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Evaluation of ethanol extract of *Artemisia maciverae* aerial part for antiplasmodial activity in mice

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Abstract

Malaria is a life-threatening disease with increased mortality rate among infants of less than five years of age, non-immune travellers and pregnant women especially in sub-Saharan Africa. In Nigeria, the most common causative malaria parasite, *Plasmodium falciparum* rapidly develops resistance to most classes of conventional drugs and this has led researchers to source for new antimalarial drugs from different sources, including higher plants. The causative malaria parasite *Plasmodium falciparum* rapidly develops resistance to most classes of conventional drugs and this has led researchers to source for new antimalarial drugs from different sources, including higher plants. A decoction of *Artemisia maciverae* Linn. (Asteraceae) aerial part is used for the treatment of malaria in some parts of Northern Nigeria. The aim of the study was to evaluate the oral acute toxicity and *in vivo* antiplasmodial effect of an ethanol extract of *Artemisia maciverae* aerial part. Oral acute toxicity of the extract was evaluated in Swiss albino mice using modified Lorke's method and the *in-vivo* antiplasmodial effect against early, established and residual infections in chloroquine-sensitive *Plasmodium berghei berghei* NK65- infected Swiss albino mice. Chloroquine (5 mg/kg) and pyrimethamine (1.2 mg/kg) were used as positive controls. The oral median lethal dose of *A. maciverae* in mice was determined to be greater than 5000 mg/kg body weight. The extract at the doses (25, 50 and 100 mg/kg^{-1} orally) used produced significant ($P< 0.0001$), dose-dependent effect against the parasite in the suppressive, curative and prophylactic tests. The extract showed the highest *in-vivo* antiplasmodial activity at 100 mg/kg^{-1}. The observed effects were also comparable with those produced by the reference drugs used as controls. *Artemisia maciverae* extract is practically non-toxic following acute, oral administration and possesses antiplasmodial effects. This suggests that it may be considered for further development as a safe and effective anti-malaria phytomedicine.

Key words: *Artemisia macivera, Plasmodium berghei berghei*, antimalarial activity.

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Introduction

Malaria is an increasingly worldwide deadly disease and Africa bears the greatest burden. According to recent World Health Organization reports, an estimated 212 million cases of people were infected with malaria in 2015 alone and an estimated 429 000 malaria – associated deaths globally [1]. Malaria remains a major killer among infants of less than 5 years of age, non-immune travellers and pregnant women, probably due to progressive spread of *Plasmodium falciparum* resistance to the most commonly used and affordable anti-malarial drugs such as chloroquine [2]. Malaria in tropical developing countries is seen as one of the major causes of illness and death. Malaria is a life-threatening disease caused by various species of *Plasmodium* parasites *(P. falciparum, P. ovale, P. vivax and P. malariae),* transmitted via the bites of infected female Anopheles mosquitoes and this transmission aids the rapid spread of the disease [3, 4]. Recently, drug resistance is extending to new geographical areas and affecting other species [2, 5]. This situation is further worsened by the circulation of fake and sub-standard anti-malarial drugs particularly in Nigeria alongside other African and Asian nations [4, 6]. Most people in developing countries use traditional medicines for the treatment of malaria because it is affordable, easy to access and this practice has led researchers to determine the efficacy of these medicinal plants [3, 7]. Medicinal plants have in the past been the source of some of the most successful anti-malarial agents such as the quinolines and the endoperoxides/artemisinin derivatives [8, 9]. Artemisinin isolated from *Artemisia annua* has demonstrated greater success in the treatment of malaria when compared with all known anti-malarial drugs that have showed resistance [10].

