ORIGINAL RESEARCH

Evaluation of the Antimalarial Activity of Ethanol Extracts of the Leaves of Three Plant Species Collected from Yayu Coffee Forest Biosphere Reserve, Southwest Ethiopia

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Objective: In the attempt of searching for potential plant derived antimalarial medicines, the aim of the present study was to examine In vivo antimalarial efficacy of crude ethanol extracts of the leaves of *Croton macrostachyus, Ruta chalepensis* and *Vernonia amygdalina* using chloroquine (CQ) sensitive strains of *Plasmodium berghei* in Swiss albino mice.

Methods: To ascertain the safety level of the plant materials, crude extracts underwent oral acute toxicity assessments whereby mice received up to a single dose of 3000 mg/kg. Peter's four day standard antimalarial suppressive test was carried out to determine growth inhibition of parasitemia at tested doses of 400, 600, and 800 mg/kg of the extracts. Survival time of experimental mice and preliminary phytochemical screenings of the extracts were also done according to the standard procedures.

Results: Extracts of the plant materials did not produce severe acute toxic effects in mice that received up to 3000 mg/kg in a single dose. Although complete clearance was not recorded, extracts of the plant materials produced dose dependent suppression of the parasitemia. The highest growth inhibition recorded was by extract of *V. amygdalina* (61.44%) followed by *C. macrostachyus* (59.3%) at 800 mg/kg of tested doses. Whereas, complete parasitemia clearance was attributed in mice treated with 25 mg/kg of CQ. In addition, survival time of experimental mice was recorded and the result showed mice treated with the extracts lived longer than the corresponding negative controls. The phytochemical screening of the extracts revealed the presence of antimalarial active constituents such as alkaloids, saponins, cardiac glycosides, flavonoids, terpenoids, steroids, phenols, and tannins.

Conclusion: The present study, therefore, suggests that crude ethanol extracts of *C. macrostachyus, R. chalepensis*, and *V. amygdalina* are safe and rich with active secondary metabolites which have promising antimalarial effects.

Keywords: Antimalarial activity, In vivo, Yayu Biosphere Reserve

Introduction

Malaria diseases remain one of the most significant causes of morbidity and mortality in resource poor countries, especially in sub-Saharan Africa. Children below the age of five years and pregnant women living in these areas are vulnerable.[1,2] According to WHO malaria report in 2019, malaria was endemic in 90 countries of the tropical and semitropical areas and it was a cause of death of nearly half a million yearly.[3]

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In Ethiopia, malariais among the leading public health problems and it is the main causes of morbidity and mortality. *Plasmodium falciparum* and *P. vivax* are the two main species accounting for 60% and 40% of malaria cases, respectively.[4] Approximately 75% of the country is malarious and about 68% of its population live in areas at risk of malaria.[5]

Drug-resistant *Plasmodium* sp. nowadays is a major problem in malaria control campaign.[6] Resistance of *Plasmodium* parasites to the currently available antimalarial medicines has been reported from different parts of the planet. It has been documented for *P. falciparum, P. vivax* and *P. malariae*. However, *P. falciparum* resistance has been reported in all available antimalarial drugs (amodiaquine, chloroquine, mefloquine, quinine, sulfadoxine-pyrimethamine, and artemisinin derivatives).[7–9]

Therefore, it is necessary to develop immediate antimalarial treatments hopefully with the understanding of the mode of actions. One of the best sources for developing new and effective antimalarial drugs that can be easily affordable by the poor living in the malarious endemic tropical countries is traditional medicinal plants and other natural products.[10]

