



IN VIVO ANTI-PLASMODIAL ACTIVITY OF ADHATODA SCHIMPERIANA LEAF EXTRACT IN MICE

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Summary

Malaria is a major public health problem in the world particularly in developing countries. It causes half a billion cases of mortality and about three-million deaths. Not only about three billion people are at risk of infection but also there is an increasing drug resistance. Therefore, the need for alternative drugs is essential. The study aims at investigating *in-vivo* anti-malarial activity of crude extracts of the leaves of *Adhathoda schimperiana* to ascertain its traditional medicinal use in Ethiopia. The hydro-alcoholic extracts of leaves of *A. schimperiana* were investigated for anti-plasmodial activity against *Plasmodium berghei* infections in Swiss albino mice. The anti-plasmodial activities during early, residual and established infections were investigated. Chloroquine (10 mg/kg) was used as positive control, and Tween 80 was used as a solvent and a negative control. Thin blood films prepared from the tail blood of each mouse were used to assess the level of parasitaemia of the mice. The crude extract of the leaves of *A. schimperiana* dose-dependently reduced parasitaemia induced by chloroquine-sensitive *Plasmodium berghei* infection in suppressive, curative and prophylactic models in mice. These reductions were statistically significant ($p < 0.05$). It also improved mean survival time relative to the control ($p < 0.05$) and it was comparable to chloroquine, the standard drug. The study could partly confirm the local anti-malaria use of *Adhathoda schimperiana* in Ethiopian traditional medical practice. Thus, there is a need to initiate further in-depth investigation by using variable experimental models.

Key words: *Adhathoda schimperiana*, *In-vivo*, Anti-malarial activity, *Plasmodium berghei*

Introduction

Malaria is an infectious disease caused by the parasite Plasmodium. The four identified pathogenic species of this parasite includes *Plasmodium vivax*, *P. falciparum*, *P. ovale* & *P. malariae*, transmitted by female anopheles mosquito (1). It is a major public-health problem and the most important tropical disease responsible for significant morbidity and mortality in the world where 3.3 billion people are estimated to be at risk (2). An estimated half a billion clinical cases every year with a corresponding mortality rate of 2–3 million annually, 90% of these being in sub-Saharan African region (3).

Drug resistant strains of *P. falciparum* have been found and majority of conventional modern anti-malarial drugs have been associated with treatment failure including the resistance to artemisinin based treatment (4). In addition, unavailability and unaffordability of antimalarial drugs (5), increasing resistance of mosquito vectors to insecticides (6), the challenge of having effective vaccines (3) coupled with adverse effects of the existing drugs (7) underlies the urgent need for novel, well tolerated and more effective and affordable anti-malarial drugs from claimed medicinal plants used in unconventional medicine.

Medicinal plants have been the focus of many anti-infective drugs and alternative sources of anti-malarial agents in various parts of the world since long ago (8). To this effect, studies have been conducted on traditionally claimed medicinal plants in Ethiopia and elsewhere for scientific validation. This is because they have been part of human life since time immemorial; and a number of plant products have been in extensive use in ethnomedicine (9).

Adhatoda schimperiana (Family: Acanthaceae) is a common plant that is relatively fast growing and prefers an altitude of 2,000 ms and above. It is an erect shrub up to 4m high; usually much branched from the base. It grows abundantly in the highlands of Ethiopia and other countries of East Africa (10). In Northern part of Ethiopia, *Adhatoda schimperiana* alone or in combination with other plants is used for

various diseases such as cough and malaria (11). The crude-hydroalcoholic extract of *A. schimperiana* was found to have bronchodilatory and respiratory distress protective effect (12). But its anti-malarial activity has not been evaluated.

Since large portions of the plant remedies in the health care system of Ethiopia are not yet well explored, effort to scientifically evaluate herbal remedies of traditionally used plants is very important for their possible application (9). In view of these facts, the aim of the present study is to evaluate the anti-malarial effect of crude hydroalcoholic extract of the leaves of *Adhatoda schimperiana*. The study addresses the need for safe and effective alternate source of anti-malarial products from traditionally claimed medicinal plant.

Materials and Methods

Preparation of crude extract: Leaves of *Adhatoda schimperiana* were collected in October 2009 around Beehere-Tsige area in Addis Ababa, Ethiopia. Voucher specimen was deposited in the herbarium of Ethiopian Health and Nutrition Research Institute (EHNRI), Ethiopia.