Although Africa has an abundance of flora, their potential as sources of malaria remedies or lead compounds for anti-malarial drugs has not been sufficiently explored, partly due to lack of scientific validation of traditional medicinal uses [9]. *Artemisia maciverae* Linn. is a small herbaceous plant belonging to the family *Asteraceae*, commonly found among the Hausa speaking people of the northern Nigeria and known locally as '*Tazargade*'. A decoction of the whole plant material is prepared locally by boiling in a mixture of water, lemon and red gypsum or soaked with locally made gin for the treatment of malaria [7]. This plant has been reported to have anti-malaria and anti-trypanosomal activities [9, 11]. It is also rich in phytochemicals such as flavonoids, triterpenes, phlobatannins, tannins, anthraquinones, steroids, saponins and alkaloids [12]. The acclaimed efficacy of *Artemisia maciverae* in traditional medicine makes it a popular home remedy for malaria in Northern Nigeria. There is limited documented literature on the safety and toxicity of *A. maciverae*. Ene et al. [13, 14] and Atawodi et al. [15] reported on the safety profile of sub-chronic administration of chloroform extract of *A. maciverae* on major organs and blood cholesterol level. Therefore, this present study is aimed at investigating *A. maciverae* whole plant extract for its acute toxic effects following oral administration and antiplasmodial effects in murine models of malaria infection.

Materials and Methods

Chemicals and Test Agents

Ethanol and chloroquine were purchased from Sigma-Aldrich representative in Nigeria (Zayo International Ltd, Jos, Nigeria). Pyrimethamine was purchased from a pharmacy store.

Plant material

Powdered whole plant of *A. maciverae* used for this study was purchased by an ethno botanist, Mallam Muazzam, from a market in Kano state, Nigeria. The powder was stored in an air tight container and kept in a cool place.

Extraction of Plant Material

Two hundred grams (200g) of powder plant material was weighed and soxhlet-extracted with 70%v/v ethanol for 48 hrs. The extract obtained was evaporated to dryness on a hot water bath at to obtain the dry extract. The extract was freshly reconstituted in aqueous tragacanth (1%w/v) prior to each experiment.

Animals

Swiss albino mice (18 - 22 g) of both sexes obtained from Animal Facility Centre, National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria were used for the study. They were housed in polypropylene cages, and given standard laboratory diet and water *ad libitum* and maintained under laboratory conditions of temperature (22 ± 1°C), relative humidity (14 ± 1%) and 12 h light and 12 h dark cycle. The study was carried out in accordance to the NIH guide for use of animals [16] and NIPRD standard operating procedures.

Rodent parasite (*Plasmodium berghei berghei NK65*)

The rodent parasite was sourced from National Institute for Medical Research (NIMR), Lagos, Nigeria and maintained in NIPRD by continuous intra-peritoneal passage in mice [17]. Prior to the start of the study, one of the infected mice was kept and observed to reproduce signs similar to human malarial infection [18].

Method

Acute toxicity study (LD_{50})

The oral acute toxicity of the extract was carried out in mice using modified biphasic method as described by Lorke [19]. In the phase one, nine mice were randomized into three groups of three mice each and were given 10, 100 and 1000mg/kg^{-1}body weight (b.wt.) of the extract orally; the mice were observed for signs of toxicity including paw-licking, stretching, respiratory distress and mortality for the first critical 4 h and subsequently for 14 days. In the second phase of the study, another nine set of fresh mice were randomized into three groups of three mice each and were given 1600, 2900 and 5000mg/kg^{-1}b.wt of the extract orally based on the result of the first phase. They were again observed for signs of toxicity and mortality for the first critical 4h and subsequently for 14 days. The oral median lethal dose (LD_{50}) was calculated as the square root of the product of the lowest lethal dose and highest dose that produced nil mortality i.e. geometric means of doses of the maximum dose producing 0% and the minimum dose producing 100% mortality:

LD_{50}= √minimum toxic dose × maximum tolerated dose

Antiplasmodial studies

Parasite Inoculation

The inoculum consisted of approximately 5 × 10^7 of *Plasmodium berghei berghei* parasitized erythrocytes per ml. This was prepared by obtaining from a donor mouse, 2 ml of infected blood which was diluted in 5 ml of normal saline. Each study mouse was inoculated intraperitoneally with 0.2 ml of infected blood.

