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In vivo antimalarial activity of the crude extract and solvent fractions of the leaves of *Zehenria scabra* (Cucurbitaceae) against *Plasmodium berghei* in mice

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Zehenria scabra **is among the Ethiopian folk medicine for malaria like fever and other infectious diseases. But it lacks apposite pharmacological investigation. This study aimed at evaluating the antimalarial activity and safety profile of *Z. scabra*. *Plasmodium berghei* was used for induction of malaria, kept in a refrigerator and maintained by serial passage of blood from mouse to mouse. The crude extract, chloroform and ethylacetate fractions were obtained from air dried aerial part of *Z. scabra*. Activity was tested *in vivo* against chloroquine-sensitive strain of *P. berghei* by measuring the parasite load using light microscope. 80% methanolic extract yield was 18%. The 2000 mg/kg body weight of the crude extract was devoid of any signs of toxicity. Crude extract of the plant provided 62.5, 72.85 and 76.01% suppression with increasing doses of 100, 200 and 400 mg/kg, respectively. Two solvent fractions of the plant have been also assessed for the same parameter. The ethyl acetate fraction was found to be the most active of all with suppression of 71.88% for 25 mg/kg, 62.47% for 50 mg/kg and 77.62% for the 400 mg/kg dose. Similarly, the 25, 50 and 100 mg/kg of chloroform fractions had also shown 53.57, 73.95 and 61.31%, respectively. In the case of survival of the animals, after 7 days of treatment groups, the ethyl acetate groups had shown better outcome which was 100% for the medium (50 mg/kg) and maximum doses (100 mg/kg). Activities as well as safety studies of this plant confirm the ethnopharmacological usefulness as antimalarial, thus its usage by the folkloric medicine. It can also be used as a base for characterization of some active compounds that could be used as markers for standardization of the extracts for use as traditional antimalarial.**

Key words: *Antimalarial acitivy*, *Plasmodium berghei, Zehneria scabra,* parasitemia.

INTRODUCTION

Malaria infection and associated complications continue to be a major health problem in many parts of the world including the America, Asia and Africa. It is one of the leading causes of morbidity and mortality in some of the poorest tropical and subtropical regions. Particularly it remains to be one of the most important illnesses in sub-Saharan Africa where 20% of children under the age of 5 die as a result of this infection. The World Health

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Organization (WHO) estimates that every year 250 million people become infected and nearly one million die (Agusto et al., 2013, Yetein et al., 2013).

In Ethiopia, malaria constitutes a major public health preoccupation particularly for children and pregnant women. In 2005, the Ethiopian government launched a massive expansion of the malaria prevention and control program aimed mainly at the reduction of malaria in populations living below 2,000 meters above sea level. However, global warming has been implicated in the increase in prevalence of malaria in some highlands of the country (Woyessa et al., 2012). In the past decades, the situation has been aggravated by increasing spread of drug-resistant *Plasmodium falciparum* strains. In Ethiopia, *P. falciparum* and *Plasmodium vivax* account for 60 to 70% and 30 to 40% of malaria cases, respectively (Kinfu et al., 2012). *P. falciparum* has been the major cause of epidemics, and of most malaria deaths and drug resistance are common for this particular protozoa species (Kiszewski and Teklehaimanot, 2004).

Despite challenges of drug resistance, modern chemotherapeutic agents are the main stay of treatment for patients having access to health services, but majority of the population in the rural areas use herbal remedies to treat malaria like fever. New antimalarial drug leads are therefore urgently needed. Traditional healers have long used plants to prevent or cure infections. Herbal medicine or phytomedicine refers to the use of any plant seeds, berries, roots, leaves, bark, or flowers for medicinal purposes (Barrett et al., 1999). Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many of which are based on their use in traditional medicine. It has been noted that the original source of many important pharmaceuticals currently in use have been plants used by indigenous people (Kirby, 1996; Yetein et al., 2013).

Countries in Africa, Asia and Latin America use herbal and traditional medicine to help meet some of their primary health care needs. In Africa, up to 80% of the population uses traditional medicine for primary health care (Kaur and Jaggi, 2010). In industrialized countries, adoption of traditional medicine termed ''Alternative medicine'' is getting much higher attention in recent years. Current research shows medicinal plants continue to play an important role in health aid (Edwards, 2012).

