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In Vivo Antimalarial Activity of Solvent Fractions of *Echinops kebericho* Roots against *Plasmodium berghei* Infected Mice

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Abstract

The search for plant-derived agents against drug-resistant malaria infection is urgently needed for safe, effective and affordable usage. *Echinops kebericho* has been commonly used in Ethiopian folk medicine for the treatment of malaria. Therefore, this study was designed with the objective of evaluating the antimalarial activities of solvent fractions of the root of *E. kebericho* in animal model.

E. Kebericho roots were macerated using 70% ethanol and further fractionated in hexane, dichloromethane, and n-butanol. The crude extract was also examined for some secondary metabolites. The safety profile of the extract in mice was also assessed using acute oral toxicity study. The protective effects of the fractions in *Plasmodium berghei* infected mice were evaluated using the four-day suppressive test. Animals were allocated into twelve groups (n = 5) and were treated with 200, 350 and 500 mg/kg b.wt doses of solvent fractions of the plant. Percentage parasitemia suppression, survival time, changes in body weight and packed cell volume were determined according to the protocols.

Oral administration of the n-butanol and aqueous fractions demonstrated significant ($P < 0.001$) and dose-dependent parasitemia suppression (ranging from 31 - 36% and 27 - 36% for n-butanol and aqueous fraction, respectively) at 350 and 500 mg/kg compared with the negative control. The percentage of mice survived on the 10^{th} day were also significantly higher ($P < 0.05$) for the plant treated groups. However, the test fractions did not prevent the reduction of body weight and packed cell volume associated with increased parasitemia. The phytochemical investigation revealed that the root of *E. kebericho* contains flavonoids, alkaloids, triterpenoids, resine, saponins and steroids. Moreover, the oral LD_{50} of the plant was higher than 5,000 mg/kg.

This study indicated that butanol and aqueous fractions of *E. kebericho* roots are effective in reducing parasitemia of mice infected with *P. berghei*. Further studies are required to isolate the active ingredient (s) and to identify the possible mechanism (s) of action.

Keywords: *Antimalarial activity; Echinops kebericho; Parasitaemia; Plasmodium berghei*

Introduction

Malaria is a major global public health problem, affecting over 300 million people every year [1]. It is among the major causes of morbidity and mortality; resulting in an annual death of nearly 1 million [1,2]. The re-emergence and distribution to previously non-affected areas become a major concern [3]. Chloroquine and primaquine were commonly used to control the causative agents of malaria, however, the emergence of resistant malarial parasites reduced the effectiveness of these drugs [4,5]. Consequently, new antimalarial drugs with novel actions are required.

Medicinal plants are being commonly used to treat malaria because of accessibility and affordability compared with modern drugs. The development of artemisinin and quinine from *Artemisia annua* and Cinchona tree, respectively; and widespread uses of medicinal plants against malaria necessitates detailed *in vitro* and *in viv*o investigation on the antimalarial potentials of medicinal plants [6]. Interestingly, several East African medicinal plants are under investigation for the development of active antimalarial compounds [7].

*Echinops kebericho (*local Amharic name: *kebercho*) belongs to the genus *Echinops*; is traditionally used in the highlands of Ethiopia against fever, diarrhea, malaria and typhus [8-11]. Burning the plant and using the smokes as mosquitoes repellant is commonly practiced by the society. Especially in the Northern part of Ethiopia, burning of the plant and inhalation of the smoke is practiced to treat headache, fever and "evil eye" (possession by evil spirits). Oral chewed preparations are also used for cases of malaria, diarrhea and stomach ache [9,10]. Despite the widespread use of *E. kebericho in Ethiopian folk medicine, only few studies have been conducted especially in animal models.* Tariku., *et al.* [11], and Ashebir and Ashenafi [12] investigated the *in vitro* antimicrobial and toxicity of crude extracts and essential oils of *E. kebericho. Our* previous study [13] also exhibited the antimalarial effects of crude ethanol extracts of *E. kebericho root in mice. Therefore, the current* study was designed to further evaluate the antimalarial effects of solvent fractions of *E. kebericho* root *in Plasmodium berghei* infected *mice*.

Methods and Materials

Chemicals

The chemicals used were absolute ethanol, hexane, butanol (Reagent Chemical-Ltd, China), Giemsa stain 10% (Shenyang XinvGuang, China), chloroquine phosphate (Ethiopian Pharmaceutical Manufacturing, Ethiopia).

