Original article

In vivo anti-malarial activity of hydroalcoholic extracts from A*sparagus africanus* Lam. in mice infected with *Plasmodium berghei*

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Abstract

Background: Malaria is a major public health problem in the world in general and developing countries in particular, causing an estimated 1-2 million deaths per year, an annual incidence of 300-500 million clinical cases and more than 2 billion people are at risk of infection from it. But it is also becoming more difficult to treat malaria due to the increasing drug resistance. Therefore, the need for alternative drugs is acute.

Objective: This study aims at investigating the *in vivo* antiplasmodial activity of extracts of the roots and area parts from traditionally used medicinal plant, named *Asparagus africanus* (Liliaceae).

Methods: A rodent malaria parasite, *Plasmodium berghei,* which was maintained at the Ethiopian Health and Nutrition Research Institute (EHNRI) laboratory, was inoculated into Swiss albino mice. The mice were infected with $1x10^7$ parasites intraperitoneally. The extracts were administered by an intra gastric tube daily for four days starting from the day of parasite inoculation. The control groups received the same amount of solvent (vehicle) used to suspend each dose of the herbal drug. Chloroquine was used as a standard drug, and was administered through the same route.

Results: Extracts from the roots and aerial parts of *A.africanus* were observed to inhibit *Plasomodium berghei* parasitaemia in the Swiss albino mice by 46.1% and 40.7% respectively.

Conclusion: The study could partly confirm the claim in Ethiopian traditional medicine that the plant has therapeutic values in human malaria. There is, thus, the need to initiate further in-depth investigation by using different experimental models. [*Ethiop.J.Health Dev.* 2006;20(2):112-118]

Introduction

Malaria is an infectious disease caused by the parasite Plasmodia. Four identified species of this parasite exist, which cause different types of human malaria, namely; *Plasmodium vivax, Plasmodium falciparum, Plasmodium ovale & Plasmodium malariae,* all of which are transmitted by the female anopheles mosquito (1). Although all the four species of malaria parasites can infect humans and cause illness, only the malaria type caused by *Plasmodium falciparum* is known to be potentially life threatening. Infection with *P.falciparum* is, therefore, a medical emergency. About 2% of persons infected with falciparum malaria die, usually because of delayed treatment (2). The present global situation indicates a recent resurgence in the severity of the disease and that malaria could still be described as one of the most important communicable diseases, with an annual incidence of 300-500 million clinically manifest cases and a death toll of 1-2 million people (3-6).

In earlier times the common oral treatment for uncomplicated falciparum malaria was limited to chloroquine, in chloroquine sensitive cases of malaria, and to quinine sulfate or mefloquine, tetracycline or doxcycline, sulfadoxine/pyrimethamine (SP) in chloroquine resistant cases (7-8). Chloroquine - a prominent antimalarial drug - has been the first line drug used for the treatment of uncomplicated malaria, over the last forty years in Ethiopia. The first report on chloroquine failure to clear asexual *P. falciparum* parasites from the blood of patients infected in various parts of Ethiopia appeared in 1986. Since then the resistance has been exponentially increasing in Ethiopia as well its neighboring countries (9-13).

One of the areas for the search for new antimalarials is the traditionally claimed antimalarial plant from the African flora (14,15). Medicinal plants have been the focus of many anti-infective drugs and alternative sources of animalarial agents in various parts of the world since long ago (16). Studies have been conducted on traditionally claimed medicinal plants in Ethiopia and elsewhere for scientific validation (17-19). 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 ethno medicine (20,21).

One of the most impressive developments in the history of medicine is the emergence of the Peruvian (Cinchona) bark (Rubiaceae) coupled with its pharmacologically active substance, - the quinine. Quinine is a classic antimalarial drug found in the bark of a tree that is native to South America (6,22). Artemisinin derivatives taken from the leaves of the Chinese herbal drug *Artemisa annua*, for instance, are plant products, which have been in use as traditional medicine for centuries for the treatment of malaria. The standard antimalarials, chloroquine and mefloquine, were also derived by modifying the basic chemical structure of quinine (22,23). The development of plant-based drugs that are

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used to treat this devastating disease, therefore, should be given utmost priority.

