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Evaluation of *In Vivo* Antiplasmodial and Toxicological Effect of *Calpurnia aurea, Aloe debrana, Vernonia amygdalina* and *Croton macrostachyus* Extracts in Mice

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Abstract

Malaria is still a major public health problem in the world causing high morbidity and mortality in sub-saharan Africa mainly on less than five years of age. Thus, there is still a need to search for new and more effective antimalarial drugs against drug-resistant Plasmodium strains. Natural products have been the main source for the search of new antimalarial drugs. This study evaluates the antiplasmodial activity of some selected medicinal plant traditionally used for the treatment of malaria in Ethiopia. The aqueous and methanol crude extracts were prepared by cold maceration technique. The acute toxicity of the extracts was conducted on mice according to OECD guide line. The antiplasmodial activity of each plant extract was assessed by the classical 4day Suppressive test and The body weights and survival time was also recorded throughout the follow-up period. The result of 4-day suppressive test revealed that the methanol and aqueous extracts of *Croton macrostachyus*, *Vernonia amygdalina, Calpurnia aurea*, and, the leaf latex of *A. debrana* had antiplasmodial activity. All the extracts showed a significant ($p<0.01$) parastemia inhibition at a dose of 400 mg/kg compared to negative control group. The highest antiplasmodial activity was recorded from the leaf extracts of *V. amygdalina* (42.26%) at 400 mg/kg dose. All the mice treated with the tested plant extracts showed protection of the mice from body weight loss. The acute toxicity study showed there was no mortality recorded at a dose of 5000 mg/kg. The result of this study indicates that the plants have antimalarial activities. This confirms the traditional claims utilization of the plant for malaria treatment. It needs further activity guided isolation and characterization and antimalarial activity and toxicity studies.

Keywords

Amidalina • Medicinal plants • Anti malaria

Introduction

Malaria is a life-threatening mosquito-borne infectious disease causing a major public health problem in the world mainly on children under five years of age [1,2]. The disease is widespread in the tropical and subtropical regions that exist in a broadband around the equator. This includes much of Sub-Saharan Africa, Asia, and Latin America [1] Nearly half of the world's population is at risk of malaria with around 212 million new cases and 429,000 deaths annually approximately 90% of both cases and deaths occurred in Africa [1,3]. In Ethiopia malaria is ranked as the leading communicable disease resulting in 29% deaths in most part of the country [1,3-6]. Malaria has a major negative effect on economic development [7]. In Africa, it is estimated to result in losses of US$12 billion a year due to increased healthcare costs, lost ability to work, and negative effects on tourism [8].

Expanding access to effective antimalarial drugs worldwide has helped reduce the impact of malaria; however, the emergence and spread of drug resistance to commonly used chemotherapeutics are contributing to rising burden [1,9]. Parasite resistance to artemisinin and mosquito resistance to insecticide has been detected in various countries [10-12]. In Ethiopia, malaria control has been complicated due to the resistance of the parasite to the currently available drugs [4,13].

Due to the devastating nature of malaria and the rapid spread of resistance parasite, there is still an urgent need to search for new and more effective antimalarial drugs. The WHO has warned that no alternatives to artemisinin are likely to become available for several years [1]. Medicinal plants have been used to treat malaria for thousands of years and are the source of modern antimalarial drugs, many of the present antimalarial drugs were discovered from plants such as Chloroquine and Artemisinin [14]. Until now, the world has relied on medicinal plants for the best malaria drugs, mainly in developing countries where modern drugs are not often available the majority of the populations use medicinal plant in many health care systems. Thus, new drugs are required against drug-resistant Plasmodium strains.

Therefore, the present study evaluates the *in vivo* antiplasmodial activities and toxicological effect of Aloe debrana, *Vernonia amygdalina, Calpurnia aurea*, and *Croton macrostachyus* crude extracts in experimental animals.

Materials and Methods

Reagent and chemicals

Absolute methanol (Reagent Chemical Limited, China), Giemsa stain 10% (Shenyang Xin Guang, China), Chloroquine phosphate pharmaceutical standard (Sigma-Aldrich) were used in the study. All chemicals used compiled with the required standard and were of analytical grade.