Plant material

Fresh *E. kebericho* roots were collected from Masha Woreda, South Ethiopia. Botanical authentication was obtained and a voucher sample (AL 001) was deposited in the National Herbarium of the Addis Ababa University (AAU), Ethiopia. Prior to extraction, the roots were washed with distilled water and dried under shade.

Crude extraction and solvent fractionation

The powdered roots of *E. kebericho* were extracted by maceration using 70% ethanol and filtered through Whatman filter paper (No. 1). A rotary evaporator was used to remove the solvent from the extract. The extracts were separated using solvents of different polarity with minor modifications of a protocol described in Seidel [14]. Accordingly, 10% (w/v) of crude extract of was diluted with distilled water. The dissolved extract was separated in a separatory funnel with 50 ml of hexane (3 times), dichloromethane (3 times) and n-butanol (3 times) successively. Finally, the solvents were removed by a rotary evaporator and a water bath at 40°C. However, a lyophilizer was used to dry the aqueous residues.

Experimental animals

Swiss albino mice of 6 to 8 weeks of age, weighing 25 - 29g, were maintained in a standard room and acclimatized to the laboratory condition for 10 days. They were housed in a polypropylene cage and a standard pellet diet and water were given on *ad libitum*. The animals were handled according to the international guidelines for the maintenance and use of experimental animals [15]. Moreover, ethical approval was obtained from the ethical committee of Hawassa University, Ethiopia.

Parasite strains and grouping of animals

Chloroquine-sensitive *P. berghei* strain (ANKA) was obtained from the Department of Biomedical Sciences, Addis Ababa University. Viable strains of *P. berghei* was maintained by a weekly passage of blood from an infected donor mouse (with a rising parasitemia of 30%) to non-infected mice. Blood from infected animal was collected through cardiac puncture. Taking into account the level of parasitemia of

infected mous and the erythrocyte count of non-infected mouse, the blood was diluted to get 1×10^7 *P. berghei*- infected RBCs in 0.2 ml normal saline diluted blood [16]. On the first day (D0), mice were intraperitoneally injected with the 0.2 ml of infected and diluted blood. Then, infected mice were allocated randomly into fourteen groups of 5 mice each. The first 12 groups received an oral dose of 200, 350 and 500mg/kg b. wt of n-butanol, hexane, dichloromethane and aqueous fractions. The other two groups were served as positive and negative control groups and received an equal volume of 3% of Tween 80 (vehicle) and chloroquine (25 mg/kg/day; orally) (Ethiopian Pharmaceutical Manufacturing, Ethiopia), respectively.

The 4 days suppressive test

The antiplasmodial effect of the fractions in infected mice was conducted according to the previously described protocol [17]. Three hours after infection, 200, 350 and 500 mg/kg/day oral doses of hexane, dichloromethane, n-butanol and aqueous fractions were given to the test groups. The control groups were treated with chloroquine (25 mg/kg/day, orally) and an equal volume of 3% Tween 80. Treatment of the test and control groups was conducted for four consecutive days (day 0 to day 3). On day 4, blood samples were collected from the tail of each mouse. Thin blood smears were made and stained with 10% Giemsa stain. The numbers of parasitized RBCs out of 100 RBCs in random fields were used to determine the parasitemia level. Then, average percentage parasitemia was determined using the following equation:

$$\%\ Parasitemia = \frac{Number\ of\ infected\ RBCs}{Total\ number\ of\ RBCs} \times 100$$

In addition, the average percentage of parasitemia suppression was calculated using the following formula:

$$\%\ suppression = \frac{Parasitemia\ in\ the\ negative\ control\ group - Pasitemia\ in\ test\ group}{Parasitemia\ in\ the\ negative\ control\ group} \times 100$$

Determination of body weight changes and packed cell volume

The body weights and packed cell volume (PCV) of mice were taken on day 0 and 4 to observe the effect of the test fractions on PCV and body weight. Blood samples were collected from the tail of each mouse in heparinized capillary tubes (¾th of the total volume), sealed with clay and centrifuged for 5 min at 10,000 rpm. PCV was determined using a Micro-Hematocrit reader taking the following formula into consideration:

$$PCV = \frac{Total\ volume\ erythrocytes\ in\ a\ given\ volume\ of\ blood}{Total\ volume\ of\ blood} \times 100$$

Determination of survival time

The curative potential of the fractions was assessed by monitoring the mortality starting from the day of treatment in infected mice, daily. The number of animals survived from the time injecting blood containing the parasite up to 10th day was determined for each group. The comparison was made based on the percentage of animals survived on the 10th day after treatment.