Zehneria scabra (L.F.) Sond is an important climber which belongs to the family Cucurbitaceae, which has been used in many traditional medicinal systems. It has many medicinal values such as to cure fever, diarrhea, skin diseases, stomach pain and treat livestock. Extracts of *Z. scabra* is mentioned in Ethiopian folklore for the treatment of a variety of diseases. The 80% methanolic extract of *Z. scabra* exhibits antimicrobial activity against common bacterial pathogens such as *Staphylococcus aureus* and *Escherichia coli*. The plant is also used traditionally for the treatment of inflammation and pain management (Bruck, 2004; Berhanemeskel, 2007). Medicinal uses of this plant have also been mentioned in other African regions such as Tanzania and Cameroon. Although there are no experimental studies conducted on the antimalarial activity of *Z. scabra,* yet studies on plant species of Cucurbitaceae family demonstrated significant (30%) reduction in parasitemia levels (Amorim et al., 1988; Bickii, 2007; Moshi et al., 2012).

In vivo antimalarial tests generally assess the development of rodent specific parasites *Plasmodium berghei* Vincke and Lips, *Plasmodium yoelii* Landau, Michel and Adam or *Plasmodium chabaudi* Landau in mice, after subcutaneous and/or oral administration. Activity is expressed as a decrease of parasitemia after a certain time which is examined in smears or as survival time. These parasite models are indispensable for the development of antimalarial even if they do not always perfectly mirror *Plasmodium falciparum* infection in human (Fidock et al., 2004; Sullivan et al., 2011). Despite widespread development of resistance, currently used and potent antimalarial drugs such as artemether, chloroquine and quinine are obtained from plant sources. Hence, it is imperative to focus on traditionally used medicinal plants for the discovery of possible new innovative antimalarial sources for the future. In line with this, the antimalarial activity of different solvent fractions of leaves of *Z. scabra*, a traditionally used plant for malaria like fever in Northwest Ethiopia has been assessed in mice models.

METHODS

Chemicals and materials used

Normal isotonic saline, 80% methanol, hexane, ethyl acetate, chloroform, tween 2%, citrate dextrose, giemsa, distilled water, 1 ml syringes, needles, feeding tube, vials, electronic balance, stopwatch, gloves, laboratory glass wares, microscope were used.

Collection of plant material

Large amounts of the leaves of *Z. scabra* were collected from the suburb of Gondar, Azezo district, 10 km away from the city, on the way to Addis Ababa. It was identified by Mr. Abyot Endale in Pharmacognosy Department, University of Gondar. The dried voucher specimen (No. SoP UoG21001) was deposited in the herbarium of the School of Pharmacy. The plant material has been collected from communal grazing land, for which specific permission was not required. The plant is not considered as endangered species, since it grows in many parts of the country, both in private and communal lands. Moreover, the amount of used in this study is small in quantity, and it does not affect the environment.

Extraction and fractionation

The leaves of the plant were air-dried at room temperature under the shade and pulverized. Subsequently, the pulverized leaves were extracted using 80% methanol. A total of 200 g dried leaves

were extracted by maceration (100 g of dried leave in 600 ml of 80% methanol) for 72 h. The extraction process was facilitated by using a shaker at 120 rpm. The mixture was first filtered with Whatman filter paper No. 1 (Whatman®, England). The residue was re-macerated twice for the same duration of hours and then filtered. The combined filtrates were then dried by Rota evaporator (Buchi Rota vapor, Switzerland) at a temperature of 40°C. After drying, a total of 36 g of dry extract was harvested (18% yield) and the dried extract was kept at -20°C until use. The crude 80% methanol extract was then successively partitioned using hexane, ethyl-acetate, chloroform and water. The fractions collected were dried and kept in the same condition as the crude extract at the same temperature.

Acute toxicity study

Acute toxicity study was conducted for the active extracts (Single dose 2 g/kg) using Organization for Economic Cooperation and Development (OECD, 2008) guidelines. The first animal was given with a limit dose of 2000 mg/kg, and then four other mice were sequentially treated based on the outcome of the first animal. The animals were observed for toxicities like diarrhea, weight loss, absence of tremor, lethargy and paralysis periodically for the first four hours during the 24 h period and later followed for 14 days for any lethality (OECD, 2008).