Plant collection and extraction

Plants have been selected based on their use in folk medicine for malaria treatment and their promising effect from previous studies. The Fresh exudates of *A. debrana* and the bark of *C. macrostachyus* were collected from north shewa, Amhara reigon whereas the leaf of *V.*

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amygdalina and *C. aurea* was from Delo menna, Oromia and Sidama Zone of SNNP region, Ethiopia respectively. The species were identified and voucher specimens were deposited at the herbarium of Traditional and modern medicine research directorate, Ethiopian Public Health Institute EPHI, where the study was conducted. The plant materials were air-dried at room temperature under shade and ground into powder by using a grinding mill whereas the leaf latex of *A. debrana* was collected in a clean plastic container and air dried at room temperature under shade.

The powdered leaves of *C. macrostachyus*, *C. aurea*, and *V. amygdalina* were exhaustively extracted with 80% methanol and water separately by a cold maceration method. The alcoholic extracts after being filtered, got concentrated under reduced pressure by Rota evaporator (Buchi Rota vapor vacuum pump, Germany) at 40°C to generate the crude ethanolic extract. The concentrated crude extracts were kept in an oven (Gallenkamp, England) at the temperature not exceeding 40°C to dry them. The water extract was filtered with cotton and Whatman filter paper (15.0 cm size). The filtrates were freeze-dried in a lyophilizer to yield a crude extract. All the extracts were stored in screw cap vials and kept in desiccators until they were used for the experiment.

Experimental animal

Healthy adult Swiss albino mice weighing between 25 g and 28 g used for this study were obtained from animal breeding unit of Ethiopian Public Health Institute. The animals were housed under standard laboratory conditions, provided with standard diet and drinking water ad libitum. They were allowed to acclimatize for one week. Animal experiments were approved by Scientific and Ethical Review Office (SERO) of Ethiopian Public Health Institute (EPHI) and carried out in accordance with international guidelines for care and use of laboratory animals

Acute toxicity test

Acute oral toxicity was done according to OECD guide line [15]; female Swiss albino mice were randomly divided into five groups of three animals per cage. Animals were fasted for four hours prior to dosing. Then, the mice were given the extract orally at a dose of 5000 mg/kg. The animals were observed individually after dosing during the first 30 minutes, periodically during the first 24 hours and daily thereafter for a total of 14 days. The mice were observed for gross behavioral changes such as changes in skin and fur, eyes and mucous membranes, convulsions, salivation, diarrhea, lethargy, sleep and other signs of toxicity manifestation [15].

In vivo antiplasmodial assay

The antiplasmodial activity of each plant extract was assessed by the classical 4 day Suppressive test to evaluate the antimalarial activity of each extracts on early infections. In this study *in vivo* model was employed as it takes into account the possible pro-drug effects and possible involvements of immune system in eradication of infection [16, 17]. Chloroquine sensitive Plasmodium berghei (ANKA strain) was used hence it was an appropriate parasite for the study. The parasite was maintained by serial blood passage in mice and blood sample was collected from donor mouse by cardiac puncture with a parasitaemia of about 25%-30%. Then each mouse was inoculated intraperitoneally with 0.2 ml of blood suspension containing about 106 or 107 infected erythrocytes. The infected animals were randomly divided into four groups for each plant, containing five mice per cage. Plant extracts were administrated within 3 hours of post-inoculation of mice with the parasite at a dose of 200 mg/kg and 400 mg/kg, the extract was dissolved with 0.5 ml of distilled water, orally given via gavage daily for 4 consecutive days. Chloroquine was used as a standard drug and negative controls were given distilled water as a vehicle.

On the fifth day (day 4) blood smear was prepared from sample taken from tail of each mouse, fixed with methanol and stained with 10% Geimsa solution. Blood films were examined under microscope at 100 × magnification to evaluate the parasitemia suppression of each extract with respect to the negative control groups. The percentage parasitaemia was determined by counting the number of parasitized erythrocytes out of 300 erythrocytes in random fields of the microscope in triplicate. The average percentage of suppression was calculated for each extract by comparing the parasitaemia present in infected controls with those of test mice.

Body weight and survival time

The body weights of the mice were taken to find out whether the test extract prevented the weight loss that is commonly observed with increasing parasitaemia in infected mice. The weight was taken at the first (Day 0) and last day (Day 4) of experiment. Mean survival time was also determined by recording the number death from the time of inoculation of the parasite for each mouse in the treatment and control groups throughout the follow-up period.