Many plant species continue to be used in traditional medicine for the treatment of malaria and many people depend upon such remedies because of unaffordability and inaccessibility to standard antimalarial drugs.[11] Since the history of malaria, plants used to play critical roles in treatments of the disease and related pathologies. Peruvian bark (*Cinchona* spp.) being the first effective sources of plant-based antimalarial drug and the source of the artemisinin (*Artemisia annua*) demonstrates that plant species are important sources for the discovery of new antimalarial agents.[12] WHO estimated 80% of people worldwide rely on herbal medicines for some part of their primary health care; especially in developing countries where malaria is endemic; they strongly depend on traditional medicine as a source for inexpensive treatment of this disease. However, scientific data to validate the antimalarial properties of these herbal remedies are scarce.[13,14]

Since malaria is the number one public health problem in Ethiopia and the majority of people are living in malaria risk rural areas coupled with the inaccessible and unaffordable antimalarial drugs; they strongly depend on traditional medicines particularly herbal remedies to treat the disease and disease related pathologies.[15,16] There are huge number of plant species used by the people to treat malaria in different parts of the country. Consequently, it is important that traditional medicinal plants should be investigated in order to establish their safety and efficacy as well to determine their potential as source of new plant-based antimalarial drugs.[17,18] This study thus aimed to examine the potential antimalarial activity of ethanol crude extracts of the leaves of three plant species namely *Croton macrostachyus, Ruta chalepensis* and *Vernonia amygdalina* which are collected from Yayu Coffee Forest Biosphere Reserve In vivo using CQ-sensitive strain of *Plasmodium berghei* in Swiss albino mice.

Materials and Methods
Plant Collection and Authentication
The fresh leaves of the plant material were collected from Yayu Coffee Forest Biosphere Reserve, which is located in Ilu Aba Boor zone, 550 km southwest from the capital Addis Ababa, Ethiopia during the month of January 2018. Identification and authentication of the collected plant specimen (*C. macrostachyus, R. chalepensis* and *V. amygdalina*) were done at the Ethiopian National Herbarium, Addis Ababa University, Ethiopia. The specimens were then deposited in the herbarium with the collection number of (Meu 01, 02, and 03/2018), respectively.

Preparation of the Crude Extracts
The collected fresh leaves of the plant was cleaned, cut into pieces and dried under shade. The dried leaves were then ground into coarse powder using an electrical cross bitter mill (IEC, 158VDE0660, Germany). Then, preparation of the crude extracts was carried out by cold maceration technique; soaking the plant powder in 1:12 w/v ratios with ethanol using Erlenmeyer flasks. The flasks containing the plant powder dissolved in ethanol were placed on an orbital shaker (ThermoFisher, USA) at 120 rotations per minute (rpm) for 72 h. The mixtures were filtered using gauze and then the filtrates were passed through Whatman paper number 1 (Wagtech international Ltd, England). The filtrate of the extract was removed under reduced pressure using rotary evaporator (Buchi type TRE121, Switzerland) at 60 rpm and 30°C to obtain the crude extract. Then, all the extracts were stored separately in screw capped glass bottles at −4°C until used.

Experimental Animals
Swiss albino mice 6 to 8 weeks old and weighing 25 to 32 g were obtained from Addis Ababa University and Ethiopian Public Health Institute, Ethiopia. Female mice

were used for In vivo acute toxicity test and males were used for antimalarial assay. These experimental animals were used according to the NIH Guide for the care and use of laboratory animals.[19]

Experimental Pathogen and Infection Procedure

The antimalarial evaluations of the extracts were performed using CQ-sensitive strain of *Plasmodium berghei* of the rodent malaria parasite. It was collected from Ethiopian Public Health Institute and kept alive by continuous intraperitoneal passage in mice on weekly basis. Percent parasitemia of the donor mouse was first determined; about 20 to 30% parasitemia and blood was collected through gentle cardiac puncher from the donor mouse using syringe after it has been sacrificed by chloroform. Then, 1 mL of blood was diluted with 4 mL of physiological saline (0.9%) and 1 mL of the dilution expected to contain 5×10^6 of infected erythrocytes. Therefore, each mouse was infected on day zero (D_0) intraperitoneally with 0.2 mL dilution of infected blood (standard inoculum) containing approximately 1×10^6 *P. berghei* parasitized red blood cells.[20,21]

