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THE EFFICACY OF *AZADIRACHTA INDICA* (LINN) LEAF EXTRACTS ON *PLASMODIUM BERGHEI (*LINN) INFECTED MICE.

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ABSTRACT

Malaria is the world's most important parasitic disease and endemic in about 100 developing counties. Resistance to the most severe form of the etiologic agent in human, *Plasmidium falciparum*, to commonly used antimalarial drugs is one of the factors contributing to high prevalence rate of malaria in Nigeria as well as other sub-Sahara region. Hence, new and effective antimalarialdrug candidates are of essence. The antiplasmodial efficacy of *Azadirachta indica* was investigated in this study using the ethanol and methanol leaf extracts on *Plasmodium berghei* infected swiss albino mice at 100 and 200mg/kg/day dosage. The 5 day curative test assay revealed that the administered dosages (100 and 200mg/kg/day) of *Azadirachta indica* ethanol extract caused chemo suppression of 75 % and 90 % respectively on day three and chemo suppression of 98.4% and 99.03% on day five. Similar dosages of methanol leaf extract caused chemosupression of 70 % and 93.54 % respectively on day three and chemo suppression of 82% and 92.77 % on day five. These values were statistically significant ($P<0.05$) when compared with the positive control. Haematological parameters of swiss albino mice such as erythrocyte sedimentation rate, packed cell volume, red blood cell and white blood cell investigated before, during and after infection showed significant variation in values. The study showed that *Azadirachta indica* leaf contained active antiplasmodial compounds and therefore might be a potential antimalarial drug.

Keywords: Antimalarial drug, resistance, chemo suppression and antiplasmodial efficacy

INTRODUCTION

Malaria is the world's most important parasitic disease and endemic in about 100 developing counties. It is transmitted by mosquitoes which serve as vectors as well for other human infectious diseases such as dengue fever, yellow fever, encephalitis, West Nile fever, lymphatic filariasis etc. Therefore they continue to pose a serious public health problem throughout the world (Das and Mukherjee, 2006). According to Kamalinder *et al.* (2008), malaria, the 'king of diseases' is re-emerging as world's number one killer infection, and every year, malaria is reported to claim about three million lives worldwide. Malaria is endemic in most African countries and one of the major causes of sickness and death in sub-Saharan Africa. Hence, a major hurdle in public-health. In Nigeria, malaria is endemic and constitutes the major cause of death in children. Although it affects all ages, cases in children under the age of five are more likely to be serious, reflecting their relative low level of immunity to the disease compared with adults (Amodu *et al*., 2005). More than 80 percent of malaria cases are caused by *Plasmodium falciparum*, while the rest are caused by *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium vivax* or a combination. There is a wide variation in the clinical manifestation of the disease, ranging from asymptomatic malaria, mild/uncomplicated malaria and with the most severe forms characterized by life-threatening complications;

severe malaria, anaemia and cerebral malaria (Amodu *et al*., 2005).

In addition to its burden in terms of morbidity and mortality, the economic effects of malaria infection can be tremendous. These include direct costs for treatment and prevention, as well as indirect cost such as lost of productivity; time spent in seeking treatment and diversion of household resources. Some analysts have estimated the annual economic burden of malaria to be at least US$ 12 billion per year of direct losses, plus many times more than that in lost economic growth (WHO, 2010). This heavy toll can hinder economic and community development activities especially in malarious areas of the world.

Multi drug resistance has become one of the most important problems impeding malaria control effort (Htut, 2009). As such, there is need for continuous search for newer drugs that can retard or reverse this resistance. This has led to the increase in the search for antimalarial plants. Since many modern drug such as quinine and artemisinin originate from plants, it is imperative that other traditional medicinal plants which have antimalarial properties are investigated, in other to establish their safety and efficacy and to determine their potential as source of new antimalarial drugs (Gessler *et al*., 1994). *Azadirachta indica* commonly referred to as Neem plant and known locally as dongoyaro (Yoruba dialect in Nigeria) is one of the most commonly used and well known medicinal plants used in traditional medicine. All parts of the tree are said to have medicinal properties; seeds, leaves, flowers and bark are used for preparing many different medical preparations. Neem bark is cool, bitter, astringent and refrigerant. It is useful in cough, fever, skin diseases and diabetes (Odungbemi, 2006). This study therefore investigated the antimalarial activity of *Azadirachta indica* extracts on *Plasmodium berghei* and its effect on the infected albino mice.