A number of studies have been conducted on the *in vitro* evaluation of the anti-malarial activity of Ethiopian traditional medicinal plants. It has been reported that extracts from plants such as *Hagenia abysinica, Berssama abysinica*, *Artemesia afra*, *Artemesia rehan* and *Ajuga remota* (24), as well as *Withania somenifer*, and *Vernonia amygdalina* (25), have significant antimalarial activity against *P.falciparum.* An *in vitro* study previously conducted on extracts from the root of *Asparagus africanus* has shown a counter activity against four different malaria schizont strains (26). Two antiprotozoal compounds, a sapogenin (muzanzagenin) and lignan ((+) nyasol) were also isolated and were reported to be responsible for the antimalarial activity (26). Other *in vitro* studies on African medicinal plants have also indicated promising antiplasmodial activities (27, 28) though no remarkable *in vivo* studies have been reported so far to strengthen the preclinical study profile. Therefore, this study aims at investigating the *in vivo* antiplasmodial activity of extracts from a traditionally used medicinal plant, *Asparagus africanus* Lam. which is extracted from Liliaceae roots and aerial parts

Methods

***Plant collection and sample preparation*:** The roots and aerial parts of *Asparagus africanus,* were collected from the vicinities of Shashamane and Awassa towns (about 270 km south of Addis Ababa), where they are used as traditional treatment for malaria. The Voucher specimens (Herb No, AA-2163) were deposited at the Herbarium of the Department of Drug Research (DDR) at the Ethiopian Health and Nutrition Research Institute (EHNRI). The plant parts were garbled and dried in the processing room, and were then powdered and kept at room temperature in a well-closed and amber coloured bottle until extracted.

The air-dried and powdered plant materials (100g) were extracted by maceration with 80% methanol for three consecutive days at room temperature. The extracts were then filtered and concentrated under vacuum in a rotary evaporator to yield 13%- 28% of gummy residue, as extract of plant parts. The extracts were kept in a tightly closed bottle in a refrigerator until used for anti-malarial testing.

***In vivo antimalarial tests*:** Tests were performed in a 4-day suppressive standard test using the methods of David, Thurston and Peters (2,6). The plasmodium species, that is most widely employed in rodent malaria parasite-*Plasmodium berghei* (chloroquine sensitive strain), was used to infect Swiss albino mice for a four-day suppressive test. The *P. berghei* was subsequently maintained in the laboratory by serial blood passage from mouse to mouse. For the study, a donor mouse with a rising parasitemia of 20% was sacrificed and its blood was collected in a slightly heparinized syringe from the auxiliary vessels. The blood was diluted with Trisodium Citrate (TC) medium so that each 0.2 ml contained approximately 10^7 infected red cells (2,6). Each animal received inoculums of about 10 million parasites per gram body weight, which is expected to produce a steadily rising infection in mice.

Male Swiss albino mice belonging to strains maintained at the EHNRI animal sanctuary were used. The mice were allowed to acclimatize to the laboratory environment under a controlled temperature of 20^0 C and at optimum humidity for at least three days before being subjected to the experiments (2). All the experiments were carried out in a calm laboratory setting that has ambient illuminations and a temperature that is close to those in the animal sanctuary (2). The mice were divided into five groups of six each, and which were all infected with malaria parasites. Three of the groups were made to receive extract treatments, while the fourth group received the vehicle (negative control) and the fifth received chloroquine (the standard antimalarial drug).

The infection of the recipient mice was initiated by needle passage of the above mentioned parasite preparation, from the donor to healthy test animals via an intraperitoneal route (2,6). Therefore, *P. berghei* infected red blood cells were intraperitoneally injected into the mice from the blood diluted with TC medium so that each 0.2 ml had approximately 10^6- 10^7 infected red cells (parasite per kg of body weight). Each mouse was infected with single inoculums of 0.2 ml blood.

The 4-day procedures were started on Mondays while blood smears for parasitemia were collected on Fridays. The infected mice were weighed, randomly divided into treatment, vehicle and standard drug groups. The treatment group, sub divided into sub groups 1, 2 and 3, received extracts from the *A. africanus* root (200 mg/kg, 400 mg/kg and 600 mg/kg). Group 4 received the vehicle (5 drops 10% Ethanol in double distilled water); while the fifth group received chloroquine 10 mg/kg, daily for 4 days, starting on the same day as that of the parasite inoculation. The same study was repeated on *A. africanus* aerial parts (200 mg/kg, 400 mg/kg and 600 mg/kg).