Evaluation of extract in early malaria infection (4-day Suppressive Test)

The 4-day suppressive test model was adopted [20]. Thirty adult Swiss albino mice

of either sex were inoculated as described above. These mice were randomly divided into 5 groups of 6 mice per group and treated orally for 4 consecutive days. Group I mice were given 10ml normal saline/kg body weight. Groups II, III and IV were given 25, 50 and 100mg extract/kg body weight respectively, while group V mice received 5mg chloroquine/kg body weight daily respectively. On day 5 of the experiment, blood was collected from the tail of each mouse and smeared on to a microscope slide to make a thin film. The blood films were stained with10% Giemsa at pH 7.2 for 10 minutes and examined microscopically. The number of parasites per field was counted for ten fields on each slide. The percentage (%) suppression of parasitaemia for the each group was calculated as:

% suppression= 100 – [(mean parasitemia treated/mean parasitemia control) × 100].

Test in established infection (Rane test)

Evaluation of the curative potential of *A. maciverae* was carried out as described by Ryley and Peters [21]. On day 0, thirty adult mice were inoculated as described above and left untreated. Three days later, the mice were randomized into five groups of six mice each. Group I mice were given 5 ml normal saline/kg body weight orally. Groups II, III and IV were treated with 25, 50 and 100 mg extract/kg/day orally respectively, while group V mice received 5 mg chloroquine /kg daily orally for 5 days. On each day, about 3 drops of blood were collected from the tail of each mouse smeared unto a microscope slide to make a thin film, stained with 10% Giemsa stain and examined microscopically to monitor the parasitaemia level. The average percentage (%) parasitaemia was evaluated for each of the doses using the formula above.

Test for repository antiplasmodial activity

The prophylactic activity of the extract was tested using the residual infection procedure [22]. Adult mice of both sexes were weighed and randomized into five groups of six mice each. Group I mice were given 10ml normal saline/kg body weight. Groups II, III and IV were given 25, 50 and 100 mg extract/kg body weight orally respectively while group V mice received 1.2mg pyrimethamine/kg body weight orally daily for 5 days. After treatment on the fifth day, all the mice were inoculated with standard inoculum containing approximately 0.1×10^7 *P. berghei berghei* NK 65 infected erythrocytes. Thin films of blood smears were then made from each mouse 72 h after last treatment, examined microscopically for parasitaemia level and mean parasitaemia level determined for each group.

Statistical analysis

Results obtained were expressed as mean ± standard error of mean. Graph pad prism version 5.02 was used for data analysis. The differences between means were compared using one way analysis of variance (ANOVA) followed by Dunnet's post hoc test. Differences in group mean values relative to the control were considered significant at $p \leq 0.05$.

RESULTS

Acute toxicity study

The physical signs of toxicity observed included excitation, paw lick and stretching at 5000 mg/kg. No mortality was recorded. The oral median lethal dose (LD_{50}) was estimated to be ≥ 5000 mg/kg b. wt.

Antiplasmodial suppressive activity of the extract

The extract significantly ($P < 0.0001$) decreased the parasite count in treated mice when compared with the normal saline treated mice. The average percentage

inhibition of parasitaemia produced was 27, 43 and 73% at the doses of 25, 50 and 100 mg extract/kg body weight respectively. The effect produced by 100 mg extract/kg body weight was comparable with that produced by 5 mg/kg chloroquine (90 %, Table 1).

Antiplasmodial effect on established infection

The extract showed a dose-dependent schizonticidal effect on the parasite *in vivo*. This effect was statistically significant relative to the control ($p < 0.001$). The average percentage inhibition of parasitaemia produced by of 25, 50 and 100 mg/kg doses of extract was 31.67, 55.83 and 85.83% respectively. The effect produced by 100 mg extract/kg body weight was comparable with that produced by 5 mg/kg chloroquine (94.42 %, Table 2).

Repository antiplasmodial activity of extract

The extract showed a dose-dependent prophylactic antiplasmodial effect. This effect was statistically significant relative to the control ($p < 0.001$) at 100mg/kg dose of extract. Similarly, 1.2 mg pyrimethamine/kg body weight produced significant prophylactic effect relative to the control. The mean percentage inhibition produced was 15, 20 and 50% by extract doses of 25, 50 and 100 mg/kg body weight respectively. A dose of 1.2 mg pyrimethamine/kg body weight produced 80% inhibition of parasitaemia (Table 3)

DISCUSSION

The results of this study showed that the extract produced significant suppressive effect against early infection, curative effect against established infection and prophylactic effect against residual infection at safe doses. The oral median lethal dose of the plant extract was greater than 5000 mg/kg body weight, which suggests that orally administered whole plant extract of *A. maciverae* is practically non-toxic. This high safety profile of the extract may be responsible for its wide spread use in different ethno-therapeutic interventions.

