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In vivo Antiplasmodial Activity of Ethanol Leaf Extract of *Marrubium Vulgare* L. (Lamiaceae) in *Plasmodium Berghei-Berghei* Infected Mice

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ABSTRACT

Malaria is one of the most widespread infectious diseases, taking the lives of almost one million people a year, most of them in sub-Saharan Africa. *Marrubium vulgare* L. (Lamiaceae) is a flowering plant in the family of *lamiaceae* and is used in traditional medicine in the treatment of malaria, tuberculosis and other febrile conditions. The aim of this study is to evaluate the *in vivo* antiplasmodial activity of the ethanol leaf extract of *Marrubium vulgare* in mice. The antiplasmodial activity was evaluated in mice infected with chloroquine-sensitive *Plasmodium berghei-berghei* using curative, suppressive and prophylactic experimental animal models. Preliminary phytochemical screening and intraperitoneal median lethal dose (LD_{50}) estimation of the extract were carried out. Data were analysed using ANOVA followed by Dunnett's post hoc test. The extract at all doses tested (75, 150 and 300 mg/kg) produced significant ($p < 0.001$) curative (percentage parasite clearance: 60, 58 and 64%) and suppressive (chemosuppression: 66, 71 and 73) effects with minimal prophylactic (chemoprophylaxis:19, 24 and 23%) effect. The extract also significantly prolonged the survival time of the treated mice up to 22 days compared to the negative control group (11 days). The results of this study suggest that the ethanol leaf extract of *Marrubium vulgare* possesses curative and suppressive antiplasmodial activity in mice at the tested doses.

Keywords: Antiplasmodial, *Marrubium vulgare*, *Plasmodium berghei-berghei*, Chloroquine.

Introduction

Malaria is a parasitic disease transmitted by the bites of female *Anopheles* mosquitoes infected with *Plasmodium falciparum* (most fatal), *P. vivax, P. malariae, P. ovale and P. knowlesi* The disease primarily affects poor populations in tropical and sub-tropical areas where temperature and rainfall are suitable for the development of vectors and parasites.[1] Malaria due to *P. falciparum* is the leading cause of morbidity and mortality in Sub-Saharan Africa, especially in children under the age of five years, where a child dies every 30 seconds.[2] In 2016, Nigeria accounted for the highest proportion of malaria cases globally (27%), followed by the Democratic Republic of Congo (10%), India (6%) and Mozambique (4%).[3]

Herbal medicines have been used to treat malaria for thousands of years in various parts of the world. The first antimalarial drug, quinine, was extracted from the bark of Chincona plant (Rubiaceae). Infusions of the plant bark were used to treat human malaria as early as 1632.[4] *Marrubium vulgare* is a flowering plant in the mint family (Lamiaceae) native to Europe, Northern Africa, and South Western and Central Asia. It is also widely naturalized in many places, including most of North and South America.

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Previous scientific work on *Marrubium vulgare* revealed that the plant possesses antibacterial activity,[5] antifungal and cytotoxic properties,[6] hypoglycaemic and antioxidant activities,[7] mulluscicidal and insecticidal activities,[8] and hepatoprotection against cyclophosphamide toxicity in rats.[9] Marrubiin isolated from the plant possessed antidiabetic property.[10] Resistance has been documented to all classes of antimalarial drugs, including the artemisinin derivatives, and it is a major threat to malaria treatment, control and eradication.[11] The disease remains a major public health concern in spite of the scale-up in control interventions[12] due partly to the development and spread of antimalarial drug-resistant parasite.[13]

Treatment failure is dangerous for the patient and also for the community, as it increases malaria transmission and facilitates emergence and spread of antimalarial drug resistance.[14] Traditional medicine practitioners in Kano State, Nigeria, have been using infusion of *Marrubium vulgare* leaves to treat malaria, pulmonary tuberculosis and other febrile conditions, but its efficacy as an antimalarial remedy has not been proven scientifically. Several conventional antimalarial agents including chloroquine, halofantrine, mefloquine and artemisinin derivatives were identified from medicinal plants using rodent malaria models.[15,16] The aim of this study is to investigate the antiplasmodial potential of the ethanol leaf extract of *Marrubium vulgare*.