MATERIALS AND METHODS

Sample collection

Azadirachta indica leaves were collected from the premises of The Federal University of Technology, Akure in Ondo State, one of the states in South Western Nigeria (7^o 10' 0" N and 5^o 5' 0" E). The identification of the leaves was done at the department of Crop, Soil and Pest management of the Federal University of technology, Akure, Ondo State.

Sample preparation

Physical selection of the leaves were done, washed with tap water, shade dried at room temperature to retain their active ingredients.

Preparation of plant extracts

The dried samples were powdered in a table model grinder (model PH2024 Binatone blender) before extraction. The powdered material was extracted using different solvents including; water, ethanol and methanol. One hundred and fifty grams of the grinded samples was weighed into cleaned plastic containers and 1000ml of each of the solvent was added and left for about 72 hours. The extracts were drained out, filtered with muslin cloth and concentrated in vacuum using rotary evaporator. The semisolid extracts were lyophilized to obtain solid extracts. The stock solution was prepared by dissolving 10g of the the powdered extracts with dimethylsulphoxide (DMSO) according to the method of Ogundare (2006).

Experimental animals

Twenty albino mice (Swiss albino) of both sex were obtained from Obafemi Awolowo University, Ile Ife, Osun State, Nigeria. The mice were bred in cages made of fabricated metal iron having a measurement of 100 x 11cm in the animal house of department of Microbiology, Federal University of Technology, Akure. The mice were divided into five groups of four each; each serving for group treated with ethanol extract of *Azadirachta indica*, group treated with methanol extract of *Azadirachta indica*, group treated with water extract of *Azadirachta indica*, positive control (Artemether) and negative control (distilled water). The animal house temperature was 28 ± 2^0C during the experiment. The body weight of all the mice ranged from 18 to 25g. They were fed with sterile mice feed and water and were allowed to acclimatize for one week, after which the weight of the animals was monitored and recorded during and after treatments.

Phytochemical screening

The leaf extracts were analyzed for the presence of alkaloid, saponin, anthraquinone, steroids, flavonoid, tannin, terpenoid, phlobatannin and

cardiac glycosides according to standard methods (Harborne, 1997).

Parasite inoculation

Donor mouse infected with *Plasmodium berghei* was obtained from Animal health department of Obafemi Awolowo University, Ile-ife, Osun State, Nigeria. Infected blood sample was diluted using phosphate buffer saline to preserve the parasites at 1:10. Inoculation of the mice was carried out according to David *et al*., (2004). Each mouse was infected intra peritoneally on the day one with 0.3 ml of infected blood containing 1 x 10^4 *Plasmodium berghei* parasitized red blood cells.

Evaluation of malaria parasite

After 72 hours of inoculating the mice, their parasitemia level was evaluated by preparing thick blood film. This was done by collecting blood by transaction of the distal end of the tail, with sterile scissors. Three drops of the blood samples were placed on a clean slide, a thin blood film was made. These films were air dried, fixed in methanol for 30 seconds and stained with Gimsa stain for about 2-4 minutes (Cheesebrough, 2014). The slides were thoroughly rinsed under running tap and left to dry. The prepared slides were viewed under x 100 objective oil immersion light microscope. Parasitemia level was determined using the formula below:

$$\text{Parasitemia} = \frac{\text{Total number of parasitized red blood cell count}}{\text{Total number of red blood cell count}} \times 100$$