Acute toxicity study

In order to assess the safety profile of the solvent fractions, mice were divided randomly into four groups of six animals each (three male and three female). Mice were fasted for one night prior to administration of the test substances and provided with only water. Then,

the fractions were given with an increasing doses of 2,000, 3,500, 5,000 mg/kg using oral gavage following the standard guidelines [18]. Animals were observed for any signs of toxicity for 14 days. Observation was carried out for any signs of overt toxicity such as hair erection, salivation, lacrimation, diarrhea, tremor, convulsion, and mortality.

Phytochemical screening

Solvent fractions of *E. kebericho* roots were evaluated for the presence of different secondary metabolites such as flavonoids, alkaloids, triterpenoids, resine, tannins, saponins and steroids, according to the previously established protocols [19,20].

Statistical analysis

Data were analyzed using Windows, SPSS Version 22.0. Comparison of percentage suppression, PCV, weight changes and percentage survival among groups were made using One-way Analysis of Variance (ANOVA) followed by Tukey's HSD test. P-values less than 0.05 was considered statistically significant.

Results

Yields of plant extract

The highest yield of 6.5% and 7.7% (w/w), respectively was recorded for the n-butanol and aqueous fractions of *E. kebericho* roots. The percentage yield of hexane and dichloromethane fractions were 2.1% and 2.3% (w/w), respectively.

Effect of solvent fractions on percentage parasitemia and survival of mice

The effects of treatment with various doses of solvent fractions of on parasitemia suppression and survival of *P. berghei* infected mice are presented in table 1. The study demonstrated that the solvent fractions of *E. kebericho* roots produced a significant ($P < 0.05$) chemosuppression compared with the negative control. Interestingly, higher doses of butanol and aqueous fractions resulted significantly greater ($P < 0.01$) and dose-dependent chemosuppression (36% at 500 mg/kg dose) compared with the negative control. In addition, the results of the percentage of mice survived on the 10^{th} day after treatment with various fractions showed that the survival time of mice treated with all doses of the fractions was longer than those in the negative control group.

Treatment groups	% Survival of Animals on day 10	% parasitemia	% suppression
Hexane fraction			
200 mg/kg	70.0 ± 0.63	63.37 ± 3.70	4.46 ± 0.95
350 mg/kg	72.0 ± 0.58	62.07 ± 4.26	6.50 ± 1.24
500 mg/kg	74.0 ± 0.51	63.77 ± 3.11	3.86 ± 0.64
Dichloromethane fraction			
200 mg/kg	72.0 ± 0.49	54.06 ± 3.10*	18.50 ± 0.63*
350 mg/kg	74.0 ± 0.75	53.18 ± 2.05*	19.82 ± 0.11*
500 mg/kg	72.0 ± 0.73	52.32 ± 1.85*	21.12 ± 0.05*
Butanol fraction			
200 mg/kg	76.0 ± 0.60	52.79 ± 1.68*	20.43 ± 0.22*
350 mg/kg	72.0 ± 0.58	45.77 ± 0.86**	31.00 ± 0.55**
500 mg/kg	68.0 ± 0.50	42.38 ± 1.99**	36.11 ± 0.11**
Aqueous fraction			
200 mg/kg	74.0 ± 0.51	51.60 ± 0.68*	22.21 ± 0.62*
350 mg/kg	78.0 ± 0.80	47.98 ± 1.24**	27.66 ± 0.66**
500 mg/kg	72.0 ± 0.73	42.44 ± 2.37**	36.02 ± 0.47**
Chloroquine 25 mg/kg	100.0 ± 0.00	00.00 ± 0.00***	100.00 ± 0.00***
Negative control	66.0 ± 0.51	66.33 ± 1.90	00.00 ± 0.00

Table 1: *Suppressive effects of solvent fractions E. kebericho and percent survival of P. berghei infected mice. Data are expressed as Means ± SEM, n = 5. *P < 0.05, **P < 0.01, ***P < 0.001 Comparison was made with the negative control.*

Effect of solvent fractions on PCV and body weight loss

All doses of fractions did not prevent body weight loss (Table 2) and PCV reduction (Table 3) caused by increased parasitemia compared with mice treated with the vehicle.

