Therapeutic effects of *Azadirachta indica* A.Juss. leaves in malaria-induced male Wistar rats

[Efectos terapéuticos de hojas de *Azadirachta indica* A.Juss. en ratas Wistar machos infectadas con malaria]

Ngozi K. Achi1, Chimaraoke Onyeabo1, Daniel A. Nnate1, Chima A. Ekeleme-Egedigwe1, Igwe K. Kalu2, Ikedichim C. Chibundu1, Grace C. Wokoma3

1Department of Biochemistry, College of Natural Sciences, Michael Okpara University of Agriculture, Umudike, PMB 7267, Umuahia, Abia State, Nigeria.
2Department of Biochemistry, Faculty of Biological and Physical Sciences, Abia State University, Uturu, Abia State, Nigeria.
3Department of Veterinary Biochemistry and Pharmacology, Faculty of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria.

Abstract

**Context:** *Azadirachta indica* has long been used in herbal or folk medicine as a remedy for the treatment of malaria and the administration of herbal preparations has raised concerns on their toxicity.

**Aims:** To determine the phytochemical content of *A. indica* and its therapeutic effect on indices of clinical importance in malaria-induced male Wistar rats.

**Methods:** Plant material was extracted with ethanol, and the lethal dose (LD$_{50}$) on the rats was determined before the study. Normal and *Plasmodium berghei* infected rats were divided into eight groups of five rats each with groups 1 and 2 serving as normal and disease control respectively. Lumartem was administered twice daily at oral therapeutic doses of artemether/lumefantrine (2/12 mg/kg) and plant extract at 300 and 500 mg/kg body weight. After 5 days of treatment, all the animals were sacrificed according to their groups for the experimental analysis.

**Results:** The plant extract was considered safe with LD$_{50}$ > 5000 mg/kg body weight. Quantitative phytochemical studies showed a high concentration of alkaloids, tannin, and terpenoids. Treatment with both extracts of *A. indica* and lumartem in malaria-infected rats showed a slight reduction in triglycerides while total cholesterol, HDL, and LDL levels increased significantly (p < 0.05). Increase in body weight of rats treated with *A. indica* was dependent on the concentration of extract administered. Treatment of malaria with the extract and lumartem resulted in a slight restoration of the hematological values.

**Conclusions:** This study shows that both *Azadirachta indica* and lumartem was practically safe and well tolerated.

**Keywords:** anti-malaria; *Azadirachta indica*; lethal dose; lumartem; phytochemical; *Plasmodium*.

ARTICLE INFO

Received: August 16, 2017.
Received in revised form: February 2, 2018.
Accepted: February 12, 2018.
Available Online: March 22, 2018.

Declaration of interests: The authors declare no conflict of interest.

Funding: The authors confirm that the project has not funding or grants.
INTRODUCTION

_Azadirachta indica_ A.Juss. (Meliaceae) known by its common name as Neem tree is native to Asian countries. The people of India have long regarded the neem tree as a remedy for sickness (Tiwari et al., 2014). With each part of either its leaves, bark, stem or root having medicinal properties, a large number of biologically active compounds have been isolated from the plant (Biswas et al., 2002). It has also been reported that the leaf extract is effective as analgesic, anthelminthic, antibacterial, antifungal, antihypoglycemic, anti-inflammatory, antiviral, antimalarial, antipyretic, insecticidal, hypercholesteremic and hypoglycemic agents (Parotta, 2001; Maragathavalli et al., 2012). _A. indica_ has been extensively reported as being effective in the treatment of malaria caused by various strains of _Plasmodium_ parasite even those resistant to traditional anti-malaria (Nwafor et al., 2003). The use of herbal medications in the treatment of malaria is popular in many parts of Africa and Asia where malaria infestation is endemic. The plant is well identified for its high antioxidant activity; its active molecules could also be chemically manipulated into effective drugs, which can be wildly used in the treatment of certain ailments (Bamidele et al., 2017). Chemical compounds in plants mediate their effect on the human body through processes identical with compounds in conventional drugs. Thus herbal medicines do not differ greatly from conventional drugs regarding how they work (Vickers et al., 2001). This enables herbal medicines to have beneficial pharmacology but also gives them the same potential as conventional pharmaceutical drugs to cause harmful side effects (Ajebo and Mbagwu, 2005).

Malaria is a tropical disease that poses serious problems on human being especially in tropical countries where the environment provides conducive ground for the parasite to thrive. Malaria is responsible for at least 750,000 deaths a year, mostly in young children in Africa (Greenwood et al., 2012; WHO, 2013). It is a disease of global importance that results in 300-600 million cases annually, and an estimated 2.2 billion people are at risk of infection (Singh et al., 2010). Children under five years and pregnant women are particularly vulnerable to the disease due to their weaker immune systems (WHO, 2000; Lamb, 2012). An estimated 438,000 people died of malaria in 2015, with over 90% of these deaths occurring in sub-Saharan Africa, and nearly all of the others occurring in South-East Asia and South America (WHO, 2015). Treatment of malaria with African herbs and medicines has been in existence long before the arrival of western drugs (Ngarivhume et al., 2015). The neem tree appears to have originated in India and South East Asia and is now spread throughout tropical and subtropical regions like Africa hence the popularity in the treatment of malaria.

