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AN *IN-VIVO* EVALUATION OF ANTIPLASMODIAL ACTIVITY OF AQUEOUS AND ETHANOLIC LEAF EXTRACTS OF AZADIRACHTA INDICA IN PLASMODIUM BERGHEI INFECTED BALB/c MICE

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ABSTRACT

Malaria remains one of the most prevalent infections in the tropical regions of the world. The increased resistance of the parasite to many available antimalarials backs the need to develop novel antimalarial drugs with effective mode of action. Several plants with antiplasmodial properties have been proved as sources for novel antiplasmodial compounds. *Azadirachta indica* has widely been reported for its medicinal properties. The leaf extract is used in folklore medicine to treat malaria. Previous *in vitro* studies has shown that the leaf extract of *A. indica* possess antiplasmodial properties. In the current research, the antiplasmodial activity of both aqueous and ethanolic leaf extracts of *A. indica* (ALEAI and ELEAI respectively) were studied *in vivo* using *Plasmodium berghei* infected BALB/c mice at 50, 100 and 200mg/kg/day dosages. The extracts were also screened for phytochemicals using standard methods. Preliminary phytochemical screening revealed the presence of alkaloids, saponins, tannins, reducing sugars, flavonoids and polyphenols in both extracts. Both ELEAI and ALEAI demonstrated significant antiplasmodial activity *in vivo* against *plasmodium berghei* in a dose-dependent manner. During early infection, oral administration of 50, 100 and 200mg/kg/day dosages of ELEAI caused chemosuppression of 56.96, 63.15 and 69.60% respectively on day four and a chemosuppression of 69.65, 75.76 and 78.32 % respectively on day six. Similar dosages of ALEAI respectively caused chemosuppression of 56.96, 59.89, 69.49% on day four and 64.42, 70.23 and 77.41% on the sixth day. These values were statistically significant ($P < 0.001$) as compared to negative control. The LD_{50} of the ELEAI was found to be greater than 1g/kg body weight of naive mice. Results from the present study therefore confirm that *A. indica* leaf contains active antiplasmodial compounds and therefore can be very useful in the search for new antimalarial drugs.

INTRODUCTION: Malaria is a major disease in tropical climate with high mortality rate. Despite recent advances in the development of a wide range of antimalarials, the disease is emerging as the greatest threat to the people of tropical developing countries. According to the WHO, over 300 million individuals are affected with malaria annually with the death toll ranging between 1 and 1.5 million annually [1]. About 90% of malaria deaths in the world today occur in Africa, South East Asia and South America.

Malaria is commonly coursed by a bite from a parasite-infected mosquito. There are five species of *Plasmodium (P.)* parasites that infect people. These include *P. falciparum* which is mostly found in the tropics and sub-tropics, *P. vivax* and *P. malariae* which occur all over the tropical regions of the world, *P. ovale* found in western Africa and *P. knowlesi* found in Southeast Asia. Of the several parasites, only infections with *P. falciparum* and *P. knowlesi* can lead to life-threatening complications with *P. falciparum* being the most drug-resistant species. Effective control of the disease has been hampered by the complexity of the life cycle of the parasite, absence of vaccines, drug persistence and non-availability of prophylactic drugs [2].

The increased resistance of *P. falciparium* to limited range of antimalarial drugs in use is reducing the therapeutic arsenal for treatment of malaria at the rate that is barely balanced by the development of novel effective drugs[3]. Further to this, the unavailability of newer antimalarial drugs to the most vulnerable population coupled with high cost of available drugs prompts the need for a search on alternative, cheap and accessible remedies. In addition, the increased resistance of the parasite to many available antimalarials backs the need for new antimalarial drugs with novel mechanisms of pharmacological action.

The success of artmesinin isolated from *Artemisia annua* and its derivatives for the treatment of malaria has focused attention on plants as sources of antimalarials drugs[4]. The use of herbal remedies for malaria treatment is endemic in many African countries. A cross section of both rural and urban dwellers, literate and illiterate in many African countries rely massively on herbal preparations for the treatment of fevers suspected to be malaria despite the existence of orthodox antimalarial drugs. In spite of the wide use of herbal medicines for the treatment of malaria, only a small number of plant species have been studied and evaluated scientifically for possible antimalarial activities.

