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RESEARCH ARTICLE

PHENOLIC CONTENTS, IN-VITRO ANTIOXIDANT ACTIVITY AND IN-VIVO ANTI-PLASMODIAL ACTIVITY OF METHANOLIC LEAF EXTRACT OF *AZADIRACHTA INDICA* (DONGOYARO) AND ITS EFFECT ON SOME BIOCHEMICAL PARAMETERS IN SWISS ALBINO MICE INFECTED WITH *PLASMODIUM BERGHEI NK 65.*

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ABSTRACT

The study was conducted to determine the phenolic contents, in-vitro antioxidant activity and in-vivo anti-plasmodial activity of methanolic leaf extract of *Azadirachta indica* (Dongoyaro) and its effect on some biochemical parameters in Swiss albino mice infected with *Plasmodium berghei NK 65.* Swiss albino mice were inoculated intraperitoneally with *Plasmodium berghei NK65*. The mice were grouped into six groups, five per group. Group I were not infected with *P.berghei*, Group II and III served as both the negative and positive control while Group IV, V, and VI were treated with 200, 400, and 800 mg/kg body weight of methanolic leaf extract of *A. indica*. The qualitative phytochemicals in the extract include tannin, anthraquinone, flavonoids, phlobatanin, saponin etc. The amount of total phenol, flavonoids, alkaloids and total proanthocyanidins present in the extract are 148.22 mg PE / g DW, 1.5%, 5.3% and 0.67±0.2 mg quercetin/g of dry plant material respectively. In the in-vitro antioxidant assay, *A. indica* was found to have FRAP, DPPH and H_2O_2 scavenging activity. The median lethal dose LD_{50} was estimated to be >5000mg/Kg body weight. The extract caused 47.80%, 50.96% and 52.30% suppression in parasitaemia at 200, 400 and 800mg/kg body weight respectively while Chloroquine exerted 100% suppression at 5mg/kg body weight. The curative test shows that the different concentration of the extract exert a growth inhibition of 50.1%, 74.57% and 73.68% at 200, 400, 800mg/kg body weight respectively while Chloroquine cleared the parasites by 94.07% at 5mg/kg body weight. The Hematological parameters showed that the extract is not hematotoxic since it significantly (P<0.05) reduced WBC count, and increase RBC, HGB, and HCT values in the treated mice compared to the infected untreated mice. The liver biomarker enzymes show that there was a significant increase (P>0.05) in plasma AST, ALT, ALP and GGT in the untreated infected group compared to other groups, this signify hepatic damage in group II animals. This may also signifies hepatoprotective effect of the extract on the Swiss mice infected with *P. berghei*. The infected untreated and the infected treated animals all have higher plasma creatinine and urea values compared to normal healthy animals. There was a significant decrease (P<0.05) in total plasma protein in group II mice compared to the mice in other groups.

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INTRODUCTION

Malaria is one of the most killing disease in the world particularly in tropical countries (shiff, 2002). Malaria is a disease caused by Plasmodium species, is one of the oldest and greatest health challenges affecting 40% of the world's population (Greenwood, 2002). According to World Health Organization (WHO) report of 2008, there were an estimated 247 million episodes of malaria in 2006, with a wide uncertainty interval from 189 million to 327 million cases. Eighty-six percent (212 million) were recorded in the African regions.

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Eighty percent of the cases were in 13 countries and over half in Nigeria (WHO, 2008). *Plasmodium berghei* was used in studying the activity of potential anti-malarials in mice because it produces diseases similar to those of human plasmodium infection (Kumar *et al.*, 2006). Plant extract have been very useful sources of medication for various disease conditions (Ataman *et al.,* 2006 and Gill *et al.,* 2002). *Azadirachta indica* commonly known as Neem is one of the most useful traditional medicinal plant. Every part of the tree has been used as traditional medicine for household remedy against various human ailments. *Azadirachta indica,* is found in Nigeria and in most of the tropical and subtropical countries and is widely distributed in the world. The taxonomic classification of *Azadirachta indica* is as follows: Kingdom *Plantae*, Order: *Rutales*, Suborder: *Rutinae*, Family: *Meliaceae*, Subfamily : *Melioideae*, Genus : *Azadirachta*, Species: *indica* (Girish

et al.,, 2008). All parts of the plant are useful and have been used in treatment of diseases ranging from teeth decay, swollen liver, ulcers, dysentery, diarrhea, malaria and other bacterial infections. (Allameh *et al.,* 2002 and Mossini *et al.,* 2004). Oxidative stress involving enhanced generation of reactive oxygen species (ROS) and has been implicated in the etiology of over one hundred human diseases including inflammation, metabolic disorders, cellular aging, atherosclerosis, heart disease, stroke, diabetes mellitus, cancer, malaria, rheumatoid arthritis, HIV / AIDS, Alzheimer's disease, ulcerative colitis and Parkinsons disease (Olukemi *et al.,* 2005; Hyun *et al.,* 2006 ; Aliyu *et al.,* 2008; Smith *et al.,* 2000). Antioxidants are molecules that are capable of neutralizing the harmful effects of the ROS through the andogenous enzymatic defense system. The antioxidant effect of plant is mainly due to phenolic components, such as flavonoids, phenolic acids and phenolic diterpenes (Shahidi *et al.,* 1992). The antioxidants capacity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994).