Dried powdered leaves of the plant were macerated in 80% (v/v) methanol for two days with intermittent agitation. It was then filtered, and the residue was re-extracted with fresh solvent. The combined filtrates were concentrated under vacuum in a rotary evaporator and further dried in a water bath to yield 24% residue. The extract was kept in a refrigerator until anti-malarial activity is evaluated. For oral administration of the extract, the crude hydro-alcoholic extract was suspended in 10% Tween 80 solution to enhance its solubility.

Preparation of animals: Swiss Albino mice (20-25gm) of either sex were used for evaluation of *in-vivo* anti-malarial activity. They were obtained from EHNRI and kept in cages in an animal house under room temperature about 24°C and relative humidity of 80%. The diet was a standard pellet and there was continuous availability of clean drinking water. Prior to the experiment, the animals were acclimatized to

laboratory environment for an hour. They were randomly assigned to a control and experimental groups.

Inoculum: The chloroquine sensitive *Plasmodium berghei berghei* was obtained from EHNRI and inoculum was prepared using the standard method (13) to assess the *in vivo* anti-malarial activity of the plant extract. The stored parasitized blood in liquid nitrogen was removed and allowed to thaw. The contents were injected (0.2 mL) into three donor mice by peritoneal route and left for 5-10 days so that 20–30% parasitaemia had developed in the mice, predicted from blood sample taken from the tail of the mice.

When 20% parasitemia was reached, the mouse was killed by a head blow, and parasitized erythrocytes were obtained immediately by cardiac puncture with a sterile and apyrogenic disposable needle, and anti-coagulated with heparin (1% w/v) in phosphate buffered saline. The blood was diluted with sterile isotonic normal saline solution and the inoculum consisted of 5×10^7 *Plasmodium berghei* parasitized red blood cells per ml. Each animal used in the experiment was inoculated intraperitoneally with 0.2 ml of infected blood containing about 1×10^7 *Plasmodium berghei* parasitized erythrocytes, which produces a steadily rising infection in mice, lethal inoculums. The *Plasmodium* was subsequently maintained in the laboratory by serial passage of blood passage from an infected mouse to a naïve mouse.

Pharmacological Evaluation: *In-vivo* anti-plasmodial activity tests

I. Activity on early infection (four-day test)

An *In-vivo* anti-malarial activity test was performed in a four-day suppressive test using a standard method (14, 15) with slight modifications. A plasmodium species, which is most widely employed in rodent malaria parasite, chloroquine sensitive strain of *Plasmodium berghei*, was used to infect Swiss albino mice. Each mouse was inoculated intraperitoneally with 0.2 ml of infected blood containing about 1×10^7 *Plasmodium berghei* parasitized erythro-

cytes, which produces a steadily rising infection in mice.

Mice were randomly assigned into five groups (six mice per group). Three of the groups (test group) were made to receive crude hydro-alcoholic extract treatments (200, 400 and 600 mg/kg as suspension, as determined from pilot study), while the fourth group received the vehicle (negative control, 10% tween 80) and the fifth group received chloroquine (10 mg/kg). The treatment dose was given 3 hrs after infection on day-1 and was repeated once daily for 3 days, as a “four-day-blood schizonticidal test”. Thick and thin smears of blood films were obtained from the peripheral blood on the tail from each mouse on day-5 after infection. The smears were placed on microscopic slides, fixed with methanol and stained with Giemsa’s stain (4%, pH 7.2 for 45 min) and then microscopically examined (1000 × magnification).

Percentage parasitaemia was described as number of parasitized erythrocytes per hundred erythrocytes and it was determined by the ratio of parasitized RBC to the total number of RBC out of 3000 erythrocytes in about 30 random fields of the microscope (≈ 98100 erythrocytes per view). Percentage chemo-suppression was taken as inhibition of parasite growth/multiplication by the extract relative to the control expressed in percentage. Percentage chemo-suppression was calculated by using the following formula (8),

$$\text{Percentage Chemo-suppression} = 100(A-B)/A$$

where

A - Average parasitemia in the negative control group
B - Average parasitemia in the test group

II. Activity on established infection (Curative test)

Evaluation of curative anti-malarial potential of the extract was done using a method described Ryley and Peters (16, 17) and modified by Carvalho *et al* (18). The mice were injected by peritoneal route with standard inoculums of *Plasmodium*

berghei infected erythrocytes on the first day and infection was allowed to be established for 72 hrs. The animals were then randomly divided into three groups of six mice each.

Seventy-two hours later, one of the groups of mice was orally administered with the crude hydro-alcoholic extract of *Adhatoda schimperiana* leaves (500 mg/kg), the other group was given chloroquine (5 mg/kg) and the solvent (1ml) was given to the third group. The groups were given the extract/control solution once daily for 3 days. Thin blood films stained with Giemsa were prepared from tail blood of each mouse daily on day 3 through day 7 to monitor the parasitaemia level. The extracts were considered active when parasitaemia was reduced by $\geq 30\%$ (18).