Azadirachta indica, commonly known as *neem* is a tree in the family *Meliaceae*. It is one of the two species in the genus *azadirachta* and is native to India, Burma, Malaysia with others growing in tropical and semi-tropical regions. Various parts of the plant have been reported for their pharmacological activities. The medicinal utilities have been described, especially for leaf, fruit and bark [5]. Several compounds with demonstrated pharmacological activities have also been isolated from the plant. In folklore medical practices, the leaf decoctions are used in the treatment of malaria and this has been confirmed by several *in vitro* laboratory studies using microbes [6]. Neem extracts have also shown potential hypoglycaemic properties [7]. A leaf extract was found to lower raised levels of serum liver enzyme and paracetamol induced liver necrosis. Bhanwra *et al*. [8], observed sedative properties of *A. indica* leaf extracts *in vivo.*

Hydroalcoholic leaf extract of *A. indica* caused a dose-dependent hypotensive effect [9] and oral administration of low doses (10-200mg/kg) of neem leaf extract showed anxioltyic effect comparable to that induced by diazepam [10]. Anti-flammatory properties of the plant have also been demonstrated in various studies. The water soluble part of the alcoholic leaf extract shows antiflammatory activity in the cotton pellet granuloma assay *in vivo* [11]. The antimalarial activities of *neem* seed and leaf extracts have been studied *in vitro* using malaria parasites [12, 13]. Components of the alcoholic extracts of leaves and seeds have been found to be effective *in vitro* against both chloroquine-resistant and sensitive strains of malaria parasite [14].

Recent investigations have shown that *neem* seed extract and its purified fractions inhibit the growth and development of asexual and sexual stages of drug-sensitive and resistant strains of the human malarial parasite *P. facliparium*[15]. Despite the ever-increasing reports with regards to the antimalarial potentials of extracts of *neem* parts against known parasites, information in respect of *in vivo* evaluation of the leaf extract for its growth inhibitory activity against *Plasmodium (P.)* has been limited and scanty. The present research thus, seeks to screen the leaf extracts of *A. indica* for phytochemicals. The study will also evaluate antiplasmodial activities of both aqueous and ethanolic leaf extracts of *A. indica in vivo* using *Plasmodium berghei*-infected BALB/c mice.

The result may provide a basis for the identification of potential antiplasmodial compounds and the development of effective novel antimalarial drugs from *A. indica* leaf.

MATERIALS AND METHODS:

Reagents and solvents: All reagents and solvents used were of analytical grade unless otherwise stated. Giemsa stain, methanol and sodium chloride were obtained from British Drug House (BDH) Poole, England. Chloroquine phosphate was obtained from Troge Medical GmBH (Humburg Germany).

Plant material: *A. indica* leaves were obtained from the Plant Production Department of the Centre For Scientific Research Into Plant Medicine (CSRPM) at Mampong-Akuapem, Ghana and authenticated by Dr. Yaw Ameyaw, a botanist of the production department.

Animals: Seven-week old female BALB/c mice (30g average weight) were obtained from the Animal Unit of the CSRPM. The animals were fed on powdered feed obtained from Ghana Agro Food Company (GAFCO), Tema, Ghana. They were allowed free access to sterile distilled water.

Preparation of Extracts: To about 500g of powdered air-dried *A. indica* leaves was added 5 litres of distilled water. The mixture was boiled for 45 minutes, sieved through a wire mesh and allowed to cool. The extract was freeze dried and stored in a cool dry place. This was reconstituted in sterilized distilled water before use. To another 500g powdered sample of the leaves of *A. indica* was added 5 litres of 95% ethanol. This was mechanically shaken for 24 hours and sieved through a wire mesh. The extract was freeze dried and store in a desiccator.

Malaria Parasites: *Plasmodium berghei* NK 65 strain from the University of Copenhagen, Denmark and maintained for more than 20 years in liquid nitrogen with occasional passage in BALB/c mice in the Department of Immunology, Noguchi Memorial Institute of Medical Research (NMIMR), University of Ghana, was used for the experiment.