The plant extracts were administered through intra gastric route using the stomach tube to ensure the safe ingestion of the extracts and the vehicle. The dose levels of the extracts were selected from a pilot study carried out in mice, and which is also based on information obtained from literature on traditional methods of usage of these plants (21). The extracts were dissolved to the indicated suitable dose levels in solution and suspension, the later requiring the addition of 5 drops of 10% ethanol in 10 ml double distilled water and the vehicle (5 drop 10% Ethanol and double distilled water). All were given daily for 4 consecutive days starting 3 hours after infection, i.e. from

day 0 (D_0) to day 3(D_3), receiving a total of 4 intra gastric doses (6).

Thin smears of blood films were obtained from the peripheral blood on the tail from each mouse on day four after infection (6,29). The smears were placed on microscopic slides, fixed with methanol and stained with Gemsa at pH 7.2, for parasitemia. The microscope had an Ehrlich's eyepiece and a nose diaphragm showing about 100 red blood cells per field. The number of parasitized erythrocytes in each of the 10-50 such fields were counted three times and the average was calculated to give the Parasitemia of each individual animal. Percentage of suppression was calculated by using the following formula (2,6,29).

$$\textbf{\% Suppression} = \frac{\text{Parasitemia in negative control-Parasitemia in study group}}{\text{Parasitemia in negative control}}$$

The body weights of the mice were measured to observe whether the test extracts of *A. africanus* roots and aerial parts prevented the weight loss that is commonly reduced with increasing parasitemia in infected mice. The weights were taken on D0 and D4.

The Packed Cell Volume (PCV) was measured to predict the effectiveness of the test extracts using the modified Wintrobe's Method (29). Blood from the tail of the animals was then drawn and duplicate and triplicate determinations were done by measuring the relative volume of the blood occupied by erythrocytes, using the relation:

$$\text{Packed Cell Volume} = \frac{\text{Volume of erythrocytes in a given volume of blood}}{\text{Total blood volume}}$$

PCV is a measure of the proportion of red blood cells to plasma. This test was done from each mouse just before infection and on the 5th day of the infection.

The daily measurement of rectal temperature was also undertaken to predict the effectiveness of the test extracts. It is theoretically accepted that the body temperature of mice decreased in a rapid manner with increasing parasitemia, contrary to the situation in human subjects. The test extracts are determined by observing the protective effect of the extracts against the rapid fall in temperature (30). The study as well as the control animals were checked for a reduction in body temperature, by using rectal thermometers conducted daily for six consecutive days starting from the day prior to parasite inoculation.

Acute and sub acute toxicity tests were done for *A. africanus* roots and aerial parts. Four groups of mice, each group having 5 male & 5 female mice, for dose levels (1000 mg/kg, 2000 mg/kg and 3000 mg/kg), and one group for the highest dose (5000 mg/kg) were used for both *A. africanus* plant parts. The mice were acclimatized and fasted over night. The weight of each mouse was measured and calculated for all the dose levels. The extracts were given to the mice before they ate anything (empty stomachs). The test was repeated with lower doses (100 mg/kg, 200 mg/kg and 500 mg/kg) and finally with 1000 mg/kg intraperitoneally. Toxicity signs such as death, changes in physical appearance, behavioral change, and organ damage were observed for 72 hours.

***Data analysis*:** Data on parasitemia, body weight, packed cell volume and body temperature were analysed using Windows SPSS Version 8. The one-way ANOVA, the Student's t-test and independent comparison tests were used to compare results among and within groups for difference between initial and final results. All data were analysed at a 95% confidence interval ($\alpha = 0.05$).

Results

The results of the study indicated that *in vivo*, hydro alcoholic extracts of *A. africanus* displayed a very good activity against the *P. berghei* malaria parasite. The comparison analysis indicated that 200 mg/kg hydro alcoholic extract of *A. africanus* roots and aerial parts showed statistically significant difference on day 4 parasitemia level, compared to the negative control. The 400 mg/kg *A. africanus* roots and aerial parts also showed a statistically significant difference on the day 4 parasitemia level. A high level (46.12 % inhibition with the 600 mg/kg), *A. africanus* roots and a 40.73% inhibition of *A. africanus* aerial parts was also observed. Therefore, the highest level of inhibition (46.12 %) was observed in 600 mg/kg of *A. africanus* roots (Table 1).