The animals were observed till the 30th days. Any death that occurred during this period was noted to determine mean survival time (MST). The MST for each group was determined by the average survival time (days) of the mice (post-inoculation) over a period of 30 days post-infection. A dose that results in survival time greater than that of infected non-treated mice was considered active. Death occurring before day 5 of infected and treated mice was regarded as toxic death. The parasitaemia level of the animals that survived after the 30 days were determined from thin film prepared from tail blood of the animals.

III. Activity on residual infection (repository test)

Evaluation of prophylactic potential of the extract was done using Peters method with slight modification (19). Mice were divided into four groups' with six mice per group. Mice in the test groups were fed with the extract (400, 600 mg/kg/d) orally at a single daily dose for three days prior to infection. Mice in the positive and negative control groups were treated with pyrimethamine (1.2 mg/kg/d), and 10 % Tween 80 (1 ml), respectively.

On day-4, a standard inoculum of *Plasmodium berghei* infected-erythrocytes was administered by intraperitoneal route to each mouse. After 72 hrs

(on the seventh day), blood smears were prepared from the tail blood. Percentage parasitaemia and the percentage of chemo-suppression of parasitaemia were calculated as described above.

Statistical Analysis

The results of the study were reported as Mean \pm standard error of the mean (SEM). For comparison of average parasitaemia and mean survival time among groups, analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests using SPSS (version 13.0) and Student's t-test were used. The mean differences were considered to be statistically significant when p-value was less than 0.05 ($p < 0.05$).

Results

Anti-plasmodial effect on early infection (Four-day study)

As shown in table-1, the results of the study indicated that the hydro alcoholic (HA) extracts of *A. schimperiana* displayed anti-plasmodial activity against *Plasmodium berghei* malaria parasite in Swiss albino mice. At 200 mg/kg concentration of the crude HA extract of *A. schimperiana*, the percent parasitaemia suppression on day five parasitaemia level was not statistically significant. However, at higher concentrations (400 and 600 mg/kg), there were statistically significant parasitaemia suppression than the negative control group ($p < 0.05$).

The percent suppression tends to increase with an increase in the concentration of the extract, with a higher level of inhibition observed at 600 mg/kg concentration. The lowest effective dose of the extract from the experiment was 400 mg/kg.

see Table 1.

Anti-plasmodial effect on established infection

On established infection, there was a daily increase in parasitaemia of the control group. However, there was a daily reduction in the parasi-

taemia levels of the extract treated groups as well as that of the positive control group. As indicated on Figure-1, on day 7, the average percentage parasitaemia for the groups were 79%, 46%, 11% and 2% for the control, extracts (400 mg/kg, 600 mg/kg) and chloroquine group, respectively.

The results showed that the crude HA extract (600 mg/kg) reduced average percent parasitaemia at the seventh day (46% reduction, $p \leq 0.05$) in relation to the negative control mice. The 400 mg/kg dose of the extract reduced only 15% of the parasitaemia and was considered inactive. The positive control (chloroquine) reduced parasitaemia by 58% ($p \leq 0.05$).

As indicated in Table-2, the mean survival times (MST) were 13, 15, 28 and 30 days for the control, extracts (400 mg/kg, 600 mg/kg) and chloroquine group, respectively. The MST of the 600 mg/kg concentration of the extract treated group was significantly ($p < 0.05$) longer than that of control and was closer to that of the standard drug, chloroquine. However, the MST of the 400 mg/kg concentrations of the extract was not statistically significant.

Several mice treated with the extract were still alive at day 30 in comparison with mice in the control group, which all died after the 7th day but before the 16th day after infection and the death was preceded by convulsions, rapid breathing and high parasitemia. In the 600 mg/kg extract group, one mouse died on day-23 whereas all others survived and the average percentage parasitaemia at the end of the study was $2.7 \pm 1.5\%$. The mice which were treated with chloroquine survived throughout the study duration and their blood cleared of the parasite at the end of the study period.

see Fig. 1

see Table 2.

Anti-plasmodial effect on residual infection

The crude HA extract of the plant also showed

prophylactic suppression on residual infection. As indicated on Table-3, the 600 mg/kg/d dose of the extract and pyrimethamine (1.2 mg/kg/d) resulted in 53% and 82% chemo-suppressions respectively, which are statistically significant compared to the control ($p < 0.05$). Chemo-suppression of the 400 mg/kg/d dose of the extract was significant lower ($p < 0.05$) than chemo-suppression by pyrimethamine.