Lumartem is a fixed-dose Artemisinin Combination Therapy of artemether and lumefantrine, with each tablet containing 20 mg artemether and 120 mg lumefantrine. Artemether is a fat-soluble artemisinin derivative (Nosten and Brasseur, 2002). Artemisinin is a natural product derived from the Chinese medicinal plant _Artemisia annua_ L., known as qinghao in China and as sweet wormwood in Europe and America (Lefèvre and Thomsen, 1999). Lumefantrine, previously known as benflumetol, is a synthetic dibutyl aminoethanol substituted fluorene racemate and is structurally related to quinine (Anyasor et al., 2013). Artemether acts quickly to rapidly reduce the affliction caused by the parasite and also the resolution of clinical symptoms by interfering with parasite transport proteins, disruption of mitochondrial function, inhibition of angiogenesis and modulation of host immune function (Anyasor et al., 2013). On the other hand, lumefantrine prevents recrudescence, acts slowly and serves as a longer-acting agent to eliminate remaining parasites (Ezzet et al., 1998). This combination makes it highly effective especially in resistant strains of _P. falciparum_ (Lefèvre and Thomsen, 1999). Both artemether and lumefantrine are well absorbed after oral administration. Administration with fat-containing foods improves bioavailability by more than 2-fold for artemether and up to 16-fold for lumefantrine in adults (Djimdé and Lefèvre, 2009).

Many anti-malarial drugs like quinine and artemisinin were isolated from plants, and because of the increased resistance of the parasite to established drugs, there is need to investigate more chemical compounds within traditional plants. The use of plant extracts without a standard dosage
coupled with non-availability of adequate scientific studies on their safety has raised concerns about their toxicity (Achi and Ohaeri, 2012). Understanding the biochemical effects and safety of these medicinal plants on its users is also very important because limited toxicological information on these plants has been a source of fear for its users. Animal models have become a useful tool in the study of many anti-malarial drugs. The rodent parasite Plasmodium berghei is effective in testing and monitoring the efficacy of anti-malarial drugs against human malaria parasite (Langhorne et al., 2011). However, in this study, the authors were not concerned about the activity of A. indica against P. berghei malaria parasite on murine models as several studies have reported a positive result (Obih and Makinde, 1985; Priyanka et al., 2013) while others reported no activity against the rodent parasite (Rochanakij et al., 1985; Farahna et al., 2010). The study is aimed at giving a better understanding of A. indica in the treatment of malaria by comparing its effect with a synthetic anti-malarial drug lumartem on the body weights, lipid and hematological parameters in malaria-infected and normal Wistar rats, which will serve as a baseline for further studies in humans.

**MATERIAL AND METHODS**

Collection and identification of plant materials

Fresh leaves of Azadirachta indica were carefully collected with hands covered in gloves from the school premises of the Michael Okpara University of Agriculture, Umudike (5°28′50.057″ N and 7°32′ 45.921″ E). The plant was identified by Mr. Ibe K. Ndukwe of the Department of Forestry and Environmental Management, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria. A voucher specimen with number MOUAU/1631529 was kept at the herbarium of the same department for future reference.

Drug and chemicals

Lumartem (artemether 20 mg + lumefantrin 120 mg) was purchased from a Pharmacy in Umuehia, Abia state, Nigeria. All other chemicals used are of analytical grade.

**Preparation of plant extract**

The leaves of Azadirachta indica were sorted, washed thoroughly and air dried on a laboratory slab for four weeks at room temperature (25 – 27°C). The dry leaves were pulverized into a fine powder using an electric blender (Corona – Ref, 121, Landers). One thousand grams of the powdered leaves was extracted with 1500 mL of 88% ethanol for 48 h in a stopped bottle with occasional stirring at room temperature. It was then sieved using a muslin cloth and filtered using Whatman No. 1 filter paper (Sigma, Aldrich). This process was repeated three times. The extract was concentrated under reduced pressure at 40°C for four days using a water bath (HH-W420). Various concentrations were prepared from the resultant crude extract, dissolved in distilled water, and administered orally to determine the LD₉₀ and the treatment of animals.

**Quantitative determination of phytochemical constituents of leaves of Azadirachta indica**

**Determination of tannin**

An analytical method for quantitative determination of tannin was done according to Harborne (1973) with modification by dissolving 25 g of sodium tungstate (Na₂WO₄) in 30 mL of distilled water to form Folin-Denis reagent. To the reagent prepared above, 5 g of phosphomolybdic acid (H₃PMO₁₀O₄₀) and 20 mL of orthophosphoric acid (H₃PO₄) were added. Ninety minutes reflux of the mixture was carried out, cooled, and diluted to 400 mL with distilled water. Five grams of powder (sample) in a conical flask was added to 100 mL of distilled water. This was boiled gently for 1 h on an electric hot plate and filtered using number 42 (125 mm) Whatman filter paper in a 100 mL volumetric flask. Addition of 5.0 mL Folin-Denis reagent and 10 mL of saturated Na₂CO₃ solution into 50 mL of distilled water and 10 mL of diluted extract (aliquot volume) was carried out after being pipetted into a 100 mL conical flask for color development. The solution was allowed to stand for 45 min in a water bath at a temperature of 25°C after thorough agitation. With the aid of a Spectrum Lab 23 A spectrophotometer optical density (brand, city, company) was measured at 700 nm and compared on a