Data analysis

The Results were analyzed statistically using one-way ANOVA (SPSS 15.0 Inc., USA) and expressed as mean ± standard error of mean (M ± SEM). The data were considered significant at $P<0.05$. Percent parasitaemia and percent suppression were also calculated [17,18].

Results

Acute toxicity

The results of acute toxicity study shows the methanol and aqueous extracts of *C. macrostachyus*, *V. amygdalina*, *C. aurea*, and, the leaf latex of A.debrana caused no mortality in mice at a dose of 5000 mg/kg within the first 24 hrs as well as for the following 14 days. Gross physical and behavioral observations of the experimental animals also revealed no visible signs of overt toxicity; no lacrimation, no urination, no muscle weakness, no sedation and no convulsion, that are commonly occurred in toxicity. The mice were physically active moving and feeding. Thus, all plant extract showed no inherent acute toxicity signs at a maximum dose of 5000 mg/kg indicating that the medium lethal dose could be greater than 5 g/kg body weight in mice.

In vivo antiplasmodial activity

The suppressive effects all plants were evaluated at dose levels of 200 mg/kg and 400 mg/kg body weight using the Chloroquine diphosphate as a positive control. The result of 4-day suppressive test indicated that the methanol and aqueous extracts of *C. macrostachyus*, *V. amygdalina*, *C. aurea*, and, the leaf latex of *A. debrana* had antiplasmodial activity against chloroquine sensitive *P. berghei* infected Swiss albino mice, the percentage parasitemia determined for all tested plant extracts were low relative to the negative control showing that the plant extracts are active.

The aqueous leaf extract of *V. amygdalina* at a dose of 400 mg/kg showed the highest chemo suppressive effect (42.26%) which was statistically significant ($p<0.01$) compared to negative control group. *V. amygdalina* at a dose of 200 mg/kg also showed parasitemia suppression (10%) but it was not significant (Table 1).

Test substance	Dose (mg/kg)	%Parasitemia ± SEM	% Inhibition	Survival time ± SEM
NC	Vehicle	30.57 ± 1.79	0.00	8.60 ± 0.40
V. amygdalina	400	17.64 ± 1.25**	42.26	9.40 ± 0.68
CQ	25	0.00 ± 00***	100.00	ND

Note: *** = p-value<0.001, ** = p-value<0.01

Table 1. Anti-plasmodial activity of *V. amygdalina* aqueous leaf extract against P. berghei in Swiss albino mice.

Results are expressed as mean ± SEM (n=5). Significant *** at p-value<0.001; **p-value<0.01 compared to control group. NC: Negative control, CQ: Chloroquine phosphate, ND: No death

The level of suppression of leaf latex of *A. debrana* at concentrations of 200 mg/kg and 400 mg/kg following the 4-day test was 39.98% and 13.33%

respectively. But only *A. debrana* at concentrations of 200 mg/kg showed statistically significant difference (p<0.001) in reducing parasite load as compared to the negative control mice (Table 2).

Test substance	Dose (mg/kg)	%Parasitemia ± SEM	% Inhibition	Survival time ± SEM
NC	Vehicle	30.57 ± 1.79	0.00	8.60 ± 0.40
A. debrana	200	18.35 ± 0.89***	39.98	9.40 ± 0.51*
A. debrana	400	26.49 ± 1.97	13.33	9.20 ± 0.58
CQ	25	0.00 ± 0.00***	100.00	ND
Note:*= p-value<0.001				

Table 2. Anti-plasmodial activity of A.debrana latex against P.berghei in Swiss albino mice.

Results are expressed as mean ± SEM (n=5). Significant *** at p-value<0.001; *p-value<0.05 compared to control group. NC: Negative control, CQ: Chloroquine phosphate, ND: No death

The aqueous extract of *C. macrostachyus* bark was also significantly active (p<0.001) against P. berghei at a dose of 400 mg/kg (37.51%) compared to the negative control mice however there was no significant difference in parasitemia reduction at concentration of 200 mg/kg (Table 3).