Inoculum Preparation: A stock of parasitized erythrocytes was obtained from infected BALB/c mice, with a minimum peripheral parasitemia of 20% by cardiac puncture in heparin-coated tube. The cell concentration of the stock was determined and diluted with physiological saline such that 0.2ml of the final inoculums contained 10^6 parasitized red blood cells (RBCs).

Acute Toxicity Study: The acute toxicity of 95% ethanolic extract of *Azadirachta indica* was determined as per the OECD guideline no. 423 (Acute Toxic Class Method). It was observed that the test extract was not mortal even at 1000mg/kg dose. Hence $1/20^{th}$ (50mg/kg), $1/10^{th}$ (100mg/kg) and $1/5^{th}$ (200mg/kg) of this dose were selected for further study[16].

Treatment of Animals: Forty-five (45) mice were selected and put into five (5) groups of nine mice per group. Each mouse was inoculated intraperitonaelly with the parasite. Group 1 (Gp1) animals received 0.2ml/mouse of distilled water (negative control), group 2 (Gp 2) animals received 5mg/kg Artemeter (Art.) positive drug control, group 3 (Gp 3) animals received *Cryptolepis sanquinolenta* (C.Sa.) positive herbal control, group 4 (Gp 4) animals received ethanolic leaf extract of *A. indica* (ELEAI) and group 5 (Gp 5) animals received aqueous leaf extract of *A. indica* (ALEAI). Within each group, the animals were further divided into three sub-groups of three. The sub-groups within the groups were given different treatment dosages (50, 100 and 200mg/kg/day) respectively for six days. The drugs were administered orally. Blood counting was done for the fourth and sixth day after drug administration.

Monitoring of Parasitemia and Antiplasmodial Activity: Twenty-four hours after last drug administration, thin blood smears were prepared using blood from the tail vein of each mouse. Each smear was air-dried, fixed in methanol, air-dried again and stained with Geimsa for 10 minutes and then examined under the microscope. The slides were observed under oil immersion. Each slide was observed at three different fields and the parasitized red blood cells (RBCs) and total number of RBCs for each field was recorded.

$$\% \text{ Parasitemia} = \frac{\text{Total number of parasitized RBCs counted}}{\text{Total number of RBCs counted}} \times 100$$

$$\text{Activity (\%Chemo - suppression)} = \frac{\text{Parasitemia of control} - \text{Parasitemia of test}}{\text{Parasitemia of control}} \times 100$$

Phytochemical Screening of *Azadirachta indica* extracts: The aqueous and the ethanolic leaf extracts of A. indica and Cryptolepis sanquinolena were screened for the presence of groups of phytochemicals according to standard methods [17, 18].

Statistical analysis: Values are expressed as means±SEM and the data were statistically analyzed using student's t-test and $P < 0.05$ was considered significant.

RESULTS AND DISCUSSIONS: Medicinal plants have been the source of hope for treatment of various ailments since time immemorial. Leaf extracts of *Azadirachta indica* have been used in folklore medical practices for the treatment of malaria. Phytochemical screening of both ethanolic and aqueous leaf extracts of *A. indica* revealed the presence of alkaloids, saponins, tannins, flavonoids and polyphenols (**Table 1**). Although the mechanism of action of these secondary metabolites has not been studied in the present work, reports have shown that some of these secondary metabolites have demonstrated antiplasmodial activity either by elevating red blood cells oxidation or by inhibiting protein synthesis [19].

TABLE 1: PHYTOCHEMICAL CONSTITUENTS OF AQUEOUS AND ETHANOLIC LEAF EXTRACTS OF *A. INDICA*

	ELEAI	ALEAI
Alkaloids	+	+
Saponins	+	+
Tannins	+	+
Reducing sugars	+	+
Steroids	-	-
Terpenoids	-	-
Flavonoids	+	+
Anthraquinones	-	-
Phlobatannins	-	-
Polyuronides	-	-
Polyphenols	+	+
Cyanogenic glycosides	-	-

- = absence of secondary metabolite; + = presence of secondary metabolite

Thus the active involvement of these phytochemicals in the plant extracts antiplasmodial activity cannot be overemphasized. The LD_{50} of the ELEAI was found to be greater than 1g/kg body weight of naive mice and this proves the clinical safety of the extract.