Materials and Methods

Collection and identification of plant material
Fresh leaves of *Marrrubium vulgare* were collected from Igabi, Kaduna State, Nigeria in the month of June 2016. The plant was identified and authenticated by a botanist, Mr. Muhammad Namadi Sunusi, in the Department of Biological Sciences, Ahmadu Bello University, Zaria. A voucher specimen number (2453) was collected for future reference and deposited in the same department.

Preparation of ethanol leaf extract
Fresh leaves of *Marrubium vulgare* were sorted manually to remove dust and other unwanted particles. They were air-dried under shade at room temperature until constant weight was obtained. Seven hundred and fifty grams (750 g) of the powdered leaves was extracted with five liters (5 L) of 70% $^V/_V$ ethanol using cold maceration method for two weeks with regular shaking. The extract obtained was concentrated and evaporated to dryness using water bath at a temperature of 45°C to obtained 11.3% $^W/_W$.

Preliminary Phytochemical Screening
Preliminary phytochemical screening was carried out on the ethanol leaf extract of *Marrubium vulgare* to identify the presence of secondary metabolites using established methods.[17,18]

Experimental animals
Adult Swiss albino mice (18 – 24 g) of either sex were obtained from the Animal House Facility of the Department of Pharmacology and Therapeutics, Bayero University, Kano. The animals were housed in cages in a well-ventilated room under standard condition of temperature (20-23°C) and light. They were observed for one week, fed on standard animal feed (Vital feeds Plc, Jos, Nigeria) and allowed access to water *ad libitum* prior to the study. Ethical approval was obtained from the animal rights committee of the Department of Pharmacology and Therapeutics, Bayero University, Kano; all experiments were performed in accordance with the principles of laboratory animal care.[19]

Drugs and Chemicals
Chloroquine phosphate (Laborate Pharmaceutical, India), Normal saline (Dana Pharmaceuticals, Nigeria), Giemsa stain (Hightech Health Care, India), Ethanol (Sigma, USA), Methanol (BDH chemicals, England).

Acute Toxicity Study (Median Lethal Dose (LD$_{50}$) Determination)
The acute toxicity study was conducted in two phases using the method of Lorke.[20] In the first phase, 9 mice were divided into three groups of three mice each and were treated with the extract at doses of 10, 100 and 1000 mg/kg intraperitoneally. The mice were monitored for the first four hours and then 24 hours after treatment for signs of toxicity and death. The second phase was carried out based on the result of the first phase. Four mice were divided into four groups of one mouse each and were treated with the extract at doses of 600, 1000, 1600 and 2900 mg/kg intraperitoneally. The mice were monitored for the first four hours and then 24 hours later for signs of toxicity and death as in the first phase.

Plasmodium parasite
Chloroquine-sensitive *Plasmodium berghei-berghei* was obtained from National Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria and was maintained in mice by continuous intraperitoneal inoculation every four days in fresh mice.[21]

Parasite inoculation
An infected mouse with *Plasmodium berghei-berghei* parasite (parasitemia of 34%) was used as a parasite donor and blood was collected retroorbitally into an EDTA containing bottle. The inoculum was prepared by determining both the percentage parasitaemia and the red blood cell (RBC) count of the donor mouse and diluting the blood with isotonic saline in such a way that 0.2 mL of the blood contained approximately 1.0 X 10^7 infected RBCs. Inoculum in volumes of 0.2 mL was administered intraperitoneally to infect each mouse.[22]

Antiplasmodial activity against established infection (Curative test)
Evaluation of the curative potential of the extract against established infection was carried out as described by Ryley and Peters.[23] On the first day (D$_0$), adult mice were inoculated and left untreated for 72 hours (D$_0$-D$_3$) for infection to be established. On day 3, all inoculated mice were then randomized into 5 groups of 5 mice each and treated intraperitoneally with normal saline (10 mL/kg, group I), graded doses (75, 150 and 300 mg/kg body weight, groups II-IV) of the extract and standard drug, chloroquine (5 mg/kg body weight, group 5) respectively for 5 days (D$_3$-D$_7$). On day three post parasite inoculation, each mouse was tail-bled, thin blood smear prepared, stained with Giemsa stain after fixing with absolute methanol and baseline parasitaemia levels were determined by counting the number of parasitized erythrocytes out of 200 erythrocytes in random fields. Post-treatment parasitaemia levels were determined on day seven of the experiment using light microscope at x100 magnification. The mice were thereafter monitored and the mean survival time for each group was

determined arithmetically by finding the average survival time (days) of the mice (post-inoculation) in each group over a period of 28 days.[23]