Test substance	Dose (mg/kg)	%Parasitemia ± SEM	% Inhibition	Survival time ± SEM
NC	Vehicle	21.94 ± 1.21	0.00	9.20 ± 0.58
A. debrana	200	18.82 ± 1.14	39.98	9.00 ± 0.32
A. debrana	400	13.71 ± 0.56***	13.33	9.80 ± 0.58
CQ	25	0.00 ± 00***	100.00	ND
Note:*= p-value<0.001				

Table 3. Anti-plasmodial activity of *C. macrostachyus* aqueous Bark extract against P. berghei in Swiss albino mice.

Results are expressed as mean ± SEM (n=5). Significant *** p-value<0.001 compared to control group. NC: Negative control, CQ: Chloroquine phosphate, ND: No death

C. aurea methanol leaf extract showed relatively parastemia suppression against P. berghei compared to other plants; it have 24.04% (p<0.001) suppression at 400 mg/kg and There was no significant difference in parasiteamia suppression in mice treated with 200 mg/kg (6.94%) compared to normal control group (Table 4).

Test substance	Dose (mg/kg)	%Parasitemia ± SEM	% Inhibition	Survival time ± SEM
NC	Vehicle	25.42 ± 1.37	0.00	9.20 ± 0.58
C. aurea	200	23.66 ± 0.95	6.94	9.00 ± 0.32
C. aurea	400	19.31 ± 1.67**	24.04	9.80 ± 0.58
CQ	25	0.00 ± 00***	100.00	ND
Note:*=p-value<0.001, **=p-value<0.01				

Table 4. Anti-plasmodial activity of *C. aurea* methanol leaf extract against P. berghei in Swiss albino mice.

Results are expressed as mean ± SEM (n=5). Significant **p-value<0.01 compared to control group. NC: Negative control, CQ: Chloroquine phosphate, ND: No death

Effects of extracts on body weight

The effect plants extracts on body weight of P. berghei -infected mice before and after the administration of the plant extract are summarized in Table 5. The leaf latex of *A. debrana* and the aqueous leaf extract of *V.amygdalina* significantly protected the mice from body weight loss (12.29% and 10.53% respectively) at a concentration of 200 mg/kg as compared to negative control group (7.05%) after 4-day suppressive test while the relative body weight gain *A. debrana* at 400 mg/kg (2.4%) and aqueous extract of *V. amygdalina* leaf at 400 mg/kg (6.89%) was lower as compared with the negative control groups (7.05%). The relative body weight gain for mice treated with *C. macrostachyus* 200 and 400 mg/kg was also higher 18.34% and 17.97% respectively while the negative control was only 16.27%.

Results are expressed as mean ± SEM (n=5). NC- negative control, CQ- chloroquine phosphate, Significant *** at p-value<0.001; *p-value<0.05 compared to control group.

Test substance	Parts used	Dose(mg/kg)	Body weight ± SEM		% Change
			Day 0	Day 4	
V. amygdalina	Leaf	NC	25.54 ± 1.17	27.34 ± 1.47	7.05
		200	28.30 ± 0.88	31.28 + 0.60	10.53
		400	27.78 ± 0.68	29.72 ± 0.89	6.89
		CQ	24.90 ± 0.72	27.24 ± 0.76	9.4
A. debrana	Latex	NC	25.54 ± 1.17	27.34 ± 1.47	7.05
		200	26.68 ± 1.65	29.96 ± 1.53	12.29
		400	25.02 ± 0.90	25.62 ± 0.70	2.4
		CQ	24.90 ± 0.72	27.24 ± 0.76	9.4
C. macrostachyus	Bark	NC	26.30 ± 0.34	30.58 ± 0.68	16.27
		200	27.26 ± 0.56	32.26 ± 0.79***	18.34
		400	26.16 ± 0.32	30.86 ± 1.02**	17.97
		CQ	26.34 ± 0.50	30.44 ± 0.48***	15.57
C. aurea	*C. aurea*	NC	28.18 ± 0.84	30.54 ± 1.15	8.37
		200	29.50 ± 0.77	30.78 ± 0.85	4.34
		400	28.70 ± 0.83	30.18 ± 0.41	5.16
		CQ	24.90 ± 0.72	27.24 ± 0.76	9.4
Note:**=p-value<0.01, ***=p-value<0.001					

Table 5. Effect of plant extracts on body weight of P. berghei-infected mice before and after the administration of the plant extract.