Oral Acute Toxicity Assessments

Twenty-five healthy female mice were used to test the acute toxicity of each crude extract through oral administration. The mice were randomized into five groups of five mice per group. They were then subjected to fast overnight and the four groups were provided with 500, 1000, 2000, and 3000 mg/kg of the extract dissolving with 0.4 mL of 20% of dimethyl sulfoxide (DMSO) for each mouse respectively. The fifth group (the control) was given the same volume of the vehicle for each mouse in the group. Then, the presence and absence of acute toxic signs such as hair erection, lacrimation, reduced motor and feeding activities, urination, convulsion, and mortality within 24 h were recorded.

In vivo Antimalarial Suppressive Tests

In order to determine the antimalarial suppressive effect of the plant extracts, Peters' 4-day suppressive test was subjected using *P. berghei* infection in mice.[20] For each extract, 20 mice randomly grouped in to four categories of equal number of mice in each group were used. At the first day (D_0) of the experiment, each mouse intended for the experiment was infected by *P. berghei* parasitemia using standard inoculums (1×10^6 *P. berghei* infected RBCs) through intraperitoneal parasite inoculation.[21]

After three hours of parasite inoculation, the three groups were administered with 400, 600, and 800 mg/kg of extract by dissolving the extract with 0.2 mL of the respective vehicle for each mouse orally using a standard oral needle for four consecutive days in a 24-h schedule. The other two groups were used as a negative control and positive control which were given 0.2 mL of the same vehicle and 25 mg/kg of CQ respectively.[20,22]

Parasitological Study

On the fifth day (D_4) of the experiment, a drop of fresh blood specimen was taken from the tail snip of each mouse and thin smears using Giemsa stain were prepared for microscopy examination.[21] Parasitemia were then examined under light microscopy starting from power of 10 and then up to power 100.[22] Ten fields of visions were taken from different angles of each slide and percentage of *P. berghei* infected RBSc were calculated from the total RBSc encompassed in the fields of vision. Finally, percentage parasitemia and parasite suppression were calculated using the following two formulas as Fidock et al have described.[23]

$$\%\text{Parasitemia} = \frac{\text{Numbere of Infected RBCs x 100}}{\text{Total number of RBCs}}$$

$$\%\text{Suppression} = 100 - \left(\frac{\begin{array}{c}\text{Mean parasitemia in} \\ \text{the study group x100}\end{array}}{\begin{array}{c}\text{Mean parasitemia in} \\ \text{negative control group}\end{array}} \right)$$

Determination of Mean Survival Time of Mice

Mortality rates were supervised daily and the number of days from the time of parasite inoculation to death were recorded for each mouse in treatment and control groups throughout the follow-up observation period. Then, the mean survival time (MST) for each group was calculated using a mathematical formula as described by Mengiste et al.[24]

$$\text{MST} = \frac{\text{Sum of survival time of all mice in agroup}}{\text{Total number of mice in that group}}$$

Phytochemical Screening

The qualitative preliminary phytochemical screening of the ethanol extracts of the leaves of *C. macrostachyus, R. chalepensis* and *V. amygdalina* were made for the identification of selected antimalarial active secondary metabolites such as alkaloids, saponins, glycosides, flavonoids,

terpenoids, steroids, phenols, and tannins according to the standard procedures and operation manual.[25,26]

Statistical Analysis

SPSS version 20 statistical software was used for analysis of the data. One sample t-test was employed to calculate the mean value of parasitemia and survival time for all variables. ANOVA was also considered to compare the level of parasitemia and survival time of *P. berghei* infected mice between the control and extract treated groups at D_4 of the study, respectively. All results were presented by the mean ± SEM (standard error of the mean) and statistical significance was considered when $P<0.05$ at the 95% of confidence interval.