Administration of treatments

Treatments were administered orally through intra gastric route using the stomach tube to ensure the safe ingestion of the treatment doses to the five tested groups for five consecutive days after 72 hours of infection. The groups treated with *Azadirachta indica* extracts were orally inoculated with 100mg/kg and 200mg/kg while the positive control group was administered 5mg/kg and the negative control group was given 0.2 ml of distilled water. On the third and fifth day, blood smear was prepared and the number of parasites was counted. The percentage suppression of parasitemia was calculated in each group using the following expression;

$$\text{Average suppression} = \frac{TC - TE}{TC} \times 100$$

Where TC=Average % parasitemia in the control group, TE Average % parasitemia in treated group.

Statistical analysis

Data obtained were subjected to analysis of variance (ANOVA) using the Statistical Package for Social Sciences, Version 15.0 (SPSS, 2003). Treatment means were separated using the Duncan Multiple Range Test (Zar, 1984).

RESULTS

The result of the phytochemical analyses revealed the presence of saponin, tannin alkaloid, flavonoid etc as shown in table 1. The result of the body weight of the albino rats showed that the infection caused a decrease in the weight of the animals. There was however increase in the weight as treatment began, an indication that the treatment caused recuperation in the animals (table 2). Table 3 shows the result of the haematological analyses before infection. Group 1 has the highest mean PCV of 38.5%, while group 4 has the least (32.5%). This is to ascertain the effect of the parasites when the infection set in. During infection, the haematological parameters were generally low. For instance, the PCV of group 1 reduced to 28.5% as shown in table 4. This is to ascertain the effect of the parasites after the infection set in check the extent of damage caused by the parasites to the blood parameters. Table 5 showed the increase recorded in the haematological parameters as treatment was administered. The increase in the parameters is an indication that treatment the rats were subjected to after infection set in caused a recuperative effect in them. This analysis is to confirm the therapeutic effect of the plant extract on the mice. Table 6 show the general antiplamodial activity of the extract as the analyses progresses.

Table 1: Phytochemical screening of *Azadirachta indica* extracts

Plant extracts	Phytochemical components						
	Saponin	Tannin	Terpenoid	Phlobatannin	Alkaloid	Anthraquinone	Flavonoid
Ethanol	+	+	+	-	+	-	+
Methanol	+	+	+	-	-	-	+
Water	+	+	+	-	-	-	+
	Cardiac glycosides						
	Legal test		Killer killiani		Lieberman's test		Salkowski
Ethanol	+		+		+		+
Methanol	+		+		+		+
Water	+		+		-		+

Table 2: Body weight of the experimental animals

Groups	Weight (g) before infection Mean±SE	Weight (g) during infection Mean±SE	Weight (g) after treatment Mean±SE
Group 1	22.05± 2.21^{c}	18.08± 1.82^{b}	16.36±2.21^{a}
Group 2	21.20± 1.83^{b}	17.21±1.92^{a}	20.92±2.61^{b}
Group 3	22.08± 2.50^{c}	17.21± 2.51^{a}	20.75 ±0.42^{b}
Group 4	22.14 ± 1.20^{c}	17.01 ±2.50^{a}	19.43 ±2.01^{b}
Group 5	20.16 ± 2.01^{c}	16.25 ±1.13^{a}	18.85 ± 0.58^{b}

Values followed by the same letter in a row is not significantly different at P>0.05.