MATERIALS AND METHODS

Collection and identification of Plant extract

The leaves of *Azadirachta indica* were obtained from Ikorodu in Lagos State, Nigeria. The plant was authenticated by Mrs Shokefun a botanist from Science Laboratory Technology Department, Environmental biology Unit, Lagos State Polytechnic, Ikorodu.

Preparation of methanolic leaf extract of *Azadirachta indica*

The leaves of *Azadirachta indica* were washed, air dried under shade in the Biochemistry Laboratory, pulverised to coarse power using blender. Extraction was carried out by dispersing 200g of the grounded *Azadirachta indica* plant material in 1L of 80% methanol and shaking was done with GFL shaker for 72 hours. This was followed by vacuum filtration and concentrated by rotary evaporator at a temperature not exceeding 40°C. The concentrated extract was dried to complete dryness in an aerated oven at 40°C for 48 hours. The extract was latter stored in a refrigerator at 4°C.

Qualitative phytochemical analysis

Phytochemical analysis for phytochemical constituents were carried out on the methanolic extract of *Azadirachta indica* using standard phytochemical procedures (Sofowora (1993), Harborne (1973), Trease and Evans (1989).

Quantitative phytochemical analysis of the methanolic leaf extract of *Azadirachta indica*

Total Phenolic Content Determination

The quantitative determination of total phenolic content using Folin-Ciocalteu (F-C) reagent involves oxidation in alkaline solution of phenols by the yellow molybdotungstophosphoric heteropolyanion reagent and colorimetric measurement of the resultant molybdotungstophosphate blue according to the method of Singleton and Rosi (1965) and modified by Dogÿan *et al.* (2005). The polyphenol fraction (extract corresponding to 1 g of dry plant material) was dissolved in 5 ml of double distilled water. An aliquot of 100 µl of this solution was diluted with double distilled water to 3 ml. Afterward, the obtained solution was added to 300 µl of double distilled water and 500 µL of the F-C reagent. After shaking, the mixture was incubated for 3 min at room temperature. Then 2000 µl of 20 % Na_2CO_3 solution was added. The volume obtained was mixed vigorously, and held for 60 min in the dark at ambiant temperature. The absorbance of the solution was then measured at 650 nm against a blank in a spectrophotometer. The sample was analysed in triplicate and the average content was noted for each measurement. The total phenolic content, expressed as mg of pyrrocatechol equivalents (PE) per g of dry weight of plant material (mg PE / g DW), was calculated through the calibration curve obtained using the equation given bellow :

Absorbance = 0.0828 x C, R^2 = 0.9993

where C was the concentration (mg/l).

Flavonoid content determination

One hundred millilitres of 80% aqueous methanol was used to repeatedly extract 1 g of the defatted sample at room temperature. The solution was then filtered through Whatman filter paper. The filtrate was evaporated to dryness in a crucible over a water bath and weighed to a constant weight (Oseni *et al.,* 2013).

Alkaloid content determination

To about 1 g of the defatted sample, 80 ml of 10% acetic acid in ethanol was added. The beaker was covered and then allowed to stand for 4 hours. The suspension was then filtered and the extract concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The precipitate was collected and washed with dilute ammonium hydroxide and then filtered to obtain the alkaloid residue. This was dried and weighed (Oseni *et al.,* 2013).

Determination of total proanthocyanidins

Determination of proanthocyanidin was based on the procedure reported by Sun *et al.,* 1998. A volume of 0.5 ml of 0.1 mg/ml of extract solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid; the mixture was allowed to stand for 15 min. The absorbance was measured at 500 nm. Extract samples were evaluated at a final concentration of 0.1 mg/ml. Total proanthocyanidin content were expressed as catechin equivalents (mg/g) using the following equation based on the calibration curve: y = 0.5825x, R^2 = 0.9277, where x was the absorbance and y is the catechin equivalent (mg/g).

Determination of β-Carotene and lycopene determination

β-Carotene and lycopene were determined by the method of Barros *et al.,*. (barros *et al.,* 2007). The dried extract (100 mg)

was vigorously shaken with acetone-hexane mixture (4:6, 10 mL) for 5 minutes and filtered through a disposable filter (0.45 µm, Millipore). The absorbance of the filtrate was measured at 453, 505, and 663 nm. Contents of β-carotene and lycopene were calculated according to the following equations:

β-carotene (mg/100 mL) = 0.216 (A663) − 0.304 (A505) + 0.452 (A453);

lycopene (mg/100 mL) = −0.0458 (A663) + 0.372 (A505) − 0.0806 (A453). The assays were carried out in triplicates, the results were mean ± SD and expressed as µg of carotenoid/g of extract.