Table 1: **Parasitemia suppressive test of hydro alcoholic extracts of *A. africanus* roots and aerial parts against *P. berghei* in mice, 2005**

Test substances	Dose mg/kg	% parasitemia	% inhibition
1. *A. africanus* roots	200	35.27±0.14	13.62
	400	29.46±0.48	27.84
	600	22.00±0.11	46.12
Vehicle	1 ml (-)	40.830.29	0.00
Chloroquine	10 (+)	0.00	100.00
2. *A. africanus* aerial parts	200	45.25±0.29	13.47
	400	40.45±0.21	22.67
	600	31.00±0.24	40.73
Vehicle	1 ml (-)	52.30±0.38	0.00
Chloroquine	10 (+)	0.00	100.00

P<0.05 Each result is a mean of 6 mice ± controls

The test extracts of *A. africanus* roots and aerial parts prevented a loss of body weight in infected mice with increasing parasitemia. The comparison analysis indicated that the extracts significantly prevented weight loss at all dose levels compared to the controls. However, the increase in body weight was not found to be dependent on dose levels (Table 2).

Table 2: **Body weight of *P. berghei* infected mice after the administration of *A.africanus* hydro alcoholic extracts, 2005.**

Test substances	Dose mg/kg	Wt. D-0	Wt. D-4	% change
1. *A. africanus*	200	30.26±0.33	32.20±0.97	6.41
roots	400	27.30±0.38	28.50±0.26	4.40
	600	32.30±0.33	33.20±0.43	2.78
Vehicle	1 ml	29.40±0.34	28.64±0.47	-2.59
Chloroquine	10	29.24±0.55	29.84±0.48	1.50
2. *A. africanus*	200	29.97±1.30	31.07±0.60	3.67
aerial parts	400	29.78±0.42	30.55±0.66	2.59
	600	32.58±0.48	32.90±0.54	0.98
Vehicle	1 ml	34.38±0.61	33.20±0.43	-3.43
Chloroquine	10	29.66±0.25	30.10±0.27	1.48

Day 0 = day infection was initiated P<0.05
day 4 = 5th day of infection Each result is with a mean of 6 mice