Results

Extract Yields

Percentage yield of crude ethanol extracts of the leaves of *C. macrostachyus, R. chalepensis* and *V. amygdalina* is presented in Table 1. A 1:12 w/v ratio of cold maceration technique of extraction was employed and yields comprised of 14 to 22% of the total plant material used.

Acute Toxicity

According to the data from Table 2, crude ethanol extracts of the leaves of *C. macrostachyus, R. chalepensis* and *V. amygdalina* did not cause death of mice within 24 h after being administered with 500, 1000, 2000, and 3000 mg/kg of the extracts. Minor gross behavioral and physical changes such as decreased feeding activities, depression, and hair erection were seen for about half a day at 2000 and 3000 mg/kg dose of the extracts received by the mice. However, they returned back to normal physical and motility conditions. They were also active during five days of inspection period.

Antimalarial Suppressive Tests

Extract treated *P. berghei* infected mice produced dose dependent and varying degree of antimalarial suppressive effects. However, complete clearance of parasitemia was not recorded. The positive control group which was treated with 25 mg/kg of CQ cleared the parasitemia at D_4 of postinfection (Table 3).

Compared to the negative control, extract treated *P. berghei* infected mice showed significant suppressive effects. It was detected that the level of *P. berghei* parasitemia in the negative control group was 46.53±1.23 while the level of parasitemia in mice administered with extracts of *V. amygdalina* at the highest dose was 17.94 ±0.31 at the fifth day postinfection. Similarly, the respective crude extracts of *R. chalepensis* and *C. macrostachyus* at a dose of 800 mg/kg produced 56.72% and 59.30% parasitemia suppressive effects, respectively when comparedwith the negative control group. However, among

Table 1 Percentage Yield of Ethanol Crude Extracts of the Leaves of *C. macrostachyus, R. chalepensis*, and *V. amygdalina*

Plant Species	Dry Powder (g)	Solvent Volume (mL)	Ratio (w/v)	Yield (g)	Yield (%)
R. chalepensis	80	1000	1:12	17.76	22.2
C. macrostachyus	80	1000	1:12	11.20	14
V. amygdalina	80	1000	1:12	12.08	15.1

Table 2 Acute Toxic Manifestation Seen After Administration of Ethanol Extracts of *C. macrostachyus, R. chalepensis* and *V. amygdalina* in Female Mice

Response	Extract Treatment (mg/kg)											
	C. macrostachyus				*R. chalepensis*				*V. amygdalina*			
	500	1000	2000	3000	500	1000	2000	3000	500	1000	2000	3000
Hair erection	A	A	P	P	A	A	P	P	A	A	A	P
Lacrimation	A	A	A	A	A	A	A	P	A	A	P	P
Decreased activity	A	A	A	A	A	A	A	P	A	A	A	P
Decreased appetite	A	P	P	P	A	A	P	P	A	P	P	P
Depression	A	P	P	P	A	P	P	P	P	P	P	P
Mortality	A	A	A	A	A	A	A	A	A	A	A	A

Abbreviations: A, absent; P, present.

Table 3 In vivo Antimalarial Suppressive Effect of Ethanol Crude Extracts of *C. macrostachyus, R. chalepensis* and *V. amygdalina* in Mice

Treatments	Dose (mg/kg)	% Parasitemia	% Suppression	*P*-value
DMSO (5%)	0.2 mL	46.53±1.23	0.00	–
V. amygdalina	400	25.47±0.61	45.26	0.74
	600	21.84±0.74	53.06	0.81
	800	17.94±0.31	61.44	0.18
R. chalepensis	400	25.17±0.94	45.90	0.78
	600	23.26±0.59	50.01	0.85
	800	20.14±0.28	56.72	0.63
C. macrostachyus	400	24.51±0.37	47.32	0.32
	600	20.43±0.25	56.10	0.04
	800	18.94±0.32	59.30	0.10
CQ	25	0.00±0.00	100.00	<0.01

Abbreviations: CQ, chloroquine; ND, not done; DMSO, dimethyl sulfoxide.

the three plant crude extracts, the highest parasitemia suppression was recorded in the *V. amygdalina* extract treated group followed by *C. macrostachyus*.