In-vitro plant antioxidant assay

DPPH Radical Scavenging Activity Assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity was measured according to the previously described procedure [18] with slight modification on the basis of the method of Blois [19]. Different concentrations of ethanol dilutions of samples were mixed with 2.0 volume of 6.5×10^{-5} M solution of DPPH. The resulting solutions were thoroughly mixed and absorbance was measured at 517nm after keeping the tubes in dark for 30 minutes. The scavenging activity was determined by comparing the absorbance with that of control containing equal volumes of DPPH solution and ethanol. The radical scavenging activity was obtained by the following equation:

$$\text{Radical scavenging activity (\%)} = \frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}} \times 100$$

The IC_{50} was defined as the concentration (in μg/mL) of the extract required to deplete the amount of DPPH radical by 50%. Gallic acid (GA) and butylated hydroxytoluene (BHT) were used as positive control

Ferric reducing antioxidant power assay (FRAP)

The reducing power assay was conducted as previously described by Wang *et al.,*. (2008) and Oyaizu (1986) with ascorbic acid (AA) and tert-butyl-4-hydroxyanisole (BHA) being used as the positive controls. In brief, 2.5 ml of individual deionized water diluted *A. indica* extract (ranged from 0.1 to 1 mg/ml) was sequentially mixed with equal volume of phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1% w/v). After incubation at 50°C for 20 min, 2.5 ml of trichloroacetic acid (10 % w/v) was then added to the mixture followed by centrifuging at 3000 rpm for 10 min. Consequently, 5 ml of the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride (0.1 % w/v). After 30 min of incubation at room temperature in the dark, absorbance of the resulting solution was measured at 700 nm using a spectrophotometer. The ferric reducing power capacities of the plant extracts and standard antioxidants were expressed graphically by plotting absorbance against concentration. Samples for the assay were prepared in triplicate

Hydrogen Peroxide Radicals (H_2O_2) Scavenging Activity assay

Hydrogen peroxide scavenging activity of *A.indica* and standards was assayed by the method of Zhao *et al.,* 2006. H_2O_2 (1.0 mL, 0.1mM) and 1.0mL of various concentrations of the extract were mixed, followed by 100 μL 3% ammonium molybdate, 10mL H_2SO_4 (2 M) and 7.0mL KI (1.8 M). The mixed solution was titrated with $Na_2S_2O_3$ (5mM) until the yellow color disappeared. The percentage scavenging effect was calculated as

$$\text{Scavening rate (\%)} = \frac{V0 - V1}{V0} \times 100$$

where $V0$ was volume of $Na_2S_2O_3$ solution used to titrate the control sample in the presence of hydrogen peroxide (without extract), $V1$ was the volume of $Na_2S_2O_3$ solution used in the presence of *A.indica*. The IC_{50} was defined as the concentration (in μg/mL) of the extract required to deplete the amount of H_2O_2 radical by 50%. GA and BHT were used as positive control.

Sources of Swiss albino mice

Eight (8) weeks old Swiss albino mice weighing 24- 35g were obtained from Nigeria Institute of Medical Research (NIMR), Lagos, Nigeria. These animals were maintained under laboratory conditions of temperature (22 to 24°C), humidity (40 to 60%) and 12 h light/12 h dark regime at NIMR animal house. They were acclimatized for three days, housed in plastic cages with saw dust as beddings; they were exposed to both food and water *ad libitum* for the entire duration of the study. The mice were used in accordance with NIH Guide for the care and use of laboratory animals; NIH Publication revised (1985) NIPRD Standard Operation Procedures (SOPs).

Acute toxicity test

The acute toxicity test of methanolic leaf extract of *Azadirachta indica* was carried out using modified Lorkes method (1993). Eighteen Swiss albino mice weighing 24 to 35 grams were randomized into three groups of six mice each and were given 1600, 2900 and 5000mg/Kg body weight of the extract orally. They were observed for signs of toxicity and mortality for the first critical four hours and thereafter daily for 7 days. The oral median dose LD_{50} was calculated.

