°C and subsequently dried in a drying cabinet at 45°C and labelled methanol extract and stored in an air-tight containers in a refrigerator at 4°C.

Experimental Animals

Adult albino rats were purchased from the Animal House, Department of Biological Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria. The animals were transported in plastic cages to the Animal House, Kebbi State University of Science and Technology Aliero and were allowed to acclimatize to laboratory conditions for two weeks. The rats were allowed access to growers mash (vital feeds Ltd, Nigeria) and clean water *ad libitum*.

QUALITATIVE PHYTOCHEMICAL SCREENING

Test for Alkaloids

Five millimetres (5ml) of 1% HCl was added to 1ml of the extract and stirred on a steam bath and filtered. Three portions (1ml each) of the filtrate was treated with 3 drops of Drangendorffs, Mayers and Wagners reagents respectively. Formation of turbidity confirmed the presence of alkaloids^[10].

Test for Tannins

Three (3) drops of 5% FeCl₃ solution was added to 3mls of the extract. Presence of brownish green or a blue-black precipitate (when viewed on white paper) indicated the presence of tannins (condensed and hydrolysable)^[10].

Test for Saponins

Five millimetres (5mls) of the extract was shaken with 15mls of distilled water in a test tube. Persistent frothing on warming confirmed the presence of saponins^[10].

Test for Glycosides

Exactly 2.5ml of 50% sulphuric acid was added to 5ml of the extract in a test tube. The mixture was heated in boiling water for 15 minutes, cooled and neutralized with 10% NaOH. Then 5ml of Fehling's solution was added and mixture was boiled. A brick-red precipitate indicated the presence of glycosides^[11].

Test for Anthraquinone

Five grams (5g) of sample extract was boiled with 10ml aqueous H₂SO₄ and filtered while hot. The filtrate was shaken with 5ml of benzene. The benzene layer separated and half of its own volume of 10% ammonia solution was added. A pink, red or violet colour indicated the presence of anthraquinone glycosides^[12].

Test for Flavonoids

Two millimetres (2mls) of the extract was treated with 1mls of 5% lead acetate solution in a test tube. A yellow colour indicates the presence of flavonoids^[13].

Test for Steroids

Two millimetres (2mls) of acetic anhydride was added to 2mls of the extract followed by the addition of 2mls of dilute H₂SO₄. Violet colour which changes to blue indicates the presence of steroids^[12].

Test for Phenols

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The 2 mL of the extract was mixed with 2 mL of 1% ferric chloride. The formation of deep blue or blue-black coloration is an indication of a positive result [12].

ANTIMALARIAL ASSAYS

In vitro Antimalarial Assays

The extract (1g) was dissolved in 1ml methanol and diluted with distilled water up to 50ml. culture medium, RPMI 1640 laced with malaria screened blood (O⁺) and serum obtained from Federal Medical Centre, Birnin Kebbi. 1ml of parasitized blood from *Plasmodium berghei* infected albino rats was constituted in 50ml distilled water. The *in vitro* antiplasmodial assay was carried out in triplicates in 96 wells microliter plate. Unto each well was measured 100µl of each extract concentrations of 0.6mg, 1mg and 2mg followed by 100µl of each culture medium and 100µl parasite (isolates), *Plasmodium berghei* (NK65) respectively. This was incubated at 37°C for 48hrs under anaerobic conditions using Thelco High Performance Laboratory Incubator (Model No. 3501).

Thin smear of the harvested cells from the wells was done and stained with Leisman stain for 3 minutes. Parasite growth was counted in 10 fields using x100 objective microscope and the mean was calculated [14]. The slight modification was the use of a thin smear and Leisman stain instead of a thick smear and Giemsa stain because many parasites may be trapped using thick smear.

Artesunate (20mg/ml) was used as positive control, medium with the screened blood as normal control, and parasitized cells with normal saline as negative control.

Percentage inhibition was calculated as:

$$\%Inhibition = \frac{Parasitemia\ in\ control\ well - Parasitemia\ in\ test\ well}{Parasitemia\ in\ control\ well} \times 100$$

Concentration required to inhibit the parasite growth by 50% (IC₅₀) was determined by linear interpolation from the parasite growth inhibition curves (concentration versus percentage inhibition) generated from parasite – extraction interaction [15].

In vivo Antimalarial Assays

Parasite Inoculation

Donor Wistar albino mice blood infected with the *P. berghei* was obtained from the animal farm of Faculty of Veterinary Medicine, Usmanu Danfodiyyo University, Sokoto, Nigeria and was used for inoculum preparation. The desired blood volume was drawn from the donor mice by heart puncture and diluted serially in normal saline solution. The final suspension contained about 1×10⁶ infected RBCs in every 0.2 ml suspension. This 0.2 ml suspension was injected into the experimental animals intraperitoneally to initiate infection. Infection for malaria was confirmed after 72 hr. The inoculated animals were then randomized into five mice per cage and maintained in the Animal House, Department of Biochemistry, Faculty of Life Sciences, Kebbi State University of Science and Technology, Aliero.