Both ELEAI and ALEAI given orally at doses of 50, 100 and 200mg/kg significantly exerted *in vivo* antiplasmodial activity on the *Plasmodium berghei* induced in female BALB/c mice in a dose-dependent fashion (**Table 2**). The result supports the traditional use of the *A. indica* extracts in malarial treatment.

TABLE 2: ANTIPLASMODIAL ACTIVITY OF AQUEOUS AND ETHANOLIC LEAF EXTRACTS OF *A. INDICA*

Drug/extract	Dose	Fourth Day		Sixth Day	
	(mg/kg/day)	Average % parasitemia	Average % suppression	Average % parasitemia	Average % suppression
ELEAI	50	33.18±0.97	56.96±2.72	25.38±0.57	69.65±0.66
	100	28.25±1.05	63.15±1.66	20.27±1.47	75.76±1.72
	200	23.31±1.04	69.60±1.09	18.13±3.68	78.32±3.31
ALEAI	50	33.00±2.36	56.96±1.90	29.75±2.97	64.42±3.48
	100	30.75±2.02	59.89±1.91	24.89±0.22	70.23±0.26
	200	23.39±1.87	69.49±3.98	18.89±2.15	77.41±2.52
C.Sa	50	33.81±0.15	55.90±0.87	27.33±3.26	67.32±3.82
	100	30.33±0.27	60.44±1.07	20.76±0.90	75.17±0.89
	200	25.69±0.39	66.49±1.90	18.39±1.56	78.01±1.23
Artemether	5	20.33±0.90	73.48±2.89	11.06±0.30	86.77±2.83
Water (control)1	0.2ml	76.68±0.83	-	83.63±1.37	-

Data expressed as mean±SEM. n = 3.

During early infection, oral administration of 50, 100 and 200mg/kg/day dosages of ELEAI caused chemosuppression of 56.96, 63.15 and 69.60% respectively during the fourth day and a chemosuppression of 69.65, 75.76 and 78.32 % respectively on the sixth day. At similar dosages, ALEAI respectively caused chemosuppression of 56.96, 59.89, 69.49% on the fourth day and 64.42, 70.23 and 77.41% on the sixth day. These values were statistically significant ($P < 0.001$) as compared to negative control. The antiplasmodial activity of both ELEAI and ALEAI were comparable to those of *C. sanquinolena* which was used as one of the positive controls. The standard drug Artemether (5mg/kg/day) caused 73.48 and 86.77% chemosuppression on the fourth and sixth day respectively. Although Artemether showed superior antiplasmodial activity, the result suggests that the leaf extracts of *A. indica* are potential candidates in the search for novel antimalarial drugs.

CONCLUSION: The problem associated with increased resistance to classical drugs and the problem of recrudescence of artemisinin necessitates the need to look for new antimalarial agents [20]. Leaf extracts of *Azadirachta indica* has been used for the treatment of malaria in traditional medical settings. In the present study, both ethanolic and aqueous leaf extracts of *A. indica* have demonstrated antiplasmodial activity *in vivo* against *plasmodium berghei induced* in BALB/c mice (50, 100 and 200mg/kg) in a dose-dependent manner. The antiplasmodial activity of both extracts was found to be statistically significant compared with the negative control. The antiplasmodial activities of the extracts were also comparable to the antiplasmodial activity of the standard antimalarial herb *C. sanquinolenta*.

Result from the present study supports the use of leaf extracts of *A. indica* in traditional medical practices for malaria treatment. It is also in agreement with previous *in vitro* antiplasmodial studies on the leaf extracts [14]. In this research, we conclude that leaf extracts of *A. indica* contain active antiplasmodial compounds and therefore can be very useful in the search for new antimalarial drugs. We however recommend a further study on repository activity and mean survival time (MST).

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