Grouping of animals for infection and treatment

The parasite *Plasmodium berghei NK 65* was obtained from Nigeria Institute of Medical Research (NIMR), Lagos, Nigeria from Dr Aina, O.O. The parasites were kept alive by continuous intraperitoneal inoculation of known amount of the parasite into Swiss mice. Iml of blood was taken from donor mice and diluted with 5ml phosphate buffer; such that 0.1ml contained standard inoculum of 10^7 infected red blood cells (Maegraith *et al.,*, 1952). Thirty acclimatized Swiss albino mice were randomly selected and twenty five Swiss mice were inoculated intraperitoneally from the same source to avoid variability in parasitemia. The mice were randomly distributed into six groups of five per group as shown below:

GROUP I (Normal control) Healthy uninfected Swiss mice
GROUP II (Negative control) mice infected with *P.berghei NK65* without treatment
GROUP III. (Positive control) = *P.berghei* +5mg/kg b,wt of Chloroquine (Standard drug)
GROUP IV= *P. berghei* + 200mg/kg b.wt of *Azadirachta indica* extract.
GROUP V= *P. berghei* + 400mg/kg b.wt of *Azadirachta indica* extract.
GROUP VI=*P. berghei* + 800mg/kg b.wt of *Azadirachta indica* extract.

Anti-Plasmodium studies

Suppressive test

The Peter's 4-day suppressive test against *P. berghei NK65* infection in Swiss mice was used (Peters, 1965). Adult Swiss mice weighing between 24 to 35gram were inoculated by intraperitonial injection with standard inoculum of *Plasmodium berghei NK65* with 10^7 infected red blood cells. The mice were divided into six groups as shown above and treated for 4 consecutive days with 5mg/kg.b.wt of Chloroquine, 200, 400, and 800mg/kg body weight of *Azadirachta indica* extract orally daily. On day 5 of the experiment, blood was collected from the tail of each mouse and smeared onto microscope slide to make a film. The blood films were fixed with methanol, stained with Geimsa at pH 7.2 for 10 minutes and examined under the microscope for the presence of parasites. The parasite density was calculated for each group by comparing the parasitaemia in infected group (Group II) with those of control and treated groups.

Curative test

The Curative test of methanolic leaf extract of *Azadirachta indica* on another fresh infected Swiss albino mice were carried out according to the method described by Ryley and Peters, 1970. The Swiss mice were injected intraperitoneally with standard inoculums of 1×10^7 *Plasmodium berghei NK 65* infected erythrocytes on the first day (day 0). Seventy two hours later, thirty mice were divided into six groups of five mice per group as shown above. The treated groups were orally treated with 5mg/kg b.wt of chloroquine, 200,400 and 800mg/kg b.wt of *Azadirachta indica* respectively. The treatment was carried out once daily for 5 days, on each day of the treatment, blood was collected from the mice tail and smeared onto microscope slide to make thin and thick films. The blood films were fixed with methanol, stained with 10% Giemsa at pH 7.2 for 10 minutes and examined microscopically to monitor the parasitaemia level. The parasite density was calculated for each group over a period of six days.

Hematological analysis

The Swiss albino mice in the suppressive assay groups were sacrificed and their bloods were collected in EDTA tubes by ocular puncturing. The bloods in the EDTA tubes were assayed for hematological parameters using BC -3200 Auto Hematology Analyzer in Lagos University Teaching Hospital in Lagos- Nigeria.

Determination of liver and kidney function test

Plasma enzymes like aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) were determined by Randox diagnostic kits. The plasma total protein, urea and creatinine were also determined using Randox diagnostic kits.

Data analysis

Data analysis was done using the Graph Pad prism computer software. Student's'-test and one-way analysis of variance (ANOVA) were used for comparison. A *P*-value < 0.05 was considered significant.

RESULTS

Qualitative phytochemical constituents of the methanolic leaf extract of *Azadirachta indica*

The results obtained from the phytochemicals analysis of *Azadirachta indica* extract showed the presence of some secondary metabolite like tannins, saponins, flavonoids, protein, anthraquinone, glycoside, phlobatanin, reducing sugar and fats and oil. (Table 1). The presence of these secondary metabolites in this extract may be responsible for the anti-plasmodial activity of the extract.

Table 1. The qualitative phytochemical constituents of the methanolic leaf extract of *Azadirachta indica*

Phytochemical components	Qualitative abundance
Tannins	
Ferric Chloride test	++
Saponins	
Frothing test	++
Alkaloids	
Wagners test	+++
Terpenoids	+
Flavonoids	
Ammonium test	+
Test for protein	
Millions test	+
Biuret test	+
Test for fat and oil	++
Test for reducing sugar	++
Glycoside	+
Anthraquinone	+
Phlobatanin	+

+ (Present in low concentration), ++ (present in moderate concentration) and +++(present in high concentration).

Quantitative analysis of the phytochemical constituents of the methanolic leaf extract of *Azadirachta indica*

The results of table 2 below revealed that the level of these phenolic compounds in *Azadirachta indica* has high level of phenolic compounds.

The values are significantly lower when compared to the standard compounds used in this study.

Table 2. Quantitative analysis of the phytochemical constituents of the methanolic leaf extract of *Azadirachta indica*

Phenolics	*Azadirachta indica* extract
Total phenol[a]	148.22
Flavonoids	1.5%
Alkaloids	5.3%
Total proanthocyanidins[b]	0.67±0.2

[a]mg of pyrrocatechol equivalents (PE) per g of dry weight of plant material (mg PE / g DW).
[b]Expressed as mg quercetin/g of dry plant material.