Experimental Design

Groups	Treatment
Group 1	Administered with distilled water, no infection, no treatment
Group 2	Parasitized without treatment
Group 3	Parasitized and treated with 20 mg/kg b.w. Artesunate
Group 4	Parasitized and treated with 2% LD ₅₀
Group 5	Parasitized and treated with 4%LD ₅₀
Group 6	Parasitized and treated with 8%LD ₅₀

Determination of Parasitemia Level in Experimental Animals

The level of parasitaemia in the experimental animals was determined haematologically, using microscopic technique [16]. Thick blood smears were collected daily from tail blood, stained with Giemsa's stain and examined under low powered microscope (x10 resolution) to determine the parasitaemia level. The level of baseline parasitaemia was determined and hence, monitored once daily for 3 days.

STATISTICAL ANALYSIS

The data was analysed using Statistical Package for Social Sciences (SPSS) windows version 20.0. Data was presented as mean ± standard error of mean analysed by one way analysis of variance (ANOVA) followed by Duncan's Post Hoc Comparison test. *P*<0.05 was considered to be significant.

RESULTS

Table 1. Qualitative Phytochemical Constituents of *Indigofera tinctoria*

PHYTOCHEMICALS	INFERENCE
Saponins	+
Tannins	+
Flavonoids	+

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Alkaloids	+
Phenols	+
Cardiac glycosides	-
Steroids	+
Glycosides	-
Quinines	+

Key: + = Detected; - = Not detected

The presence of some phytochemicals detected in methanol leaf extract of *Indigofera tinctoria* is presented in Table 1. *Indigofera tinctoria* revealed the presence of saponins, tannins, flavonoids, alkaloids, cardiac glycosides, steroids and quinines. Cardiac glycosides and glycosides were not detected.

Table 2: In vitro Antiplasmodial Activity of *Indigofera tinctoria*

Treatment	DAY 1		DAY 2		DAY 3	
	Parasite Count	Percentage Inhibition(%)	Parasite Count	Percentage Inhibition(%)	Parasite Count	Percentage Inhibition(%)
NC	0.00±0.00 ^a	100	0.00±0.00 ^a	100	0.00±0.00 ^a	100
IC	440.67±40.88 ^d	0.00	412.00±23.46 ^c	0.00	453.00±21.39 ^c	0.00
DC	28.00±3.46 ^a	93.65	18.69±9.61 ^a	93.20	0.00±0.00 ^a	93.82
MLEIT 1	155.33±18.00 ^c	64.75	146.67±9.6 ^d	62.30	83.00±7.09 ^b	65.71
MLEIT 2	114.67±8.19 ^{bc}	73.98	71.67±5.78 ^c	72.17	55.33±8.51 ^b	74.69
MLEIT 3	90.33±1.08 ^b	79.50	41.67±6.64 ^{bc}	78.08	14.00±7.02 ^a	80.06

Values are expressed as mean ± standard error of mean (n = 5). Mean values having common superscript letters in a column are significantly different ($P > 0.05$) analysed one-way ANOVA followed by Duncan's multiple range test).

NC = Administered with distilled water, no infection, no treatment

IC = Parasitized without treatment

DC = Parasitized and treated with 20 mg/kg b.w. Artesunate

MLEIT 1 = Parasitized and treated with 2% LD₅₀ (100mg/kg b.w. MLEIT)

MLEIT 2 = Parasitized and treated with 4% LD₅₀ (200mg/kg b.w. MLEIT)

MLEIT 3 = Parasitized and treated with 8% LD₅₀ (400mg/kg b.w. MLEIT)

Table 2 shows the *in vitro* antiplasmodial activity of *Indigofera tinctoria* against induced albino. At Day 1 after induction, the parasite count of the induced group was significantly higher ($P < 0.05$) than that of all the other groups. The drug treated group had the lowest parasite count with a percentage protection of 93.65% while the percentage protection of the group that received the highest dose of the

extract had a percentage protection of 79.50%. At Day 2, the trend was similar to that at Day 1. At Day 3, the untreated group still had its parasite count significantly higher than that of the other groups, while the lowest percentage count was recorded in the drug treated group. The parasite count of the group that received the highest dose of the extract was not significantly different from that of the group treated with the standard drug. The percentage inhibition of the drug control at Day 3 was 93.82 while that of the group that received the highest dose of the extract was 80.06%. The parasite count before treatment (Day 0) for the normal control was significantly different ($P < 0.05$) from that of the other groups. At Day 1, the parasite count for the induced control was significantly ($P < 0.05$) higher than that of the other groups. There was no significant difference ($P > 0.05$) between the groups treated with the extract. At Day 6, the percentage inhibition for the drug control was 87.58%, while that of the extract treated groups were 39.98%, 57.86% and 76.48% respectively. At Day 9, there was no significant difference between the normal control, the drug control and the group treated with the highest group of the extract.