This showed that, though there was a low chemo-suppressive activity at the lower dose, the chemo-suppression exerted by the highest dose of the extract was comparable to that of the standard drug. The results showed that the effective dose of the extract was 600 mg/kg/d since a lower dose was almost inactive. Thus, higher doses may be required to produce chemo-suppressive effect.

see Table 3.

Discussion

Although the major plasmodium strains (*P. falciparum*, *P. ovale*, *P. vivax* and *P. malariae*) that causes malaria infection in human do not cause malaria in rodents and it is possible to conduct efficacy evaluation experiment on primate models, rodent models have also been validated for preliminary assessment of several conventional anti-malarials, such as chloroquine, halofantrine, mefloquine and more recently artemisinin derivatives (8, 20).

In-vivo murine *Plasmodium* models such as *Plasmodium berghei*, *P. vinckei* and *P. yoelii* are firmly established models in anti-malarial drug discovery (21-25). The biological activity of the *in-vivo* anti-malarial activity is expressed as a decrease of parasitaemia after a certain time which is examined in blood smear or as an increase in survival time (8, 14). These parasite models are indispensable for the development of anti-malarial drugs. The murine based parasite models of experimental malaria are used to produce the disease with a natural history similar to human cerebral malaria: namely, high parasitaemia, convulsions, respiratory distress, and

ultimately death; even if they may not always perfectly mirror *Plasmodium falciparum* infection in human (26). Although an *in-vivo* test on *P. falciparum* culture could also be done to evaluate anti-malarial activity, an *in-vivo* test will give an opportunity to take into account any pro-drug effect or the role of the immune system in controlling infection as compared to an *in-vivo* study.

Thus, in the current study to evaluate anti-malarial activity of *Adhatoda schimperiana*, *Plasmodium berghei* was used for the prediction of treatment outcomes, and hence, it was an appropriate parasite for the study. It is also logical and cost effective to conduct preliminary pharmacological screening studies of crude extracts in rodent models. When a standard anti-malarial drug is used in mice infected with *P. berghei*, it suppresses parasitemia significantly (27), which is in agreement with the effects of chloroquine in our study which achieved 98% suppression of parasitaemia.

The four-day suppressive test is a standard test commonly used for antimalarial screening, and the determination of percent inhibition of parasitaemia is the most reliable parameter (14). In the study, *P. berghei* infected mice treated with hydro-alcoholic extract of *Adhatoda schimperiana* leaves, the percentage of parasitaemia measured changed significantly from those in the control group indicating that the extract has antiplasmodial activity supporting the traditional practice as anti-malarial herbal remedy.

The mean survival time of the extract was more than twice that of the control. According to Peters (28) this is considered as evidence for the antiplasmodial activity of the extract. It is also good to note that the parasitaemia decreased with time in the extract administered group and there was an increase in the survival time of the mice at moderate doses of the extract indicating a dose-dependent pharmacological activity.

Evaluation of the residual activity of the plant extract suggested that at moderate dose level, comparable percent parasitaemia suppression was observed as the standard drug, pyrimethamine.

Considering the fact that the crude extract was used in these experiments, this plant is worthy of further investigation for isolation and characterization of active principals that have antiplasmodial activity.

A previous phytochemical study done showed that the leaves of the plant contain terpenoids, alkaloids, glycosides, polyphenols, and saponins (29). These compounds present in this plant alone or in combination might have contributed to the antiplasmodial activity observed in the study. A study done on dichloromethane extract of the leaves of a closely related plant *Adhatoda latibracteata* (family: Acanthaceae) showed a very high anti-malarial activity (30). The active antiplasmodial constituents of *Adhatoda schimperiana* leaves may be novel or similar to those active constituents of *Adhatoda latibracteata*. Other members of the Acanthaceae family, such as *Hypoestes rosea*, *Clinacanthus siamensis* and *Andrographis paniculata* contain terpenes and xanthenes with antiplasmodial activity (31, 32). Thus, compounds with antiplasmodial activity found in Acanthaceae might also be present in *Adhatoda schimperiana*.

In another study conducted to evaluate acute toxicity effect of the crude hydro-alcoholic extract of *Adhatoda schimperiana*, the extract was found to be less toxic when taken orally as evidenced by higher median lethal dose which was much higher than the effective anti-plasmodial dose, and absence of gross behavioral and physical observations of signs of toxicity (12). This may supports the safe use of the plant as an anti-malarial herb in Ethiopian traditional medical practice. This also shows that the extract has relatively safe profile of toxicity and may be a good candidate for future work-up as standardized anti-malarial herbal product and to find lead compounds.