[http://jppres.com/jppres](http://jppres.com/jppres)
standard tannic acid curve. The following equation (1) was used in the calculation.

\[
\text{Tannic acid} = \frac{C \times 100V}{\text{Aliquot volume} \times \text{weight of sample}}
\]

Where:
C was concentration of tannic acid read off the graph
V was the volume of extract

Determination of alkaloids

Quantitative determination of alkaloid was according to the methodology by Harborne (1973). Exactly, 200 mL of 10% acetic acid in ethanol was added to the powder sample of leaves of A. indica (5.0 g) in a 500 mL beaker and allowed to stand for 3 hours. The extract was concentrated in a water bath to one-quarter of the original volume followed by addition of 15 drops of concentrated ammonium hydroxide dropwise to the extract until the precipitation was complete immediately after filtration. After 3 h of mixture sedimentation, the supernatant was discarded, and the precipitates were washed with 20 mL of 0.1 M of ammonium hydroxide and then filtered using Gem filter paper (12.5 cm). Using electronic weighing balance Model B-218 (B-218 Series, Jiangsu, China), the residue was dried in an oven, and the percentage of alkaloid was expressed mathematically as equation (2).

\[
\%\text{Alkaloid} = \frac{\text{Weight of alkaloid}}{\text{Weight of sample}} \times 100
\]

Determination of flavonoids

Flavonoid determination was by the method reported by Boham and Kocipi (1994). Exactly 50 mL of 80% aqueous methanol added was added to 5.0 g of sample in a 250 mL beaker, covered, and allowed to stand for 24 h at room temperature. After discarding the supernatant, the residue was re-extracted (three times) with the same volume of ethanol. Whatman filter paper number 42 (125 mm) was used to filter the whole solution of the sample. Each sample filtrate was later transferred into a crucible and evaporated to dryness over a water bath. The content in the crucible was cooled in a desiccator and weighed until constant weight was obtained. The percentage of flavonoid was calculated as equation (3):

\[
\%\text{Flavonoid} = \frac{\text{Weight of flavonoid}}{\text{Weight of sample}} \times 100
\]

Determination of saponin

Saponin quantitative determination was carried out using the method reported by Obadoni and Ochuko (2002). Exactly, 100 mL of 20% aqueous ethanol was added to 5.0 g of the powder sample of A. indica leaves in a 500 mL conical flask. The mixture was heated over a hot water bath for 4 h with continuous stirring at a temperature of 55°C. The residue of the mixture was re-extracted with another 100 mL of 20% aqueous ethanol after filtration and heated for 4 h at a constant temperature of 55°C with constant stirring. The combined extract was evaporated to 40 mL over a water bath at 90°C. Twenty mL of diethyl ether was added to the concentrate in a 250 mL separator funnel and vigorously agitated from which the aqueous layer was recovered while the ether layer was discarded. This purification process was repeated twice. 60 mL of n-butanol was added and extracted twice with 10 mL of 5% sodium chloride. After discarding the sodium chloride layer, the remaining solution was heated in a water bath for 30 min, after which the solution was transferred into a crucible and was dried in an oven to a constant weight. The saponin content was calculated as equation (4).

\[
\%\text{Saponin} = \frac{\text{Weight of saponin}}{\text{Weight of sample}} \times 100
\]

Determination of cyanogenic glycoside

Cyanogenic glycoside (CG) quantitative determination methodology used in this research is that by Harbone (1973) with modifications. Five grams of the sample was weighed into a 250 mL round bottom flask and about 200 mL of distilled water was added and allowed to stand for 3 h for autolysis to occur. Full distillation was carried out in a 250 mL conical flask containing 20 mL of 2.5% NaOH (sodium hydroxide) in the sample after adding an anti-foaming agent (tannic acid). Cyanogenic glycoside (100 mL), 8 mL of 6 M NH₂OH (ammonium hydroxide), and 2 mL of 5% KI (potassium iodide) were added to the distillate(s), mixed, and titrated with 0.02 M AgNO₃ (silver nitrate) using a microburette against a black background. Turbidity which was
continuous indicates the end point. The content of
cyanogenic glycoside in the sample was calculated
as equation (5).

\[
CG (\text{mg/100 g}) = \frac{\text{Titre value (mL) } \times 1.8 \times \text{extract volume (mL)} \times \text{sample weight (g)}}{\text{ Aliquot volume (mL)} \times \text{sample weight (g)}}
\]

(5)