Discussion

Determination percent suppression, percent parasitaemia, and mean survival time of the mice treated with plant extract was done by using the standard 4-day suppressive test, which mainly evaluates the antimalarial activity of candidate drugs on early infections commonly used for antimalarial screening. In this method, determination of percentage inhibition of parasitemia is the most reliable parameter. In this study, the standard 4-day suppressive test was used to evaluate the antimalarial activities four selected medicinal plant which are traditional claimed to have antimalarial effect in Ethiopia.

The evaluation of antiplasmodial activity of aqueous leaf extract of *V. amygdalina* on early malaria infection, in a four-day suppressive test, have significant parasitaemia suppression at the highest dose by showing the highest parasitaemia, and chemosuppression, *A. debrana* also showed

high parasitaemia suppression with the longest survival time compared to all other doses and the negative control. Parasitaemia suppression effect of extracts is probably due to the presence of high concentration of anthraquinones and other quinoid compounds in *A. debrana* that are the characteristic constituent of the genus Aloe [18,19] and presence of flavonoids, tannins, alkaloids, terpenes, saponins and glycosides in *V. amygdalina* [20].

The antimalarial activity of *V. amygdalina* against CQ-susceptible strain of P. berghei was previously reported. The ethanolic extract of *V. amygdalina* was significantly active against P. berghei in a dose-dependent manner with maximum activity observed at 1000 mg/kg (% inhibition of 82.3 %) [21]. V. glaberrima which is in genus Aloe also showed a dose-dependent chemo-suppression by the three extract groups 300, 150 mg/Kg and 75 mg/Kg at 62.1%, 54.3% and 32.2% respectively [20]. Aloin from *A. debrana* displayed a significant antimalarial activity at doses of 25, 50 and 100 mg/kg with chemosuppression values of 48.38, 69.66 and 78.31%, respectively [22]. Another study by Deressa.T et al, reported the methanol extract of *A. debrana* induced 73.95% suppression, whereas its water extract exerted 54.36% suppression of parasitaemia [18].

The aqueous extract of *C. macrostachyus* bark was also showed significant parasitemia suppression. Previously *in vivo* antimalarial activity of methanol extract of *Croton macrostachyus* leave was 17.42% and the water extract was 4.6% [23] which is lower than the current result suggesting that the bark have more effective than the leaf. This plant possess antimalarial compounds like diterpenoids, triterpenoids, alkaloids, flavonoids, lignoids and proanthocyanidins [24]. Therefore, the antiplasmodial activity observed in this study may be because of these bioactive compounds [23,24].

Relatively *C. aurea* methanol leaf extract showed lower parastaemia suppression against P. berghei compared to other plant extract tested in this study. This species were reported to cause 36.8% ($P<0.001$) *in vivo* suppression at 60 mg/kg dose in 4-day suppressive, tests [24]. This parasitaemia suppression effect of the extract may be attributed to the presence of presence of alkaloids, flavonoids, terpenoids, phenols, phytosteriods, saponnins, tannins and cardiac glycosides.

The acute toxicity test in this work shows all tested plant extracts were non-toxic to test mice at all dose levels tested as they did not show signs of acute toxicity with in the test period. This seems to agree with other reported studies that the plant had a no sign of toxicity [18,21]. This result justifies the traditional use of the plant in malaria treatment. Further work is suggested to be performed to isolate, identify and characterize the active principles from the plant.

Conclusion

The antimalarial activities of methanol and aqueous extracts of *C. macrostachyus*, *V. amygdalina, C. aurea*, and, the leaf latex of *A. debrana* were tested. All the tested extracts showed significant antimalarial activities as compared to the negative control group ($p<0.05$). Better antimalarial activities were observed on *V. amygdalina* 400 mg/kg, *A. debrana* 200 mg/kg, *C. macrostachyus* 400 mg/kg, and *C. aurea* 400 mg/kg with mean percentage suppression of 42.26%, 39.98%, 37.51% and 24.04% respectively. Thus, this plants may possesses significant antimalarial properties which need further activity guided isolation and characterization to facilitate standardization and to determine possible new antimalarial compound.

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