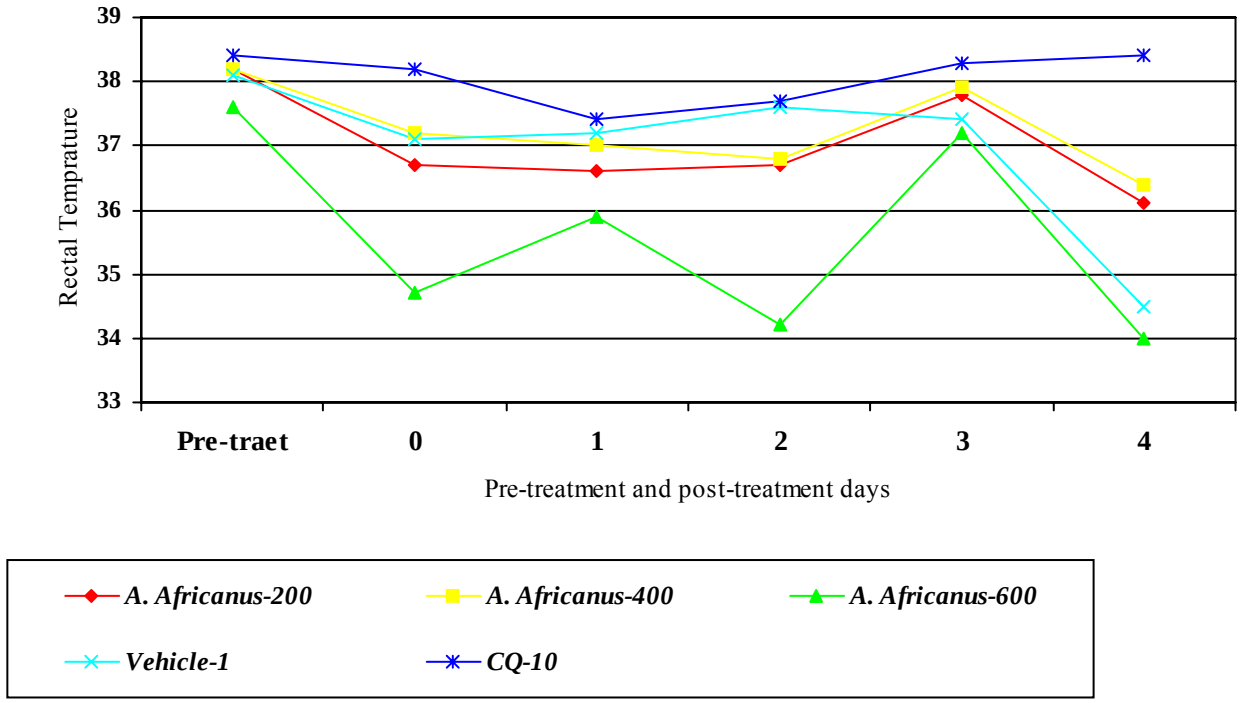

Figure 1: **Rectal temperature test for *A. africanus* hydroalcoholic extract of root against *P. bergei* in mice, 2005**

The analysis of Packed Cell Volume (PCV) on day 4, and an examinations of the rectal temperature indicated that the hydro alcoholic extract of both roots and aerial parts of *A. africanus* failed to significantly prevent the reduction in PCV and body temperature, although, some what lesser percentages of reduction and significant preventive effects were observed, compared to the controls at only a few dose levels.

The hydroalcoholic extracts of *A. africanus* roots and aerial parts showed no lethality in up to 5,000 mg/kg of doses, when administered through intragastric routes (which is 25 minimum effective dose, 200 mg/kg). Similarly, intraperitoneal administration of the same extracts in doses of up to 1000 mg/kg did not produce lethality. The mice were monitored for over a week but no sign of toxicity was observed. Gross behavioural and physical observations revealed no lacrimation, no urination, no muscle weakness, no sedation and no convulsion. They were physically active.

Table 3: **packed cell volume tests of hydro alcoholic extracts of *A. africanus* roots and aerial parts against *P. berghei* in mice, 2005.**

Test substances	Dose mg/kg	D-0 PCV	D-4 PCV	% reduction
1. *A. africanus*	200	52.61±0.32	43.24±0.42	17.81
roots	400	51.24±0.30	43.86±0.22	14.40
	600	53.42±0.31	43.73±0.41	18.40
Vehicle	1 ml	52.02±0.25	42.34±0.37	18.61
Chloroquine	10	51.38±0.34	48.27±0.45	6.05
2. *A. africanus*	200	52.83±0.03	43.37±0.31	17.91
aerial parts	400	51.80±0.44	43.6600.14	15.71
	600	53.32±0.34	43.08±0.70	19.20
Vehicle	1 ml	53.26±0.33	42.97±0.63	19.32
Chloroquine	10	52.68±0.55	48.05±0.24	8.79

P<0.05
Day 0 = day infection initiated Each result is a mean of 6 mice
Day 4=5th day of infection

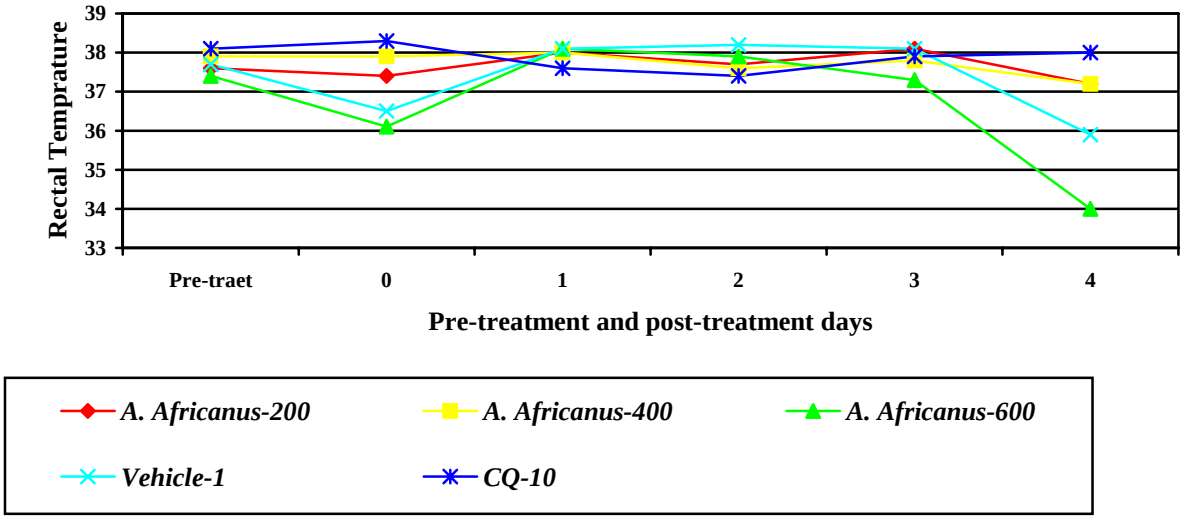

Figure 2: **Rectal temperature test for *A. africanus* hydroalcoholic extract of aerial part against *P. berghei* in mice, 2005.**