**Determination of percentage steroids**

Into a thimble connected to a Soxhlet extractor
chamber with a pre-weighed flat bottom was added
to the dry sample powder (5.0 g) and connected to a
condenser. Petroleum ether (100 mL) enough to
cause a reflux was added to the flask and the lipid
from the wood sample was extracted for 3 h by
heating on an electric hot plate at 50°C. The extractant (petroleum ether) was distilled off and
the lipid recovered by cooling the flask in a desiccator and its value calculated by reweighed flask and
content (AOAC, 1984, Khan et al., 2011). The calculated
percentage of lipid content as equation (6).

\[
\text{%Lipid} = \frac{\text{Weight of lipid}}{\text{Weight of sample}} \times 100
\]

(6)

**Determination of phenols**

Defatting of 5.0 g of the sample was carried out
for 2 h in 100 mL of ether using a Soxhlet apparatus.
The defatted sample was boiled for 15 min with
50 mL of ether for the extraction of the phenolic components. Exactly 10 mL of distilled water, 2 mL
of 0.1 N ammonium hydroxide solution, and 5 mL of
concentrated amyl alcohol were also added to 5 mL
of the extract and left to react for 30 min for color
development. The optical density was measured at
505 nm. Tannic acid (0.20 g) was dissolving in dis-
tilled water and diluted to 200 mL mark (1 mg/mL)
in preparation for phenol standard curve. Varying
concentrations (0.2 - 1.0 mg/mL) of the standard
tannic acid solution were pipetted into five differ-
ent test tubes to which 2 mL of NH₃OH, 5 mL of
amyl alcohol, and 10 mL of water were added. The
solution was made up to 100 mL volume and left to
react for 30 min for color development. The optical
density was determined at 505 nm (Edeoga et al., 2005).

**Acute toxicity study**

Healthy male Swiss mice weighing 18 - 25 g were
purchased from the Department of Veterinary, Uni-
versity of Nigeria, Nsukka (UNN). The LD₅₀ of the
ethanol leaf extract of A. indica was determined using
the method as described by Chinedu et al.,
(2013). After 2 weeks of acclimatization, the mice
were starved overnight for acute toxicity test. The study was done in two stages. In the first stage, 4
groups of 1 mouse each were orally administered 50,
200, 400 and 800 mg/kg of the extract. The mice were observed for signs of toxicity and mortality for the first 1 h and then at regular interval for 24 h. In
the second stage, 3 groups of 1 mouse each were administred 1000, 1500 and 2000 mg/kg of the ex-
tract and observed for 1 h post-administration and
then periodically for 24 h. For the third stage, 3
groups of 1 mouse each were administered 3000,
4000 and 5000 mg/kg of the extract and observed for 1 h post-administration and then every 2 h for 24 h. There was no mortality at 5000 mg/kg and a con-
firmatory test was carried-out on two mice, which confirmed the test results. The doses of 300 and 500
mg/kg extract were selected for the study.

**Experimental animals**

Forty healthy male Wistar rats weighing be-
tween (110 - 125 g) were also purchased from the
Department of Veterinary, University of Nigeria,
Nsukka (UNN). Ethical approval was obtained from the
Director, board of experimental animal research, College of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike, Abia
State, Nigeria, which was in line with the guidelines
for the care and use of laboratory animals as given
by the National Institute of Health (NRC, 1985). The
rats were acclimatized to their diets and water in
standard laboratory cages for 14 days and main-
tained under good laboratory practice (12-h light
and dark cycle, uniform temperature of 25 - 28°C)
with free access to food (Pfizer feeds, Kaduna) and
water ad libitum.
Induction of malaria parasite

*Plasmodium berghei* (NK65) used for the study was obtained from National Institute for Medical Research (NIMR), Lagos, Nigeria. The parasites were preserved by continuous intraperitoneal reintroduction in healthy Wistar albino mice (18 - 25 g) after 2 days. The inoculum was prepared as described by Akuodor et al. (2015). Blood was collected from the donor mouse by cardiac puncture. The parasitized erythrocyte was diluted in normal saline such that the blood suspension contains $1 \times 10^7$ *P. berghei* parasitized erythrocytes. Each rat was intraperitoneally infected with 0.2 mL of the standard inoculum.

**Experimental procedure**

Two days after inoculation, both normal and infected rats were randomly divided into 8 groups of 5 rats each. The ethanol extract of *A. indica* and lumartem separately were dissolved in distilled water and administered orally with the aid of an oral cannula to the experimental rats. Lumartem was administered twice daily at oral therapeutic doses of artemether (2 mg/kg body weight) and lumefantrine (12 mg/kg body weight). These doses were equivalent to the therapeutic doses of artemether-lumefantrine (20/120 mg/10 kg). The rats were treated according to their groups (Table 1). The disease control (Group 2) received 1 mL of distilled water. Treatment was continued daily according to their groups for 5 days in mg/kg body weights.

The body weights of the rats were recorded on a daily basis in order to ascertain the new amount of the extract to be administered according to their body weight.