Animals

Antimalarial activity was assessed on mice of either sex. Swiss albino mice (18 to 25 g) were maintained at a temperature of 22 ± 2°C with 12 h light/12 h dark cycle and given food and water. Animal handling and care in the experimental procedure were conducted according to local ethical guidelines. This study was approved by the Bioethics Committee of School of Pharmacy, University of Gondar. All animals were also acclimatized to the working environment one week prior to the experiments.

In vivo antimalarial activity testing

Plasmodium berghei was used for induction of malaria in experimental mice. The parasites kept in a fridge before they were administered to the mice and maintained by serial passage of blood. The infected blood was then collected in test tubes by the tail bleeding and diluted with isotonic saline before it was given to the different groups of mice. The *in vivo* antimalarial activity was evaluated using the method described by Mohanty et al. (2013). Methanol, ethyl acetate and chloroform fractions were obtained from air dried aerial part of *Z. scabra,* while the hexane fraction could not result in a yield which can be administered to the animals. The fractions were tested *in vivo* against *P. berghei* infected mice by measuring the parasite load and mean survival time. In this test, five mice in each group were infected by intraperitoneal (i.p.) injection of *P. berghei* infected erythrocytes, diluted in 0.5 ml of sterile acid citrate dextrose. The blood was then diluted with physiological saline (0.9%) based on parasitemia level of the donor mice and the red blood cell (RBC) count of normal mice in such a way that 1 ml blood contains 5×10^7 infected RBCs. Each mouse was then given 0.2 ml of this diluted blood intraperitoneally which contained 1×10^7 *P. berghei* infected RBCs (Kiszewski and Teklehaimanot, 2004).

Mice were orally treated following Peters 4-Day suppression test (Peters, 1993). Animals were infected on day 0, (D0) while treatment started 24 h after infection (D1). Malaria infection was established in mice by i.p. inoculation of 200 µl of 1×10^6 parasitized cells/ml on the first day (D0) of the experiment. The animals in the test groups have received different doses of the preparations based on their group 24 h post infection. The dose was maintained for 4 consecutive days. Drugs were administered orally. To ascertain the parasitemia, on the third day of experiment, thin blood smears were made and stained with 10% giemsa in phosphate buffer, pH 7.2 for 20 min. A blood sample for the slide preparation was taken using tail bleeding method. The slide was examined under a microscope at 100×. Percentage parasitemia was determined by counting the parasitized red blood cells on at least four random fields of the giemsa stained slide. The mean parasitemia of each group was determined and the standard deviation values were also included in the result. Each experiment had a positive control group and a negative control group. The positive control group received chloroquine (reference drug) at a dose of 25 mg/kg while the negative control group received tween 2%. *In vivo* antimalarial efficacy was examined by evaluating percent suppression, percent survival and mean survival time. Percent suppression was calculated as:

$$\text{Percent suppression} = 100 \times [(A-B) / A]$$

Where A is the mean percent parasitemia of the mice taken as negative control and B is the mean parasitemia in the test group. In addition, mortality in the mice was followed up to 28 day post-infection to evaluate the percent survival and mean survival time. According to ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guideline (Kilkenny et al., 2012), we used the humane endpoints for the survival study. The animals were humanely euthanized and the criteria used for their killing includes weight loss up to 25%, weakness or inability to obtain feed or water and when they are at their critical stage of their lives. Cervical dislocation method was used for killing the animals after the experiment. Any pain or distressed which occurred after the ends of the experiment were thoroughly followed and appropriate measures like humanely killing of the animals were used. Animals were checked for these symptoms twice a day until the end of the experiment. Nonetheless, throughout the experiments, we did not notice a distress on the animals with respect to the guideline. No anesthetic or analgesic was used as the procedure and method did not require the use of analgesics or anesthetics for such a study.

Phytochemical screening

The crude extract and solvent fractions were screened for the presence of different secondary metabolites following standard procedures (Gavamukulya et al., 2014).

Statistical analysis

Results were expressed as means ± SEM and analyzed using SPSS version 19. Comparisons were made between negative control (vehicle), positive control (chloroquine) and treatment groups of various doses using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Body weight differences were also analyzed using paired t-test. P-values of 0.05 were considered statistically significant.