Survival Time Determination

Treatment with extracts of the plant materials improved the life expectancy of *P. berghei* infected mice while the corresponding negative control were treated with 0.2 mL of 5% DMSO died sooner (Table 4). Dose of the extracts were found associated significantly with the survival time of mice in the treatment groups.

In mice, the higher parasitemia suppression recorded, the longer they lived. Thus, parasitemia-infected mice treated with 800 mg/kg of plant material crude extracts for the three species of study plant produced the

highest percent suppression of parasitemia and lived the longest time when compared with lower dose treatments and the negative control groups.

Preliminary Phytochemical Screenings

As shown from (Table 5), the presence and absence of antimalarial active secondary metabolites among extracts of the three plant materials are demonstrated. Alkaloids, tannins and terpenoids were found from all the three extracts whereas steroids from *V. amygdalina*, and flavonoids and saponins from *R. chalepensis* were not detected.

Discussion

Although medicinal plants are assumed to be safe, potentially toxic species were discovered and therefore,

Table 4 Survival Time of Crude Ethanol Extracts of *C. macrostachyus, R. chalepensis* and *V. amygdalina* Treated *P. berghei* Infected Mice

Treatments	Dose (mg/kg)	% Suppression	Survival Time/day	*P*-value
DMSO (5%)	0.2 mL	0.00	6.20±0.85	–
V. amygdalina	400	45.26	9.20±0.58	0.66
	600	53.06	11.4±1.12	0.32
	800	61.44	14.60±0.68	0.19
R. chalepensis	400	45.90	7.00±0.32	0.39
	600	50.01	9.00±0.84	0.02
	800	56.72	10.60±0.68	0.19
C. macrostachyus	400	47.32	9.80±0.58	0.66
	600	56.10	10.60±0.68	0.19
	800	59.30	12.40±0.68	0.19
CQ	25	100.00	ND	ND

Table 5 Secondary Metabolites Identified from the Ethanol Crude Extracts of *C. macrostachyus, R. chalepensis* and *V. amygdalina*

Phytochemicals	Results		
	C. macrostachyus	*R. chalepensis*	*V. amygdalina*
Alkaloids	+	+	+
Flavonoids	+	–	+
Cardiac glycosides	+	+	–
Phenols	–	+	+
Saponins	+	–	+
Steroids	+	+	–
Tannins	+	+	+
Terpenoids	+	+	+

determination of the safety level of plant extracts needs to be considered before being used for treatment purposes.[27,28] Thus, acute toxicity tests of the crude ethanol extracts of *C. macrostachyus, R. chalepensis* and *V. amygdalina* were carried out among healthy mice up to 3000 mg/kg of the extracts orally. Slight physical and behavioral changes such as depression and hair erection were found for about half a day. However, absence of severe acute toxic symptoms such as impaired movement, listlessness, reduced motor activity, and mortality within 24 h and survival of mice during the follow-up period justifies that the extracts are safe and do not produce oral toxicity at the tested dosages. It is also an indication that the estimated oral median lethal dose (LD_{50}) is not toxic and the extracts at 6000 mg/kg body weight could be nonlethal.[28] This is also one justification why these plant species are widely used in traditional treatment of malaria in Ethiopian folk medicines.[29]

In the present study, all the three crude extracts established statistically significant ($P<0.05$) and dose dependent *P. berghei* parasitemia suppression when compared with the respective negative control. These antimalarial activity of crude ethanol extracts of the plant materials supposed to be by the presence of antimalarial active phytochemical constituents such as alkaloids, saponins, cardiac glycosides, flavonoids, terpenoids, steroids, phenols, and tannins, which are attributed in the antiplasmodial activities alone or the synergetic effects.[30–33] Many other antimalarial active plant species including the source of artemisinin (*Artemisia annua*) also possess the abovementioned secondary metabolites.[34,35]