Determination of β-Carotene and lycopene determination

β-Carotene and lycopene were found in small amounts. The obtained values for β-Carotene and lycopene were 0.65 ± 0.12 µg and 0.34±0.02 µg/g respectively.

A.indica may serve has H_2O_2 scavenger has shown in the Table above.

Acute toxicity test for *Azadirachta indica*

The mice were monitored for four hours, but no signs of toxicity were observed. The behavioural and physical observations revealed no involuntary urination, muscle weakness, and convulsion. The animals were physically active for the first four hours. The median lethal dose LD_{50} was estimated to be >5000mg/Kg body weight. The methanolic leaf extract of *A.indica* caused 47.80%, 50.96% and 52.30% suppression in parasitaemia of *P.berghei NK65* infected mice at 200, 400 and 800mg/kg body weight respectively (Figure 3), while Chloroquine, a standard anti-malarial drug used exerted 100% suppression at 5mg/kg body weight. The curative test shows that the different concentration of the extract of *A. indica* exert a growth inhibition of 50.1%, 74.57% and 73.68% at 200, 400, 800mg/kg body weight of the extract respectively while Chloroquine, the standard drug, cleared the parasites by 94.07% at 5mg/kg body weight (Figure 4).

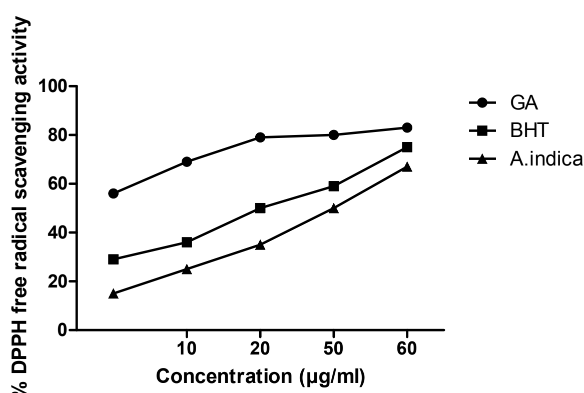

Fig. I. DPPH radicals scavenging activities of the extracts of *A.indica,* GA and BHT at different concentrations. Each value represents mean ± SD (n =6).

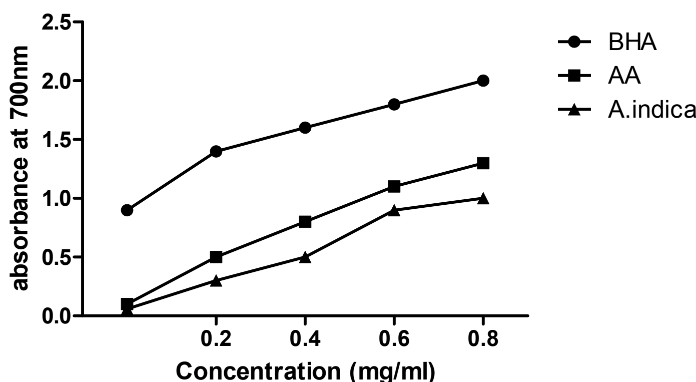

Fig. 2. Reducing power of BHA, AA and *A.indica* at different concentrations. Each value represents mean ± SD (n =6).

Table 3. Scavenging activity of H_2O_2 by gallic acid (GA), BHT and *A. indica* methanolic leaf extract

Group	Concentration (µg/ml)	Inhibition (%)	IC_{50}(µg/ml)
Gallic acid	5	12.31±1.01	36.17
	10	18.91±1.32	
	50	61.8±1.17	
BHT	5	6.34±0.21	128.13
	10	16.23±0.18	
	50	32.13±0.81	
A.indica	5	7.13±0.11	103.41
	10	15.23±0.23	
	50	41.14±0.10	

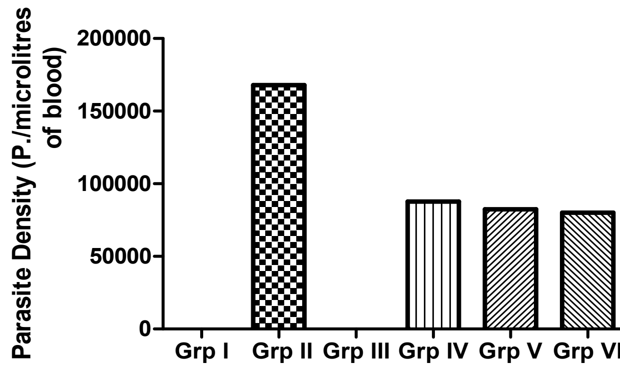

Fig. 3 Suppresive test showing the effect of methanolic leaf extract of *A,indca* on Swiss mice infected with *Plasmodium berghei NK65*.