Legends

Group 1 – Negative control treated with distilled water

Group 2 - Positive control treated with Artemether

Group 3 (AIE) – Group treated with ethanol extract of *Azadirachta indica*

Group 4 (AIM) – Group treated with methanol extract of *Azadirachta indica*

Group 5 (AIW) – Group treated with water extract of *Azadirachta indica*

Table 3: Hematological parameters before infection

GROUPS	PCV (%)	RBCx10^{3}mm^{3}	WBC	Hb(g/100ml)	LYMPH (%)	EOSIN (%	NEU (%)	MONO (%)	BASO (%)
Group 1	38.5±1.29	371.3±0.05	152.83±0.42	12.83±0.40	63.5±2.87	2.5±0.58	26.0±3.74	7.25±0.5	0.75±0.6
Group 2	36.0±2.45	372.5±0.45	134±0.82	12±0.82	64.0±2.94	3.25±0.96	25.5±2.38	6.0±2.45	0.75±0.5
Group 3	33.75±1.89	389.3±2.3	148.90±0.62	11.9±0.62	63.0±2.16	2.0±0.82	29.25±2.06	5.25±1.26	0.75±0.5
Group 4	32.5±1.91	375.1±4.32	131.18±0.62	11.18±0.62	67.25±1.71	2.5±0.58	23±1.41	6.0±2.45	0.25±0.5
Group 5	35.75±3.59	377.6±1.5	160.75±0.66	11.75±0.66	63.25±1.71	2.5±.0.58	27.5±1.29	6.25±0.95	0.5±0.58

Each value represents the Mean ± S.E

Table 4: Hematological parameters during infection

GROUPS	PCV (%)	RBCx10^{3}mm^3	WBC	Hb (g/100ml)	LYMPH (%)	EOSIN (%)	NEU (%)	MONO (%)	BASO (%)
Group 1	28.5±1.02	324.1±0.05	104.51±0.42	9.24±0.40	45.5±2.57	1.78±0.80	20.0±0.45	5.25±0.5	0.65±0.6
Group 2	30.0±1.10	325.5±0.05	109.0±0.62	9.0±0.82	59.0±2.94	2.20±0.41	20.5±1.08	5.0±1.29	0.69±0.5
Group 3	29.22±1.89	322.3±2.3	104.0±0.42	8.7±0.62	57.0±1.07	1.80±0.82	22.29±1.03	5.0±1.26	0.65±0.5
Group 4	25.5±0.21	322.0±4.32	102.09±0.60	9.16±0.72	54.25±1.71	1.67±0.48	22.0±0.22	4.82±0.04	0.50±0.5
Group 5	29.75±0.26	329.6±0.87	105.75±0.43	9.34±0.75	53.16±2.71	1.5±.0.30	19.5±1.08	4.11±0.57	0.54±0.50

Each value represents the Mean ± S.E

Table 5: Hematological parameters after treatments

GROUPS	PCV (%)	RBCx10^{3}mm^3	WBC	Hb(g/100ml)	LYMPH(%)	EOSIN(%)	NEU(%)	MONO(%)	BASO(%)
Group1	24.5±1.04	311.1±0.09	88.83±0.42	7.83±0.40	45.5±0.55	2.5±0.58	26.0±3.45	7.25±0.5	0.42±0.6
Group 2	32.05±1.99	368.5±0.02	129.0±0.82	11.8±0.82	52.0±2.00	3.25±0.96	25.5±2.38	6.0±2.45	0.63±0.5
Group 3	31.75±1.80	358.3±0.40	124.98±0.62	11.4±0.62	58.0±0.60	2.0±0.82	29.22±2.06	5.25±1.26	0.60±0.5
Group 4	30.67±0.50	352.0±1.20	118.18±0.62	10.18±0.62	67.25±1.71	2.5±0.58	23.0±1.41	6.0±2.45	0.58±0.5
Group 5	30.90±0.59	348.6±0.60	115.75±0.66	10.90±0.32	63.25±1.71	2.5±.0.58	27.5±1.29	6.25±0.95	0.60±0.58

Each value represents the Mean ± S.