Table 3. In vivo Antiplasmodial Activity of *Indigofera tinctoria*

Treatment	DAY 0	DAY 1	Percentage Inhibition(%)	DAY 6	Percentage Inhibition(%)	DAY 9	Percentage Inhibition (%)
	Parasite Count before treatment	Parasite Count		Parasite Count		Parasite Count	
NC	0.00±0.00 ^a	0.00±0.00 ^a	100.00	0.00±0.00 ^a	100.00	0.00±0.00 ^a	100.00

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IC	194.40±12.51 _b	209.00±17.72 _d	0.00	212.60±9.76 ^f	0.00	213.40±6.44 ^d	0.00
DC	203.80±21.99 _b	69.00±5.56 ^b	66.99	26.40±5.18 ^b	87.58	0.00±0.00 ^a	100
MLEIT 1	212.20±15.84 _b	143.20±7.62 ^c	31.48	127.60±8.30 ^e	39.98	96.40±6.82 ^c	54.83
MLEIT 2	211.40±12.84 _b	127.20±8.10 ^c	39.14	89.60±6.23 ^d	57.86	61.60±2.73 ^b	71.13
MLEIT 3	206.80±12.07 _b	94.40±2.98 ^b	54.83	50.00±10.42 ^c	76.48	9.60±3.93 ^a	95.50

Values are expressed as mean ± standard error of mean (n = 5). Mean values having common superscript letters in a column are significantly different ($P > 0.05$) analysed one-way ANOVA followed by Duncan's multiple range test).

NC = Administered with distilled water, no infection, no treatment

IC = Parasitized without treatment

DC = Parasitized and treated with 20 mg/kg b.w. Artesunate

MLEIT 1 = Parasitized and treated with 2% LD₅₀ (100mg/kg b.w. MLEIT)

MLEIT 2 = Parasitized and treated with 4% LD₅₀ (200mg/kg b.w. MLEIT)

MLEIT 3 = Parasitized and treated with 8% LD₅₀ (400mg/kg b.w. MLEIT)

The parasite count before treatment (Day 0) for the normal control was significantly different ($P < 0.05$) from that of the other groups. At Day 1, the parasite count for the induced control was significantly ($P < 0.05$) higher than that of the other groups. There was no significant difference ($P > 0.05$) between the groups treated with the extract. At Day 6, the percentage inhibition for the drug control was 87.58%, while that of the extract treated groups were 39.98%, 57.86% and 76.48% respectively. At Day 9, there was no significant difference between the normal control, the drug control and the group treated with the highest group of the extract (Table 3).

DISCUSSION

Many antimalarial drugs currently available on the market have been developed from plants and natural products. Antimalarial drug resistance remains a major challenge and continues to emerge creating an obstacle in malaria control and elimination^[17]. *Plasmodium falciparum* resistance to the existing antimalarials necessitates the development of improved drug interventions^[18]. At present, developing novel approaches and new alternative antimalarial drugs is pivotal to combat the disease.

The present study investigated the phytochemical and antimalarial activity of *Indigofera tinctoria* methanol leaves extract against *P. berghei*-infected rats. Phytochemical investigation of *Indigofera tinctoria* revealed the presence of some phytochemicals which are known to provide an antiplasmodial effects. This result of the phytochemical

evaluation of *Indigofera tinctoria* supports the findings of other authors on the phytochemical analysis of *Indigofera tinctoria*^{[19][20][21]}. Based on some literatures and the findings of this study, flavonoids which were found to be present in *Indigofera tinctoria* could be responsible for most anti-oxidant and anti-radical effects of *Indigofera tinctoria* that produced the anti-plasmodial activity. Antioxidant compounds can inhibit hemozoin formation, and free heme is very toxic for malaria parasite. In addition, secondary metabolites such as glycosides have been shown to possess direct antiplasmodial effects^[22].

The antimalarial activity showed that the antiplasmodial activity of *Indigofera tinctoria* used as an orthodox medicine in African region and elsewhere, against *Plasmodium berghei* malaria parasite, exerted a chemosuppression effect at different concentrations over 72 hours supporting its folk use in the treatment of uncomplicated malaria. The various doses of the extracts exhibited a good chemosuppression of *Plasmodium* multiplication compared to the negative control. According to a study conducted by 22 *et al.*^[23], the in-vivo anti-plasmodial effect of plant extracts with chemosuppression effect of up to 50% or more was seen to be very good. This implies that *Indigofera tinctoria* extract is a very good antimalarial agent.

CONCLUSION

Based on the findings of this study in which *Indigofera tinctoria* leaves extracts exhibited antiplasmodial activity, it could be concluded that the medicinal plant *Indigofera tinctoria* can be a good source of antimalarials. The presence of certain phytochemicals such as phenols, tannins, alkaloid and flavonoids in *Indigofera tinctoria* may perhaps make it a good source for antimalarial formulations. The potent antimalarial activity observed could be attributed to the presence of the secondary metabolites in *Indigofera tinctoria* leaves extracts.

CONFLICT OF INTEREST

We hereby confirm that there is no conflict of interest in the content of this article.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85- 23, revised 1985) were

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followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Author Jude Nwaogu conceptualized, designed and supervised the study. Author Benjamin Kusuma performed the experiment, collected all data, and wrote the first draft of the manuscript. Author Angela Nnenna Ukwuani-Kwaja did the literature search, performed the statistical analysis and wrote the final manuscript. All authors read and approved the final manuscript.

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