Antiplasmodial activity against early infection (suppressive test)
The 4-day suppressive test of the extract against *Plasmodium berghei-berghei* infection in mice as described by Peter [25] was employed. On the first day (D$_0$), adult mice were inoculated with *Plasmodium berghei-berghei* and thereafter randomly divided into their respective groups as described in the curative test. On the same day (D$_0$), treatment was started four hours after inoculation and continued daily for three days. Twenty-four hours (24 hours) after administration of the last dose (D$_4$), the mice were tail-bled and thin film prepared, fixed in absolute methanol and stained with Giemsa solution for parasitaemia level determination.

Prophylactic (Repository) test
The prophylactic activity of the extract was studied using the procedure described by Peters.[26] Adult mice were randomized into 5 groups of 5 mice each and treated with the graded doses of the extract and standard drug intraperitoneally for five (5) days (D$_0$-D$_4$). The standard drug used was chloroquine 5 mg/kg. On the sixth day (D$_5$), all the mice were inoculated with *Plasmodium berghei-berghei*. Smears were made from tail blood of each mouse 72 hours after inoculation and parasitaemia level was determined.

Data analysis
Results were expressed as mean plus or minus standard error of mean (Mean ± SEM) and analysed using One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for comparison among groups. Results were considered statistically significant at $p \leq 0.05$.

Results and Discussion

Phytochemical screening
The results obtained from phytochemical screening of the ethanol leaf extract of *Marrubium vulgare* showed the presence of alkaloids, glycosides, saponins, triterpenes, phenols, tannins, and flavonoids (Table 1).

Acute Toxicity Study (Median Lethal Dose (LD$_{-50}$) Determination)
The intraperitoneal median lethal dose (LD$_{50}$) of the ethanol extract of *Marrubium vulgare* in mice was estimated to be 775 mg/kg.

Curative Test
The extract produced significant ($p < 0.001$) reduction in the mean parasitaemia level in the treated groups compared to the control group. The extract produced 60, 58 and 64% parasite clearance at the doses of 75, 150, and 300 mg/kg, respectively. The standard drug chloroquine also produced 78% parasite clearance at the dose of 5 mg/kg. Mortality was recorded in the control group on the ninth day and by the fourteenth day, all mice in the group died (Mean survival time of 11.5 days). On the other hand, mice in the extract treated groups survived up to 22 days. Mice in the chloroquine treated group survived the 28-day period of observation (Table 2).

Table 1: Phytochemical Constituents of Ethanol Leaf Extract of *Marrubium vulgare* L

Constituents	Inference
Alkaloids	+
Glycosides	+
Saponins	+
Triterpenes	+
Phenols	+
Tannins	+
Flavonoids	+

+ = Present

Table 2:   Curative Effect of Ethanol Leaf Extract of *Marrubium vulgare* in Mice infected with *Plasmodium berghei-berghei*

Treatment(mg/kg)	Mean Parasitaemia		% Clearance	Survival (Days)
	D3	D7		
N/Saline 10 ml/kg	15.00 ± 1.53	26.53 ± 2.83	-	11.5 ± 0.99
ELMV (75)	13.40 ± 1.15	10.50 ± 0.82*	60	18.6 ± 1.64
ELMV (150)	12.20 ± 1.12	11.10 ± 1.98*	58	21.8 ± 1.89
ELMV (300)	12.70 ± 2.97	9.47 ± 0.94*	64	22.0 ± 2.84
CQ (5)	13.30 ± 1.91	5.83 ± 1.12*	78	28.0 ± 0.00

Values presented as Mean ± SEM. n=6, * significantly different from control at $P < 0.001$ using One-way ANOVA and Dunnett's post hoc test. ELMV = Ethanol Leaf Extract of *Marrubium vulgare*, CQ= Chloroquine, D3, D7 Indicate days 3 and 7 respectively.