Krettli et al in their review demonstrated that a plant-based compound is considered an active antimalarial when it produces 30% or more inhibition against parasitemia of *P. berghei* in mice. The above generalization supports the result of the present study in which all plant extracts produced significant parasite suppression by more than 45% although complete clearance of the parasite is not yet detected.[21] Similar studies on other plant species such as *Acacia nilotica*, *Morinda lucida*, *Otostegia integrifolia*, *Clerodendrum myricoides*, *Dodonea angustifolia* and *Aloe debrana* and *Asparagus africanus* also reported that extracts of the plant species produced dose dependent and significant parasitemia growth inhibition against *P. berghei* in mice.[36–40] The present study, therefore, could be evidence demonstrating the relevance of these herbal medicines that are promising in the search for new antimalarial medicines.

Several plant-based medicinal derivatives have identified antiplasmodial mode of actions to exert antimalarial activity by interfering with the parasite's ability to eliminate the toxic byproduct of hemoglobin digestion or directly killing young intraerythrocytic malaria parasites or inhibiting protein synthesis of the parasite or by other unknown modes of action or using synergetic mechanisms of actions of two or more.[41–43] However, plant extracts of the present study are crude; the antimalarial action could be the result of synergetic effects of different phytochemical constituents. However, the mechanisms of action have not yet been explained in the study.

Mice treated with the plant extracts lived longer compared to the negative controls. This justifies the crude ethanol extracts of the plant materials suppressed *P. berghei* parasitemia and probably reduced the overall pathogenic effect of the parasite in the experimental mice. A justification, therefore, can be provided that the plant material could probably be used to inhibit further multiplication of *P. parasitemia* until the infected individuals get modern medical treatments.[44]

Even though extract-treated mice lived longer than the ones fed with the vehicle, the highest survival times were recorded at a dose of 800 mg/kg body weight of the extracts. However, the life span of mice treated with the extracts was not significantly higher than the negative control. This is probably because the plant extracts did not clear all parasitemia from the mice. Furthermore, ethanol used for dissolving plants material confirming the traditional usage of these plant species as a malaria remedy and the use of water and ethanol (local alcohols) as common solvents in traditional medicine.

Conclusion

Crude ethanol extracts of the leaves of *C. macrostachyus, R. chalepensis* and *V. amygdalina* produced significant dose dependent antimalarial activity In vivo against *P. berghei* in Swiss albino mice. The extracts are found safe in mice that received up to 3000 mg/kg in a single dose. The phytochemical screenings of the extracts also revealed the presence of potent antimalarial secondary metabolites. Thus, these plant species could potentially be used as alternative sources for the development of a new plant-based antimalarial agent.

Limitation of the Study

This was a preliminary study which aimed to assess only the antiplasmodium potential of the crude extracts of the plant materials. The mode of action of the extracts against the parasite and the host cell was not considered in the present study. However, a recommendation of further bioactivity guided fractionation of the extracts could permit the isolation of active compound and ability to understand its mechanism of action.

Data Sharing Statement

Additional questions or information may be obtained by contact the corresponding author, Solomon Yeshanew.

Ethical Approvals

The study was carried out after having ethical clearance endorsement from Research Ethical and Technical Review Clearance Committee (RETRCC), College of Medical Sciences, Mettu University, Ethiopia. The committee approved all experimental protocols are in compliance with the NIH and ARRIVE Guidelines for the care and use of laboratory animals.

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Author Contributions

All authors conceived the study, carried out the field and laboratory works, involved for analysis and interpretation of the data and took part in drafting the manuscript. They critically read, revised for intellectually important contents, gave the final approval of the version to be published and agreed to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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