Conclusion

In the present study, the hydro-alcoholic extracts of the leaves of *Adhatoda schimperiana* has shown promising suppressive and curative anti-malarial activities using *Plasmodium berghei* infected mice in a dose-dependent manner. The results of this study

support the ethno-botanical use of the plant in the treatment of malaria in Ethiopia. Further investigation is being advocated in elucidating the principal ingredients responsible for its activities, or designing a standardized herbal preparation for local use.

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Test substance	Parasitaemia (%)	Chemo-suppression (%)
Tween 80 (Control), 1 ml	46 ± 3.2	0.0
Crude HA Extract, 200 mg/kg	42 ± 2.3	8.7
400 mg/kg	16 ± 1.9 ^a	65.2
600 mg/kg	7 ± 2.6 ^{a, b}	84.7
Chloroquine (10 mg/kg)	1 ± 1.3 ^{a, b, c}	97.8

Table-1: Parasitemia suppression of crude hydro-alcoholic extract (HA) of leaves of *Adhatoda schimperinana* against *Plasmodium berghei* on early infection (Mean ± SEM; n = 6)

a - $p < 0.05$ compared to the control (solvent)
 b - $p < 0.05$ compared to the 200 mg/kg crude HA extract
 c - $p < 0.05$ compared to the 600 mg/kg crude HA extract

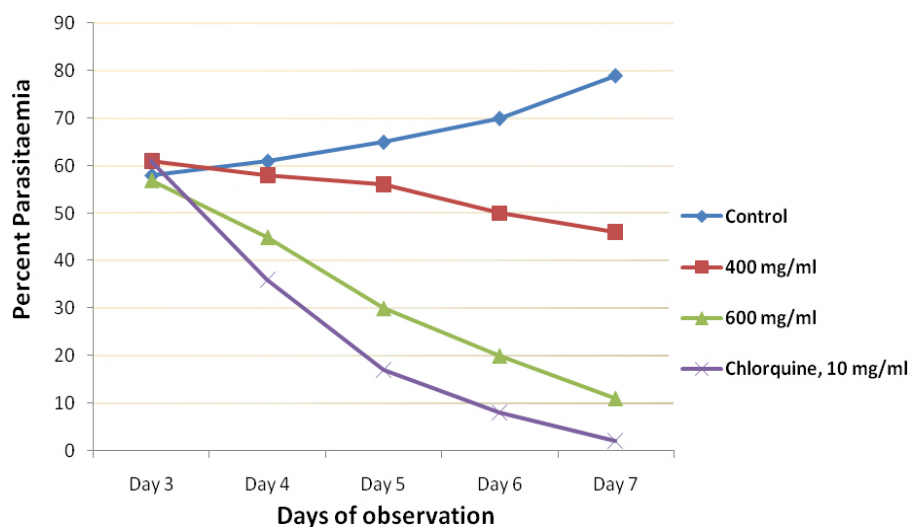


Figure 1: Antiplasmodial effect of hydroalcoholic leaf extract of *Adhatoda schimperiana* on established infection

Test substance	Mean Survival Time (days)
Tween 80 (Control), 1 ml	12 ± 3.3
Crude HA Extract, 400mg/kg	16 ± 3.7
600mg/kg	28 ± 3.2 ^{a, b}
Chloroquine (10 mg/kg)	30 ± 0.0 ^{a, b}

Table-2: Effect on mean survival time of crude hydro-alcoholic extract (HA) of leaves of *A. schimperinana* against *Plasmodium berghei* on established infection (Mean ± SEM; n = 6).

a - p<0.05 compared to the control (solvent)
b - p<0.05 compared to the 400 mg/kg crude HA extract

Test substance	Parasitaemia (%)	Chemo-suppression (%)
Tween 80 (Control), 1 ml	45 ± 2.6	0.0
Crude HA Extract, 400 mg/kg/d	38 ± 1.9 ^a	15.6
600 mg/kg/d	11 ± 2.4 ^{a, b}	75.6
Pyrimethamine (1.2 mg/kg/d)	8 ± 3.3 ^{a, b}	82.2

Table-3: Chemo-suppression of crude hydro-alcoholic extract (HA) of the leaves of *Adhatoda schimperinana* against *Plasmodium berghei* (Mean ± SEM; n = 6).

a - p<0.05 compared to the control (solvent)
b - p<0.05 compared to the 400 mg/kg crude HA extract