At the end of the 5th day, the changes in body weights of the rats were calculated using the equation (7).

\[
\%\text{Change in body weight} = \frac{\text{Final body weight} - \text{Initial body weight}}{\text{Final body weight}} \times 100
\]  

(7)

Experimental animals were sacrificed on the 6th day following the method as described by Achi et al. (2017). Blood sample for lipid analysis was collected by cardiac puncture into plain tubes and allowed to clot. Sera were harvested from the clot-

<table>
<thead>
<tr>
<th>Table 1. Experimental grouping of animals and treatment doses.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>Group 1 (normal control)</td>
</tr>
<tr>
<td>Group 2 (disease control)</td>
</tr>
<tr>
<td>Group 3 (normal)</td>
</tr>
<tr>
<td>Group 4 (normal)</td>
</tr>
<tr>
<td>Group 5 (normal)</td>
</tr>
<tr>
<td>Group 6 (malaria infected)</td>
</tr>
<tr>
<td>Group 7 (malaria infected)</td>
</tr>
<tr>
<td>Group 8 (malaria infected)</td>
</tr>
</tbody>
</table>

Erythrocyte count, total leucocyte count, thrombocyte number concentration, erythrocyte volume fraction and hemoglobin concentration were carried out by the method as described by Ochei and Kolhatkar (2008). Blood was collected in a tube containing 10% EDTA dipotassium salt, as an anticoagulant (2 mg/mL of blood). Hematological parameters were calculated using the equations (8-11) below.

Number of RBC counted

\[
\text{Total erythrocyte count} = \frac{\text{Number of RBC counted}}{\text{Volume counted} \times \text{dilution factor} \times \text{conversion factor}} \times 10^9/\text{L} (8)
\]

Number of WBC counted

\[
\text{Total leucocyte count} = \frac{\text{Number of WBC counted}}{\text{Volume counted} \times \text{dilution factor} \times \text{conversion factor}} \times 10^9/\text{L} \times 10^9/\text{mL} (9)
\]
Platelet count = \frac{\text{Number of platelet counted}}{\text{Volume counted} \times \text{dilution factor} \times \text{conversion factor}}

Where:
Volume counted = 0.1 mm \times 4 \text{ mm}^3 = 0.4 \text{ mm}^3
Platelet count (10^9/L)
Conversion factor (10^9/L = 10^3/mL)

PCV% = \frac{\text{Packed RBC column height}}{\text{Total blood volume height}}

Where:
PCV was packed cell volume or hematocrit
RBC was red blood cells

The hemoglobin (Hb) concentration was determined, using fully automated hematology analyzer (BSysmex® Automated Analyser KX-21N, Sysmex Corporation, Kobe, Japan) and results expressed in g/L (Stott and Lewis, 1995).

Statistical analysis

The experimental results were performed in triplicate, and the results were expressed as mean ± standard deviation. Data were analyzed using SPSS Statistics version 21, and one-way analysis of variance (ANOVA) was used for comparison of means. Differences between means were considered to be significant when p<0.05.

RESULTS

Quantitative phytochemical studies of the plant extract showed a high concentration of alkaloids, tannin, and terpenoids; moderate phenol and flavonoid with lower glycoside contents (Table 2). This is in line with previous studies, which reported that alkaloids and terpenoids impact the antimalarial properties on most medicinal plants (Bray et al., 1990; Saxena et al., 2003).

No symptoms similar to P. berghei infection was observed three days after infection. Two deaths in the disease control group and none in the treatment groups was recorded on the 4th day of treatment, i.e., day 6 of inoculation with P. berghei. At the end of the study, i.e., day five of treatment, infected rats were still alive as no further death was recorded. Serum lipid profile of the rats investigated in this study is shown in Figure 1. There was a non-significant difference (p > 0.05) in cholesterol level of normal rats and those treated with 300 mg/kg body weight. However, administration of 500 mg/kg body weight showed a significant decrease in total cholesterol, triglycerides, and LDL while HDL levels increased significantly compared to normal control (Fig. 1). Administration of lumartem on normal rats led to a reduction of total cholesterol, LDL, HDL and increased triglycerides. Treatment with both extracts of A. indica and lumartem in malaria-infected rats showed a slight reduction in triglycerides while total cholesterol, HDL, and LDL levels increased significantly. There was no significant difference (p > 0.05) between HDL levels of malaria-infected rats treated with lumartem and the plant extracts. Administering 500 mg/kg body weight of the A. indica to malaria-infected rats in Group 7 slightly increased the total cholesterol and LDL levels compared to Groups 6 and 8. The study also recorded a significant (p<0.05) increase in triglycerides in the disease control (Group 2) when compared with experimental groups and the control.

Table 2. Percentage phytochemical constituents of ethanol extract of Azadirachta indica leaves.

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannins</td>
<td>2.63 ± 0.07</td>
</tr>
<tr>
<td>Soluble CHO</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>1.94 ± 0.06</td>
</tr>
<tr>
<td>Glycoside</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>Phenol</td>
<td>1.48 ± 0.02</td>
</tr>
<tr>
<td>Saponins</td>
<td>1.20 ± 0.04</td>
</tr>
<tr>
<td>Cyanogenic glucosides</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>3.21 ± 0.07</td>
</tr>
<tr>
<td>Terpenoid</td>
<td>2.57 ± 0.05</td>
</tr>
<tr>
<td>Steroids</td>
<td>0.68 ± 0.10</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard deviations of triplicate analysis.