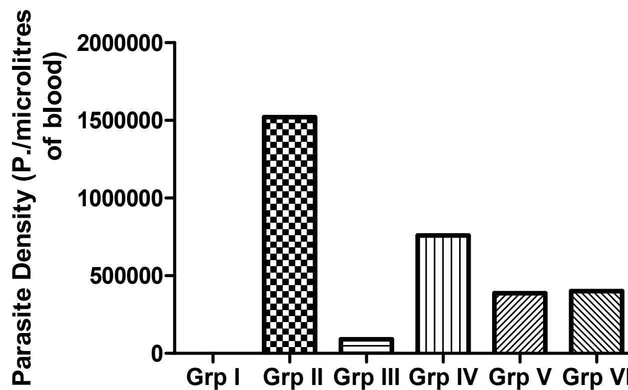

Fig. 4 Curative test showing the effect of methanolic leaf extract of *A.indica* on Swiss mice infected with *Plasmodium berghei NK65*.

Table 4. Suppressive effect of methanolic leaf extract of *Azadirachta indica* and chloroquine on hematological parameters of Swiss albino mice infected with *P. berghei NK65*

Hematological Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
WBC (X 10^9/L)	*7.6 ±1.1*	14.10 ±2.10	9.10 ±0.6*	9.61 ±0.50*	9.10 ±0.60*	8.40 ±0.70*
PCT (%)	0.458 ± 0.059	0.322 ± 0.071	0.391 ± 0.068	0.385 ± 0.084	0.435 ± 0.073	0.357 ± 0.093
MPV (fL)	6.6 ± 0.2	6.9 ± 0.2	6.7 ± 0.10	7.1 ± 0.30	6.8 ± 0.40	6.7 ± 0.10
PDW	14.20±0.10	14.50±0.20	14.1±0 .20	14.2±0.10	14.40±0.20	14.10±0.30
HGB (g/dl)	14.3±1.60*	8.7±1.2	10.6±1.20*	11.60±0.70*	12.10±0.5*	13.10±0.50*
RBC (X 10^{12}/L)	9.10±0.5*	6.80 ± 0.4	8.20±0.43*	7.80±0.40*	8.72±0.25*	8.10±0.55*
HCT (%)	48.3±0.8*	32.2±6.5	44.1±0.4*	43.2±1.4*	41.10±2.6*	40.40±5.1*
MCV (fL)	54.22±3.4	53.30±3.2	54.10±2.4	53.20±0.30	51.20±0.40	52.50±0.40
MCH (Pg)	15.7±0.7	14.8±0.4	15.0±0.4	14.90±0.55	12.90±1.10	14.20±1.20
MCHC (g/dL)	31.10±0.20	29.10±1.20	31.40±0.30	30.30±1.52	29.30±0.30	28.90±0.75
RDW-CV (%)	17.70±0.50	18.10±0.10	17.90±0.20	16.80±0.50	17.20±0.60	16.90±0.65
RDW-SD (fL)	30.40±0.20	31.40±0.50	31.10±0.30	30.10±0.40	29.90±0.50	30.45±0.40

Values are expressed in mean ± SD of five mice in each group. * indicate Significant difference when compared to negative control (P <0.05). WBC: White blood cell; PCT: Plateletcrit ; MPV: Mean platelet volume; PDW: Platelet Distribution Width; HGB: Hemoglobin Concentration; RBC: Red Blood Cell; HCT: Hematocrit ; MCV: Mean corpuscular volume; MCH: Mean Cell hemoglobin; MCHC: Mean Cell hemoglobin concentration; RDW–CV: RBC distribution width–coefficient of variation; RDW–SD: RBC distribution width–standard deviation.

Table 5. Determination of Plasma Total protein, Liver and Kidney function test of uninfected group and infected groups treated with chloroquine and the extract of *Azadirachta indica*

Hematological Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
AST (U/L)	5.20 ±1.50*	11.10 ±4.0	6.20 ± 2.10*	6.40 ±2.10*	6.10 ±2.0*	6.40 ±1.40*
ALT (U/L)	4.50±1.00*	17.10±5.10	6.80±1.10*	5.20±1.00*	7.50±1.40*	6.90±1.10*
ALP (U/L)	66.30±2.10*	109 ±4.30	68.40±1.40*	69.20±6.10*	69.30±7.4*	75.10±4.20*
GGT (U/L)	14.40 ± 3.2*	45.4 ±3.2	18.20±5.30*	19.30±3.10*	24.10±3.0*	23.10±5.10*
Creatinine (mg/dl)	0.81 ± 0.020*	1.71 ± 0.15	1.25 ± 0.20*	1.31 ± 0.22	1.49 ± 0.34	1.52 ± 0.25
Urea (mg/dl)	20.10±4.20*	29.50±1.50	24.5±2 .30*	27.20±1.20	26.20±3.50	26.30±2.90
Total protein (g/l)	76.80±4.00*	58.50±5.60	67.10±3.20*	66.20±3.60*	64.80±2.1*	70.70±4.30*

* indicate Significant difference (P <0.05) when compared to negative control animals (group).

above Table shows the effect of liver biomarker enzymes and other biochemical parameters on the infected group and the treated groups.