Treatment groups	Before treatment	Weight (g) After treatment	% Change
Hexane fraction			
200 mg/kg	27.28 ± 1.46	25.42 ± 1.63	-1.86 ± 0.17
350 mg/kg	31.60 ± 1.50	27.42 ± 0.61	-4.18 ± 0.91
500 mg/kg	24.86 ± 1.87	24.38 ± 1.21	-0.48 ± 0.03
Dichloromethane fraction			
200 mg/kg	24.56 ± 2.99	24.42 ± 1.45	-0.12 ± 0.02
350 mg/kg	26.92 ± 2.20	25.56 ± 0.91	-1.36 ± 0.45
500 mg/kg	26.28 ± 2.64	24.94 ± 1.51	-1.34 ± 0.87
Butanol fraction			
200 mg/kg	26.06 ± 2.70	25.16 ± 1.20	-0.90 ± 0.13
350 mg/kg	27.68 ± 1.51	23.34 ± 1.27	-4.34 ± 0.91
500 mg/kg	24.26 ± 2.44	23.56 ± 0.88	-0.70 ± 0.31
Aqueous residue			
200 mg/kg	28.12 ± 3.31	26.96 ± 1.48	-1.16 ± 0.23
350 mg/kg	28.48 ± 1.79	27.18 ± 1.26	-1.30 ± 0.53
500 mg/kg	26.82 ± 1.44	24.66 ± 0.56	-2.16 ± 0.75
Chloroquine 25 mg/kg	24.80 ± 2.31	31.08 ± 1.23*	+6.28 ± 0.84*
Negative control	30.60 ± 0.63	27.90 ± 0.29	-2.7 ± 0.43

Table 2: *Body weight of P. berghei infected and E. kebericho treated mice.*

Data are expressed as Means ± SEM, n = 5.

**P < 0.001 compared with the negative control*

Treatment groups	Before treatment	PCV (%) After treatment	% Change
Hexane fraction			
200 mg/kg	52.48 ± 0.65	47.03 ± 0.76	-11.69 ± 0.12
350 mg/kg	51.67 ± 1.50	48.10 ± 0.75	-7.42 ± 0.65
500 mg/kg	52.82 ± 0.78	47.52 ± 2.33	-11.15 ± 1.17
Dichloromethane fraction 200 mg/kg	52.70 ± 0.83	49.28 ± 1.10	-6.94 ± 0.14
350 mg/kg	53.26 ± 0.77	47.94 ± 1.78	-11.10 ± 0.98
500 mg/kg	53.10 ± 0.27	50.18 ± 0.78	-5.82 ± 0.17
Butanol fraction			
200 mg/kg	53.34 ± 0.31	51.65 ± 0.34	-3.27 ± 0.11
350 mg/kg	53.00 ± 0.55	47.86 ± 2.00	-10.74 ± 1.11
500 mg/kg	53.26 ± 0.97	49.62 ± 1.31	-7.33 ± 0.91
Aqueous residue			
200 mg/kg	54.46 ± 0.65	52.06 ± 0.36	-4.03 ± 0,32
350 mg/kg	54.16 ± 0.45	50.64 ± 0.54	-6.95 ± 0.01
500 mg/kg	53.56 ± 0.69	51.20 ± 0.62	-4.61 ± 0.07
Chloroquine 25mg/kg	53.21 ± 0.61	52.16 ± 0.66*	-2.01 ± 0.11*
Negative control	51.64 ± 0.82	47.15 ± 0.43	-9.51 ± 0.32

Table 3: *Effect of treatment with solvent fractions of on packed cell volume of mice.*

Data are expressed as Means ± SEM, n = 5.

**P < 0.001 compared with the negative control*

Acute toxicity study

The result of acute oral toxicity studies on the solvent fractions showed that the fractions were found to be safe up to a maximum dose of 5,000 mg/kg. Administration of any of the fractions did not produce mortality, signs of morbidity and a significant behavioral and physical changes. Therefore, the results indicate that the medium lethal doses (LD_{50}) of the fractions are greater than 5,000 mg/kg body weight.