Table 6: Antiplasmodial activity of the extracts during treatment

Treatments (Drug/extracts)	Pre-parasitemia count	Dosage(mg/kg)	Third day		Fifth day	
			Post treatment parasitemia	Average suppression	Post treatment parasitemia	Average suppression
Distilled water (negative control)	24^a±0.2	0.2ml	40^b ±2.65	0.00	62.67^c ±3.05	0.00
Artemether (postive control)	21^b±0.6	5	7.5^c±0.00	81.6	0±0.0	100
AIE	20^c±0.6	100	10^a ±0.33	75	1^b ±0.43	98.4

		200	$4^{c} \pm 0.76$	90	$0.54^{c} \pm 0.04$	99.03
AIM	$22^{c} \pm 0.4$	100	$12.0^{a} \pm 2.43$	72.5	$2.78^{b} \pm 0.08$	95.55
		200	$6.0^{c} \pm 0.78$	87.5	$1.80^{c} \pm 0.17$	97.09
AIW	$21^{b} \pm 0.6$	100	$11.6^{a} \pm 2.34$	70	$4^{b} \pm 0.22$	93.54
		200	5.23^{c} 0.16	85	$2^{c} \pm 0.22$	96.77

The mean values with the same letter within the same row are not significantly different from each other at $P<0.05$

Legend

AIE - Group treated with ethanol extract of *Azadirachta indica*

AIM – Group treated with methanol extract of *Azadirachta indica*

AIW– Group treated with water extract of *Azadirachta indica*

DISCUSSION

The phytochemical screening of the different extracts of *Azadirachta indica* leaves indicated the presence of some secondary metabolites (Table 1). There was variability in the secondary metabolites in different extracts. More secondary metabolites were identified from ethanol extracts. This include saponnin, tannin, alkaloid, steroid, terpenoid. Our findings is in agreement with Nwanjo *et al*, (2006), while anthraquinone and phlobatannin were absent in all the extracts tested. The cardiac glycosides test results for legal test, keller killiani test and salkowski test were present in all the extracts.

The mean body weight of the mice ranged from 20.16 ± 2.01 to 22.14 ± 1.20 before infection. There was a general decrease in the body weight during infection while during treatment there was an increase in the body weight except in the negative control. The increment was pronounced in the treatment group 2 and group 3 (Table 2). This observation could be due to increase in the feeding rate of the mice during treatment with leaf extracts of *Azadirachta indica*. Badam *et al*. (1999) reported that *Azadirachta indica* has the ability to lower the fever and increase one's appetite, enabling a stronger body to fight the malaria parasites and therefore hasten the recovery rate. Tables 3, 4 and 5 showed variation in the Haematological parameters of the infected albino mice before, during infection and after treatment. The result showed gradual reduction in the RBC, PCV, WBC, hemoglobin counts and the differential counts during infection. The decrease in values was high in group 1 followed by group 5 while a slight difference was noticed between group 2 and group 3. After treatment increment in the haematological parameters was also recorded in all the groups except in group 1 where all the values decreased. The ability of *Azadirachta indica* to boost the body's macrophage response, which stimulate the lymphocytic system and increase the production of white blood cells is believed to be a major factor in *Azadirachta indica* effectiveness against malaria infection (Saxena *et al*., 2003).

The Antiplasmodial activity of the extracts showed that the 200 mg/kg ethanol extract was not statistically different from the positive control (artemether) while the negative control group was significantly different from other treatments. Also there was significant difference in the parasitemia level on the day 3 and day 5. The average suppression of *Azadirachta indica* is dose dependant. Highest average suppression recorded was in 200 mg/kg ethanol extract of *Azadirachta indica* at day 3 and day 5 (90% and 99.03% respectively). And 75% and 98% average suppression was recorded in 100 mg/kg ethanol extract of *Azadirachta indica* at day 3 and day 5 respectively (Table 6). Some studies have reported that both the water, ethanolic and methanolic extracts of the plant are proven effective against malaria parasite (Abatan and Makinde, 2006). The suppression ability of neem plant confirmed the reason why it is used traditionally in the treatment of malaria and fever. This study therefore conclude that the water, ethanol and methanol extracts of *Azadirachta indica* have shown decrease in parasitemia level of *Plasmodium berghei* infected albino mice in a dose related manner. However the toxicity assessment of over dosage of the plant extracts is an area under research.

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