Discussion

Although primate models provide a better prediction of efficacy in human than the rodent models, the latter have also been validated through the identification of several conventional antimalarials, such as chloroquine, halofantrine, mefloquine and more recently artemisinin derivatives (6). *P. berghei* are used in the prediction of treatment outcomes. Hence, it was an appropriate parasite for the study. As this parasite is sensitive to chloroquine, this drug was employed as a standard drug in this study.

The 4-day suppressive test is a standard test commonly used for antimalarial screening, and the determination of percent inhibition of parasitemia is the most reliable parameter. A mean group parasitemia level of less than or equal to 90% that of mock-treated control animals usually indicates that the test compound is active in standard screening studies (2). Therefore, it is clear from the result (table 1) that in *P. berghei* infected mice treated with the extracts of *A. africanus* roots and aerial parts, the percentage of parasitemia measured changed significantly from those in the control animals. This significant suppression of parasitemia by the hydroalcoholic extract of *A. africanus* on day 4 was also in agreement with that shown for a water extract employed against four different malaria schizont strains *in vitro* (26). The observed antimalarial activity is consistent with the traditional use of the plant as a herbal medication against the disease in south Ethiopia (21).

When a standard antimalarial drug is used in mice infected with *P. berghei*, it suppresses parasitemia to non-detectable levels (23), which is in agreement with the effects of chloroquine in this study. The observed protection of the infected animals from loosing body weight by the roots extract in the present study is in conformity with previous studies (30). The much higher increment in body weight as compared even to that of

chloroquine might be attributed to the presence of several nutrients and immunomodulatory substances, in addition to the anti-parasitic activity (31).

However, although the results clearly indicated that the test extracts of *A. africanus* roots and aerial parts significantly prevented weight loss at all dose levels compared to the controls, the increase in body weight is not consistent with increasing dose of the extracts. The percentage change (% preventive effect) declines with increasing dose, which is consistent with both plant parts. It may possibly indicate the appetite suppressive effects of the extracts, which would reasonably increase with increasing dose. This in turn can affect the feeding capability of the mice and could cause a relative reduction in body weight, which could be indicative of toxicity. However, the toxicity tests of the present study do not indicate any other toxicity manifestation at reasonably higher doses and no death was observed before five days.

In untreated mice, the parasite count increased and the hematocrit packed cell volume (PCV) decreased markedly from day to day until the death of the animal, which was also observed in previous studies (15). Although lesser percentage reduction in PCV and rectal temperature were observed in the present study with the plant extracts and significant preventive effects were observed at some dose levels, the overall effects were not consistent and conclusive, both in the case of PCV and the rectal temperature.

In general, if the lethal dose (LD_{50}) of the test substance is three times more than the minimum effective dose (MED), the substance is considered a good candidate for further studies. It was also suggested that oral administration is about 100 times less toxic than intraperitoneal (30). However, the hydro alcoholic extracts showed no lethality to mice at 5,000 mg/kg, which is 25 times the MED and no gross behavioural and physical changes were revealed. Intraperitoneal administration also did not produce lethality at doses of up to 1000 mg/kg. It was difficult to give higher doses because of the viscosity of the extracts (being unable to pass through intraperitoneal needle). Therefore, observations that no death with upto an oral dose of 5000 mg/kg and an intraperitoneal dose of 1000 mg/kg could indicate that the test extracts are very safe. This could also explain the safe use of the plant by the local people, who have been using it in traditional treatment of malaria, in Southern Ethiopia.

From the present study, it can be concluded that the hydroalcoholic extracts of the root and aerial parts of *Asparagus africanus* have shown parasite suppressive effects on *P.berghei* infected Swiss albino mice in a dose-related fashion, whereas the effects on Packed Cell Volume and body temperature are inconclusive.

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