The body weights of the rats investigated in this study are shown in Table 3. There was a significant (p < 0.05) weight difference in all groups compared to normal control. The disease control experienced a greater weight loss. Normal rats treated with A. indica experienced an increase in weight while administration of lumartem led to a decrease in weight when compared to normal control. The same trend was also observed in malaria infected.
rats when compared to disease control. Increase in body weight of rats treated with *A. indica* was dependent on the concentration of extract administered.

The results of hematological parameters of the rats investigated in this study are shown in Table 4. A reduction in all hematological values was observed in normal rats after the administration of extracts and lumartem compared to normal control. Malaria infection gave rise to a reduction of Hb, RBC, PCV, and platelets while an increase in WBC was observed. Treatment of malaria with extracts and lumartem resulted in a slight restoration of the hematological values. An inverse proportionality was observed between WBC and platelets in all treatment groups except for lumartem (Group 5), which recorded the highest number of platelets.

### DISCUSSION

Malarial is characterized by anemia resulting from the destruction of infected as well as uninfected RBCs. This massive destruction leads to a decrease in erythroid precursors and erythropoiesis inhibition, usually resulting in the death of the patient (Coronado et al., 2014). In this experiment, an inoculum size of $10^7$ parasitized erythrocytes was used, which resulted in less than 50% death in the disease control. During the erythrocytic cycle of *P. falciparum* from the trophozoite stage to a schizont, about 80% of the host-cell hemoglobin is ingested and is degraded by the developing trophozoite. Anemia is a preventable cause of death in malaria-infected children under five years and pregnant women (WHO, 2006).

![Figure 1](http://jppres.com/jppres/J Pharm Pharmacogn Res (2018) 6(3): 198)

Figure 1. Lipid profile of animals administered extract of *Azadirachta indica* and lumartem.

Results expressed as mean ± SD (n = 3). *significant values at p < 0.05 relative to group 1, # not significant at p > 0.05 relative to group 1.

Group 1: (normal control); Group 2: disease control; Group 3: extract 300 mg/kg; Group 4: extract 500 mg/kg; Group 5: lumartem (artemether 2 mg/kg + lumefantrine 12 mg/kg); Group 6: disease induction + extract 300 mg/kg; Group 7: disease induction + extract 500 mg/kg; Group 8: disease induction + lumartem (artemether 2 mg/kg + lumefantrine 12 mg/kg).
Table 3. The effect of ethanol extract of *Azadirachta indica* leaves on the body weight of normal male albino Wistar rats.

<table>
<thead>
<tr>
<th>Group/treatment</th>
<th>Body weight (g)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial weight (g)</td>
<td>Final weight (g)</td>
<td>Change in weight (%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>114.15 ± 1.05</td>
<td>121.02 ± 3.36</td>
<td>5.68</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>116.78 ± 4.13</td>
<td>112.50 ± 1.85</td>
<td>-3.80</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>122.48 ± 1.85</td>
<td>124.91 ± 1.12</td>
<td>1.95</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>118.60 ± 2.30</td>
<td>120.42 ± 2.63</td>
<td>1.51</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>113.22 ± 1.84</td>
<td>111.83 ± 3.00</td>
<td>-1.24</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>120.52 ± 3.76</td>
<td>123.00 ± 2.22</td>
<td>2.02</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>115.61 ± 1.36</td>
<td>118.34 ± 2.41</td>
<td>2.31</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>124.20 ± 3.00</td>
<td>121.11 ± 1.17</td>
<td>-2.55</td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as mean ± SD (n = 3). *a* *P* < 0.05 in comparison with normal control; *b* *P* < 0.05 in comparison with disease control. Group 1: (normal control); Group 2: disease control; Group 3: extract 300 mg/kg; Group 4: extract 500 mg/kg; Group 5: lumartem (artemether 2 mg/kg + lumefantrine 12 mg/kg); Group 6: disease induction + extract 300 mg/kg; Group 7: disease induction + extract 500 mg/kg; Group 8: disease induction + lumartem (artemether 2 mg/kg + lumefantrine 12 mg/kg).

Table 4. Ethanol extract of *Azadirachta indica* and lumartem on the hematological profile.

| Group/treatment | Hematological parameters |  |  |  |  |
|-----------------|--------------------------|----------------|----------------|----------------|
|                 | Hb (g/L) | WBC (10^9/L) | RBC (10^12/L) | Platelet (10^9/L) | PCV (%) |
| 1               | 14.95 ± 0.39 | 7.65 ± 2.57 | 9.40 ± 1.63 | 581 ± 0.08 | 42.27 ± 0.96 |
| 2               | 9.02 ± 0.59 | 13.17 ± 2.58 | 6.20 ± 2.33 | 361 ± 1.00 | 25.35 ± 3.53 |
| 3               | 12.13 ± 1.32 | 7.30 ± 3.10 | 7.06 ± 1.51 | 473 ± 0.77 | 37.67 ± 1.15 |
| 4               | 11.23 ± 0.45 | 6.53 ± 2.28 | 7.80 ± 2.60 | 433 ± 0.10 | 32.01 ± 1.73 |
| 5               | 10.83 ± 1.29 | 9.00 ± 1.64 | 6.61 ± 2.40 | 610 ± 0.87 | 29.00 ± 2.20 |
| 6               | 11.93 ± 0.73 | 11.90 ± 3.31 | 6.74 ± 1.19 | 421 ± 0.60 | 35.62 ± 1.51 |
| 7               | 10.01 ± 0.90 | 9.41 ± 5.10 | 7.30 ± 1.87 | 480 ± 0.39 | 27.77 ± 2.28 |
| 8               | 9.76 ± 1.43 | 10.53 ± 4.66 | 7.17 ± 3.00 | 466 ± 0.38 | 15.73 ± 1.65 |