Rodent models continue to be relevant in discovery of effective anti-malarial agents. Rodent models have been validated through the identification of several conventional anti-malarials such as chloroquine, halofantrine, mefloquine and more recently artemisinin derivatives [6]. *Plasmodium berghei berghei* parasite (NK 65 strain) is used in predicting treatment outcomes of any suspected anti-malaria agent because of its susceptibility to chloroquine. It also provides valid symptoms of the disease condition similar to those produced in man, making mouse *P. berghei berghei* infection model an appropriate model for this study [4, 23].

The significant suppressive antiplasmodial effect produced by the extract is consistent with the traditional use of the plant as a herbal medication against malaria in Northern part of Nigeria.

In the curative study, a daily increase was observed in the parasite count of the negative group, which is consistent with normal proliferation of the parasite. The antiplasmodial effect produced by the extract was similar to that produced by chloroquine in this study. This implies that the extract may act through mechanisms which are similar to those through which chloroquine acts. Chloroquine is lethal to malaria parasites by causing the accumulation of toxic heme in the parasitic acidic food vacuole [24]. Heme may be toxic to the parasite by its interference with the nucleic acid biosynthesis [25]. The present finding is consistent with earlier study by Ene et al. [7] who reported that a chloroform extract of the

A. maciverae and *A. maritima* possess potent antimalarial activity. Its prophylactic effect may also be likened to that of pyrimethamine, which acts by inhibiting dihydrofolate reductase (DHFR), an important enzyme in the parasite's folate biosynthetic pathway [26].

In the prophylactic study, the whole plant extract of *A. maciverae* exerted significant activity only at a dose of 100 mg/kg. In a study on multidisciplinary approach of evaluating antimalarial activity of medicinal plants, it was concluded that a 50 per cent (%) *P. berghei berghei* inhibition at *in vivo* dose of 100mg/kg/day indicates a very good antimalarial activity [27].
The mechanism of action of the plant extract is yet to be determined. Saponins, tannins and alkaloids in plants have been suggested to act as free radical scavengers or primary antioxidant that can counteract the oxidative damage induced by malaria parasite [28]. Notably, the results presented in this study suggest that the extract of *A. maciverae* possesses potent anti-malaria activity which justifies its continuous use in folk medicine as an anti-malarial remedy. Further studies are necessary to determine specific components of the plant responsible for the observed effects and identify possible mechanism(s) of antiplasmodial effects.

Conflict of Interest
The authors declare that there is no conflict of interest.

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Table 1: Suppressive effect of *A. macivera* in *P. berghei berghei* – infected mice

Treatment	**Mean ± SEM**	**Suppression (%)**
10ml normal saline/kg	10.00±0.58	-
25 mg extract /kg	7.30±0.49***	27.00
50mg extract /kg	5.70±0.21***	43.00
100mg extract/kg	2.70±0.33***	73.00
5 mg chloroquine/kg	1.00±0.26***	90.00

***Significantly different from control at $p < 0.0001$, n=6.

Table 2: Curative effect of *A. maciverae* in *P. berghei berghei* – infected mice

Treatment	Mean ± SEM	Inhibition (%)
5ml normal saline/kg	12.00±0.61	-
25mg extract /kg	8.20±0.70***	31.67
50 mg extract /kg	5.30±0.80***	55.83
100 mg extract/kg	1.70±0.56***	85.83
5 mg chloroquine/kg	0.67±0.21***	94.42

*** Significantly different from the control at $p < 0.0001$, n=6.

Table 3.0: Prophylactic effect of *A. macivera* in *P. berghei berghei* – infected mice

Treatment	Mean ± SEM	Suppression (%)
10ml normal saline/kg	10.00±0.73	-
25 mg extract /kg	8.50±0.76	15.00
50 mg extract /kg	8.00±0.37	20.00
100 mg extract/kg	5.00±0.52***	50.00
1.2 mg pyrimethamine/kg	2.00±0.52***	80.00

*** Significantly different from the control at $p < 0.0001$, n=6.