Phytochemical screening

Phytochemical investigation of *E. kebericho* demonstrated the presence of flavonoids, alkaloids, triterpenoids, resine, saponins and steroids; however, the extract was found to be negative for tannins.

Discussion

In vivo model using rodents is commonly applied to evaluate the antimalarial activity of various compounds [16]. It is also preferable because of similarity with human malaria infection and the sensitivity of *P. berghei* to chloroquine [21]. The model is well known for the development of most antimalarial drugs, including artemisinin derivatives [22]. The suppressive test was used to evaluate the effectiveness of the fractions and; parameters such as percent parasitemia suppression, survival time, PCV and Body weight changes were compared among groups.

A compound is considered to be an active antimalarial agent when it produces a minimum of 30% parasitemia suppression or when it produces greater percent survival compared to infected non-treated mice [23,24], which is in agreement with the findings of the current study. The results of the four-day suppressive test indicated that high doses of butanol and aqueous fractions of *E. kebericho* demonstrated significant parasitemia suppression. The findings suggest that butanol and aqueous fractions of the plant are endowed with antimalarial activity with an optimal dose of 500 mg/kg body weight. Moreover, relative to the negative control group, treatment with various doses of solvent fractions significantly enhance the survival of mice on the 10^{th} day, which could be attributed to the suppressive effect of the extracts on parasitemia. These findings are in agreement with previous studies conducted on *Croton Machrostachyus* [25] and *Nigella sativa* [26]. Our previous study on the crude ethanolic extract of *E. kebericho* roots also confirmed that the plant has a significant effect in reducing percentage parasitemia and extending the survival time of mice [13]. Nevertheless, 100% survival was recorded in chloroquine treated groups; the difference could be as a result of the fast elimination phase of the fractions [27].

Phytochemical constituents of plant extracts are frequently indicated for the therapeutic benefits of traditional remedies of plant materials [28]. Secondary plant metabolites such as alkaloids, sesquiterpenes, flavonoids, triterpenoids, quassinoids, xanthones, quinines and phenolic compounds have shown very significant inhibitory activities [8,21,28-30]. Therefore, it is probable that the antimalarial activity of *E. kebericho* could be as a result of the individual or synergistic effect on the phytoconstituents present. Free radical scavenging [31] and immunomodulatory [32] activities of phytoconstituents are frequently reported for the antimalarial activity of herbal remedies. Inhibition of protein synthesis and prevention of invasion of new RBCs by plasmodium parasites [33,34] could also be considered as a possible mechanism. Moreover, crude extract and essential oils of the plant have shown significant antibacterial activity [11,12]. Tetracycline and related antibacterial drugs have been indicated in malarial treatment. Therefore, compounds in the nonpolar and semipolar fractions of the plant could be responsible for the antimalarial effect of *E. kebericho* [25].

A compound derived from plant is considered to have best antimalarial activity when it prevents loss of body weight and anemia in *P. berghei* infected mice [30]. In our four-day suppressive test, the significant body weight gain was recorded only in chloroquine-treated mice. Similar to our previous finding in the crude ethanol extracts of this plant [13], all the animals treated with different solvent fractions demonstrated variable degrees of body weight loss. This might be due to the presence of appetite-suppressant metabolites in the fractions, which is in agreement with earlier studies on other plant extracts [21,25,33,35,36].

P. berghei infection in mice is correlated with increased erythrocyte fragility, reduced PCV [37,38], and life-threatening anemia [24,33]. Unlike to chloroquine, all the solvent fractions of *E. kebericho* failed to prevent PCV reduction. The ineffectiveness of the solvent fractions in preventing reduction of PCV might be associated with the presence of saponins, which are reported to cause hemolysis of erythrocytes [39].

Mortality was not observed in mice receiving the ethanolic extract of the plant at an increasing dose of up to 5,000 mg/kg b. wt. Any orally administered test substance with LD_{50} greater than 1,000 mg/kg [39] or higher than three times the minimum effective dose can be considered as nontoxic and can be considered for further studies [23]. Therefore, acute oral exposure to *E. kebericho* at 5 g/kg can be considered safe.

Conclusion

The n-butanol and aqueous fractions of *E. kebericho* roots have good antimalarial activity. The study also demonstrated that the plant fractions are safe in acute exposure. The results also consolidate the previous *in vivo* study on crude extracts as well as the claims made by the traditional medical practitioners in Ethiopia.

Competing Interests

There are no conflicts of interest.

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