Results expressed as mean ± SD (n = 3). *a* *P* < 0.05 in comparison with normal control; *b* *P* < 0.05 in comparison with disease control; *c* *P* > 0.05 in comparison with normal control; *d* *P* > 0.05 in comparison with disease control. Group 1: (normal control); Group 2: disease control; Group 3: extract 300 mg/kg; Group 4: extract 500 mg/kg; Group 5: lumartem (artemether 2 mg/kg + lumefantrine 12 mg/kg); Group 6: disease induction + extract 300 mg/kg; Group 7: disease induction + extract 500 mg/kg; Group 8: disease induction + lumartem (artemether 2 mg/kg + lumefantrine 12 mg/kg).

Hb: hemoglobin; WBC: white blood cell; RBC: red blood cell; PCV: packed cell volume or hematocrit.

The onset of anemia is as a result of a decrease in hemoglobin due to a decrease in the total number of erythrocytes. Most medicinal plants have a protective effect against anemia and can be used for the management of severe damage of erythrocytes in malaria infection (Onyeabo et al., 2017). In this study, the plant extracts resulted in a slight restoration of the hematological values.

Malaria parasites are unable to synthesize organic nutrients required for their growth. To ensure their survival and propagation, parasites must scavenge host cell nutrients such as lipids, which they cannot synthesize (Sherman, 1979). The rapid rate of replication of the parasite within the erythrocyte demands a considerable amount of cholesterol for membrane formation (Visser et al., 2013). It has also
been reported that these host lipids have also been implicated in the formation of hemozoin *in vivo* (Bendrat et al., 1995; Fitch et al., 1999). The hem, ferriproporphyrin IX (hematin) that is released upon digestion of the globin chains is lethal to the parasite and is converted into a crystalline substance called hemozoin (HZ) that is harmless to the parasite (Bray et al., 2005). Since the transformation of heme into hemozoin is an essential process for the survival of the malaria parasite, this molecule has become a target for antimalarial drugs (Monti et al., 1999).

Another phytochemical with antiparasoidal activity in the plant material is azadirachtin a tetranortriterpenoid (Jones et al., 1994). Limonoids like gedunin and meldenina isolated from medicinal plants have also been demonstrated to impact antimalarial activity on *A. indica* (MacKinnon et al., 1997). However, gedunin did not inhibit *P. berghei* in mice (Odetola and Bassir., 1986; Bray et al., 1990) while meldenina was very much active against *Plasmodium falciparum* (Joshi et al., 1998). Gedunin has been proposed to be responsible for the alkylation of hem and specific proteins, which leads to the death of malaria parasite (Arnason et al., 2004). Azadirachtin, on the other hand, inhibits the formation of mobile microgametes *in vitro* (Jones et al., 1994).

*A. indica* has been reported to be effective against *P. falciparum* in humans (El-Tahir et al., 1999; Alshawsh et al., 2009; Adebayo and Krettli, 2011). The dilemmas with *A. indica* on rodent models is because the causative agent of malaria infection in humans do not cause infection in rodents while *P. berghei* only causes infection in murine models but not in humans (Farahna et al., 2010). However, artemether/lumefantrine seems to be effective against both human and rodent parasites (Abolaji et al., 2012; Otuechere et al., 2012). Previous studies showed that Wistar rats inoculated with 107 parasitized red blood cells by intraperitoneal route showed 25% mortality, with a reduction in parasitemia and complete eradication of parasitemia around the 18th day of post inoculation (Dow et al. 1999; Pedroni et al., 2006). This could be responsible for the survival of the experimental animals after inoculation with *P. berghei* parasite. Therefore, despite the high dose of extract administered, it is not certain that the survival of experimental animals was as a result of the anti-plasmodial activity of *A. indica*.

The significant increase in the level of HDL-cholesterol and reduction of LDL-cholesterol in the groups administered with *A. indica* compared to normal control showed that the extract could be of great importance in the treatment of hyperlipidemia and cardiovascular diseases. Studies have shown that high concentrations of HDL have a protective effect against cardiovascular diseases such as ischemic stroke and myocardial infarction (Sacco et al., 2001). The result obtained in this study shows that ethanol extracts of *A. indica* and lumartem are favorable against atherogenesis, because of the ability to maintain a normal level of TAG and cholesterol.