The total WBC counts of the treated mice were significantly lowered following the administration of Azadirachta indica extract and chloroquine. The mean HGB, RBC and HCT values of the infected untreated mice (group II) were significantly ($P<0.05$) lowered when compared to group I and all other treated groups (Table 4). The other Hematological parameters show no significant differences between the untreated group and other groups.

DISCUSSION

The result of the qualitative phytochemical analysis shows that the methanolic leaf extract of *A. indica* contain some secondary metabolites like phlobatanin, tannin, anthraquinone, saponin, glycoside, reducing sugar, flavonoids etc. (Table 1). The amount of total phenol, flavonoids, alkaloids and total proanthocyanidins present in the leaf extract are 148.22 mg PE / g DW, 1.5%, 5.3% and 0.67±0.2 mg quercetin/g of dry plant material respectively (Table 2). Several recent studies showed that phenolics and flavonoids constitute the main powerful antioxidant compounds (Ksouri *et al.,*2008 and Braca *et al.,* 2003). Most of the antioxidant potential of medicinal plants is due to the redox properties of phenolic compounds, which enable them to act as reducing agents, hydrogen donors and singlet oxygen scavengers (Hakkim *et al.,* 2007). The antioxidant, anti – inflammatory, antifungal and healing properties of some plant extracts have been attributed to the presence of tannins (Araújo *et al.,* 2008). β-Carotene and lycopene were found in small amounts. The obtained values for β-Carotene and lycopene were 0.65 ± 0.12 µg and 0.34±0.02 µg/g respectively. The presence of these compounds in the extract indicates the anti-oxidant potential of the plant.

DPPH is a free radical compounds and has been widely used to test the free radical scavenging ability of various samples (Sakanaka *et al.,* 2005 and Shimoji *et al.,* 2002).The free radical scavenging activity is usually expressed as percentage of DPPH inhibition but also by the antioxidant concentration required for 50 % DPPH reduction (IC_{50}). Basically, a higher DPPH radical scavenging activity is associated with a lower IC_{50} value. IC_{50} value was determined from plotted graph of scavenging activity against the different concentrations of *A. indica* extracts, gallic acid (GA) and butylated hydroxytoluene (BHT). The highest percent DPPH radical-scavenging activities were observed in the GA, followed by BHT whilst the extract showed lowest scavenging activity. At a concentration range of 10–50 μg/mL, the IC_{50} of GA was 3.60 μg/mL that of BHT was 35.49 μg/mL and *A.indica* has a value of 65.49 μg/mL (Figure 1). This result suggested that the extract is a fairly good scavenger for DPPH radicals. The ferric reducing antioxidant power (FRAP) assay measures the reducing ability of antioxidants against the oxidative effects of reactive oxygen species. This assay is based on the ability of antioxidants to reduce the ferric form (Fe^{3+}) to the ferrous form (Fe^{2+}). Prussian blue colour complex is formed by adding $FeCl_3$ to the ferrous (Fe^{2+}) form. Therefore, reduction can be determined by measuring the formation of Perl's Prussian blue at 700 nm (Chung *et al.,* 2002; Gülcin *et al.,* 2010). In this assay, the colour of the test solution changes to blue colour because of the reducing power of antioxidant samples. A higher absorbance indicates a higher ferric reducing power. Increase absorbance indicates an increase in reductive ability. Figure 2 presents the dose dependent ferric reducing powers of the extracts of *A.indica,* BHA, and AA respectively. The reducing power of the extracts is lower compared to other standard used (AA and BHA). The H_2O_2 scavenging activity of GA was stronger than that of BHT and the extract. Ability of the extract to scavenge H_2O_2 was determined and the extract was found to dose dependently scavenge H_2O_2 as well with IC_{50} of 103.4. μg/mL (Table 3).

The result obtained from this analysis shows that *A.indica* extract is a better scavenger of H_2O_2 compared to one of the standard BHT with IC_{50} of 128.13. μg/mL. The presence of these secondary metabolites in *A. indica* may be responsible for the plant anti-*plasmodium* activity. It is evident by these findings that *A. indica* possessed anti-plasmodium activity justifying its usage in the management of malaria. Milliken, 1997, Christensen and Kharazmi, 2001 showed clearly that anti-plasmodial screening of plant substances may be caused by terpenes, flavonoids and alkaloids. These compounds could be acting singly or in synergy with one another to exert the anti-plasmodial activity observed in this study. No death occurred during toxicity test at all the dose level used which shows that the extract does not have any toxic effect that can lead to the death of the animals. The median lethal dose LD_{50} was estimated to be >5000mg/Kg body weight. Behavioural signs of toxicity like salivation, paw licking, restlessness, reduced activities and stretching were observed.