Table 3:   Suppressive Effect of Ethanol Leaf Extract of *M. vulgare* in Mice infected with *Plasmodium berghei-berghei*

Treatment (mg/kg)	Mean Parasitaemia	% Chemosuppression
N/Saline 10ml/kg	19.30 ± 1.70	-
ELMV (75)	6.60 ± 1.10*	66
ELMV (150)	5.70 ± 0.82*	71
ELMV (300)	5.20 ± 0.72*	73
CQ (5)	3.90 ± 1.71*	80

Values presented as Mean ± SEM. n=6, * significantly different from control at $p < 0.001$ using One-way ANOVA and Dunnett's post hoc test. ELMV = Ethanol Leaf Extract of *Marrubium vulgare*, CQ= Chloroquine.

Table 4:   Prophylactic Effect of Ethanol Leaf Extract of *M. vulgare* in Mice infected with *Plasmodium berghei-berghei*

Treatment (mg/kg)	Mean Parasitaemia	% Prophylaxis
N/Saline 10ml/kg	10.70 ± 2.72	-
ELMV (75)	8.70 ± 1.31	19
ELMV (150)	8.13 ± 1.24	24
ELMV (300)	8.30 ± 1.11	23
CQ (5)	4.30 ± 0.56*	60

Values presented as Mean ± SEM. n=6, * Significantly different from control at $p < 0.05$ using One-way ANOVA and Dunnett's post hoc test, ELMV = Ethanol Leaf Extract of *Marrubium vulgare*, CQ= Chloroquine.

Suppressive Test
The ethanol leaf extract of *M. vulgare* produced significant ($p < 0.001$) chemosuppression of 66%, 71%, and 73% in a dose-dependent manner compared to the control group. The standard drug chloroquine showed 80% chemosuppressive effect (Table 3).

Prophylactic Test
The ethanol leaf extract of *M. vulgare* produced insignificant ($p > 0.05$) chemoprophylaxis at the doses (75, 150 and 300 mg/kg) used in this study. The extract produced 19%, 24% and 23% chemoprophylaxis. However, chloroquine produced significant ($p < 0.05$) chemoprophylaxis of 60% (Table 4).
Preliminary phytochemical screening of ethanol leaf extract of *Marrubium vulgare* revealed the presence of alkaloids, glycosides, flavonoids, phenols, saponins, tannins and terpenoids. These are secondary metabolites and constitute an integral part of medicinal plant having numerous biological activities. Physical signs of toxicity observed within the first four hours of extract administration are restlessness, hyperventilation and later reduced physical activity. The LD$_{50}$ of 775 mg/kg body weight after intraperitoneal administration of ethanol leaf extract of *Marrubium vulgare* implied that the extract is slightly toxic to the mice.[20]

Flavonoids were reported to exert antiplasmodial activity by chelating with nucleic acid base pairing of the parasite thereby killing the parasite.[27] Some plants are known to produce antiplasmodial activity either by causing elevation of red cell oxidation[28] or by inhibiting protein synthesis.[29] The ethanol leaf extract of *Marrubium vulgare* may be acting through one of these mechanisms or by different mechanism. The results obtained from this study showed that the extract has significant curative (blood schizonticidal) and suppressive (tissue schizonticidal) activities with minimal prophylactic effect in mice infected with *Plasmodium berghei berghei*. Significant parasite clearance observed by the extract in curative test correlates positively with an increase in survival time in the treated groups. The ethanol leaf extract of *Marrubium vulgare* showed significant and similar pattern of activity in both curative and suppressive models which are commonly used for antimalarial drug evaluation. The ethanol leaf extract of *Marrubium vulgare* produced minimal chemoprophylaxis at the doses tested (75, 150 and 300 mg/kg), which might be due to the short half-life of the extract with the inability to achieve a steady-state antiplasmodial concentration in the plasma or low volume of distribution or both**.**

Conclusion

The results of this study revealed that the ethanol leaf extract of *Marrubium vulgare* has significant curative and suppressive antiplasmodial activity at the tested doses in mice and thus support the ethnomedicinal use of the plant in treating malaria.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

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