Artemether has a rapid schizontocidal action (Lefèvre and Thomsen, 1999). It is primarily metabolized by cytochrome P450 (CYP) 3A4/5 but also by CYP2B6, CYP2C9, and CYP2C19. Metabolism through CYP3A4 produces an active metabolite, dihydroartemisinin that contributes significantly to its antimalarial activity (Cousin et al., 2008). The endoperoxide bond (O–O) is crucial for expression of antiparasitic activity. Its activation involves an initial chemical decomposition induced by ferrous hem Fe(II) produced from 1-electron reduction of oxidized ferric hematin within the malaria parasite and is responsible for the bioactivation of the endoperoxide bridge to potentially toxic free radicals in the food vacuole of the parasite (Bray et al., 2005). The oxy radical initially produced subsequently rearranges into carbon-centered radical intermediates (O’Neill and Posner, 2004), which gives off a high-valent iron-oxo species to produce a stable end-product (Posner et al., 1995). Nnate and Achi (2016) have reported that the radical complex formed by the reaction of Fe(II) with oxygen is suitable for the formation of another radical intermediate in the presence of a radical electron. Free-radical mediated autoxidation of membrane lipids is triggered by abstraction of a weakly bonded allylic hydrogen by some strong oxidant, which might be the hydroxyl radical (•OH), the perhydroxyl radical (HO•), a chelated iron-oxo complex such as ferryl (Fe•O) or perferryl (Fe•O2) compounds (Girotti, 1985).

A Fenton-like reaction has also been proposed where the highly reactive endoperoxide moiety in artemisinins undergo homolytic cleavage to form hydroperoxide, which is then degraded to produce reactive oxygen species and carbon-centered radical
molecules that modify the proteins of malaria parasites (Haynes et al., 1999). The endoperoxide bond is crucial for expression of antiparasitic activity, which is mediated through this reactive species produced. L umefantrine is metabolized primarily by CYP3A4 and then undergoes glucuronidation (Hietala et al., 2010; Mwesigwa et al., 2010). The mechanism of action of lumefantrine is equivalent to quinine, mefloquine, and halofantrine. Lumefantrine inhibits the formation of non-toxic hemozoin crystals by interfering with hematin, the toxic intermediate step produced during hemoglobin breakdown. Accumulation of hematin generates free radicals, which destroys the membranes by a lipid peroxidation mechanism and results in parasite death (Byakika-Kibwika et al., 2010).

Interaction of lipid solubilized hem with artemisinin followed by ferrous-mediated generation of oxyly and carbon radicals makes these reactive intermediates available to the allylic hydrogens of unsaturated lipid bilayers. Hydrogen abstraction and allylic carbon radical formation with subsequent triplet ground state oxygen capture result ultimately in the formation of lipid hydroperoxides. Studies by Berman and Adams (1997) demonstrated that artemisinin causes an increase in hem-mediated lipid membrane damage. This is in agreement with recent studies on artemether-lumefantrine and artesunate-amodiaquine combination (Otuechere et al., 2012). The decrease in weight of infected rats could be due to the breakdown of hemoglobin and lipids by malaria parasite; while that observed in groups treated with lumartem was as a result of the destruction of lipid membranes. The induction of malaria parasite in this study did not affect lipid profiles of rats. A similar trend was also recorded by Abolaji et al. (2012) without P. berghei infection in the rats.

The action of ROS on lipids leads to the activation of blood platelets (Sener et al., 2005). Thus, administration of lumartem caused an elevation in lipid peroxidation and activation of blood platelet. This enhances platelet-leukocyte aggregate formation and triggers inflammatory responses (Graff et al., 2001) as a result of increased leucocyte formation. The elevated leucocyte and platelets observed in the group treated with lumartem were as a result of lipid peroxidation. The administration of A. indica in normal rats reduced platelet count. Tohti et al. (2006) have a previously reported a decrease in platelet count as a result of the presence of phytochemicals such as saponins and cardiac glycosides malaria infection. Changes in WBC and platelets in P. falciparum malaria infection has also been reported by Guerra et al. (2008) and Karanikas et al. (2004).

CONCLUSIONS

Azadirachta indica leaf extract was practically safe, non-toxic and well tolerated at the administered doses. The extract of Azadirachta indica displayed a considerable anti-malaria property by having a higher efficacy and could be a template for short-term anti-malaria drug development.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENT

The authors confirm that the project has no funding or grants.

REFERENCES


http://jppres.com/jppres


http://jppres.com/jppres

Anti-malaria studies on *Azadirachta indica* leaves in rats


Author contribution:

<table>
<thead>
<tr>
<th>Contribution</th>
<th>Achi NK</th>
<th>Onyeabo C</th>
<th>Nnate DA</th>
<th>Ekeleme-Egedigwe CA</th>
<th>Kalu IK</th>
<th>Chibundu IC</th>
<th>Wokoma GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concepts or ideas</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Design</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Definition of intellectual content</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Literature search</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Experimental studies</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Data acquisition</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Data analysis</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statistical analysis</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Manuscript preparation</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Manuscript editing</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Manuscript review</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>