The four day suppressive test is a standard test commonly used for anti-malarial screening, and the determination of percentage inhibition of parasitaemia is the most reliable parameter. The results obtained from this study showed significant decrease in parasitaemia of *P.berghei* after treatment with the extract of *A. indica.* The significant decrease in parasitaemia observed in this study was dose dependent. The methanolic leaf extract of *A. indica* caused 47.80%, 50.96% and 52.30% suppression in parasitaemia of *P.berghei* at 200, 400 and 800mg/kg body weight respectively while chloroquine, a standard anti-malarial drug used exerted 100% suppression at 5mg/kg (Figure 3). When a standard anti-malarial drug is used in mice infected with *P. berghei*, it suppresses the parasiteamia to a non-dectable level (Kiseko,*et al.,* 2000). The curative test shows that only chloroquine cures the parasites from day 3 of treatment while the different concentration of the extract of *A. indica* exert a growth inhibition of 50.10%, 74.57% and 73.68% respectively (Figure 4). Studies showed that the *in vitro* anti-malarial activity of *A. indica*, previously reported by El-Tahir *et al.,* in 1999, indicate that an aqueous extract of Neem leaves showed an IC_{50} value <5 µg/ml against *P. falciparum*. Similar results were obtained by Alshawsh and colleagues in 2009, where they reported that aqueous *A. indica* extracts inhibited the development of the ring stage of *P. falciparum*.

Hematological and biochemical indices have been reported to be a reliable parameter for assessment of the health status of

animals (Sexena ,*et al.,* 2011 and Ohaeri, 2011). WBC count functions primarily in body defence against foreign bodies and this is often achieved through leucocytosis and antibody production. There is a significant increase (P < 0.05) in the WBC count in the untreated group (group II) compared to group III and all the groups treated with *A.indica* (Table 4). Leukocytosis observed in group II may be due to bone marrow tumor, luekemia, tissue damage, and inflammatory disease of the mice infected with *P.berghei NK65*. The primary reason for assessing the RBC is to check the level of anemia and to evaluate normal erythropoiesis. HGB level shows the amount of intracellular iron present, while HCT, indicates the volume of RBC in 100ml of blood and it helps to determine the degree of anemia or polycythaemia. The extract prevented a drastic reduction in HGB, RBC and HCT values, features typical of infection due to malaria. This observation is supported by a report stating that anaemia is characterized by decreased values of HGB, RBC and hematocrit (HCT) (Aleksandro *et al.,* 2009). There were no significant change (P<0.05) in the MCV, MPV, PCT, MCH, MCHC, PCT, PDW, RDW-CV.and RDW-SD. This study shows that the methanolic leaf of *A.indica* extract does possess hematopoietic activity and is not hematotoxic.

Liver is the major organ used for removing xenobiotic substances from the body and as such it possesses a high metabolic rate and is subjected to many substances causing oxidative stress. Table 5 shows that there is a significant increase (P>0.05) in plasma AST, ALT, ALP and GGT in the untreated group compared to other groups. This shows that the animals in this group (group II) have liver impairment or haptocellular damage compared to other animals in other groups. This may also signifies hepatoprotective effect of *A.indica* extract on the Swiss mice infected with *P. berghei*. Increase in the serum levels of AST and ALT (especially ALT) are reported to be associated with liver damage (Mukherjee, 2003). Momoh *et al.,*, 2014, Momoh and Manuwa, 2014 showed clearly that increase in plasma ALP and GGT levels are associated with liver damage. The positive correlation found between gestational malaria and liver enzyme, suggest that the latter increased in malaria parasitaemia to a level dependent on the degree of parasitaemia, and also suggest that the liver is involved in the pathophysiology of malaria. There were no significant difference (P>0.05) in the plasma creatinine and urea values obtained in the infected untreated group compared to the treated groups (group III to VI). The infected untreated and the infected treated animals all have higher plasma creatinine and urea level compared to normal healthy animals, this is due to the presence of *Plasmodium berghei NK65 i*n their system. The decrease in the plasma total protein in group II compared to other groups may be due to the reduction in protein synthesis. This may indicate that malaria parasite causes the destruction of cells that are responsible for protein synthesis. This finding agrees with an earlier report that showed chronic infections and autoimmune diseases may lead to reduced protein synthesis (Bygbjarb 2001).

Conclusion

The result obtained from this study reveals that 200, 400 and 800mg/kg body weight of methanolic leaf extract of *Azadirachta indica* possess antioxidant properties, is not hematotoxic, may prevent hepatic damage and suppresses *Plasmodium berghei NK 65* and could be used in the management of malaria.

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