RESULTS

Extraction

The dried pulverized leaves of 200 g of *Z. scabra* were extracted by maceration with 80% methanol and the yield

Table 1. The average percentages of parasitemia, chemosuppression and survival effect of the different extracts, standard drug and negative control in mice (N = 5).

Plant ZS extract	Doses (mg/kg)	Parasitemia% (Mean ± SEM)	Chemosuppression% (Mean ± SEM)	Survival (%) on day 10
80% methanolic ZS	100	29.05 ± 7.7	62.54 ± 9.9	40
	200	21.05 ± 2.5	72.85 ± 3.17 [b***]	40
	400	18.6 ± 2.9	76.01 ± 3.75 [b***]	60
TWEEN 2%		77.55 ± 2.4	0 ± 0 [a***]	0
Chloroquine	25	0.0000	100 ± 0 [b***]	100
Chloroform	25	36 ± 6.4	53.57 ± 8.26	40
	50	20.2 ± 6.3	73.95 ± 8.17 [b***]	40
	100	30 ± 8.2	61.31 ± 10.58	60
Ethyl acetate	25	21.8 ± 5.6	71.88 ± 7.25 [b***]	60
	50	29.1 ± 4.1	62.47 ± 5.23	60
	100	17.35 ± 3.9	77.62 ± 5.13 [b***]	60

a - against Chloroquine, b - against Tween 2 %, * - p<0.05, 2 - **<0.01, 3 - ***<0.001.

was 36 g (18%), slightly higher than the findings of Akele (2012). There was no yield for n-hexane while the yields for chloroform and ethyl acetate were approximated to 10% each.

Acute toxicity

The 2000 mg/kg body weight of the crude extracts was devoid of any signs of toxicity in the first mouse. The other four animals also showed a similar outcome. These were evidenced by the lack of signs of toxicities like diarrhea, weight loss and absence of tremor, lethargy and paralysis during the first four hours, within the 24 h period and throughout the 14 days. Although there are no studies which supports the safety of the plant studies elsewhere (Moshi, 2007; Daniel, 2008) showed the relative safety of the plant. This study was also in line with the previous reports and supports the usage of the plant by the society without great concerns of safety.

Parasitemia and chemo-suppression

Malaria mortality was slightly but not significantly reduced by extracts of *Z. scabra* leaves in relation to the mortality in non-treated mice (P > 0.05). However, the mice survival time was prolonged due to the different extracts of the plant (Table 1). The 80% methanolic extract of the plant provided 62.5, 72.85 and 76.01% suppression with increasing doses of 100, 200 and 400 mg/kg, respectively. Two of the most active fractions (choloroform and ethyl acetate) of the plant have been also assessed for the same parameters. The ethyl acetate fraction was found to be the most active extract with suppression of 71.88% for (25 mg/kg), 62.47% for (50 mg/kg) and 77.62% for the maximum dose (100 mg). Similarly, the 25, 50 and 100 mg/kg of chloroform fractions also showed 53.57, 73.95 and 61.31% suppression, respectively. In the case of survival time for the animals, after ten days of treatment, the ethyl acetate group had shown a better result in maintaining the life of the mice, i.e. 100% for the 50 and 100 mg/kg.

Weight variation of animals

As shown in Table 2, the body weight of the animals in different groups was measured before the experimentation and afterwards. The chloroquine group together with the ethyl acetate fractions had kept the initial body weight, while the chloroform fractions, i.e. 25 mg and 100 mg/kg doses, and the negative control (Tween 2%) did not show any tendency of prevention of weight loss.

Phytochemical screening

Table 3 shows preliminary phytochemical tests and it has been revealed that a variety of secondary and primary metabolites were detected in the crude extract.

DISCUSSION

Nature has been the source of medicine for thousands of

Table 2. The variation in body weight of the animals in different groups before and after the experiment (N=5).

Group	Body wt. before (Mean ± SEM)	Body wt. after (Mean ± SEM)	*P*-value
ZS100	26.3 ± 0.72	24.4 ± 0.51	0.035
ZS200	25.9 ± 0.57	22 ± 1.04	0.071
ZS400	26.2 ± 1.16	25 ± 1.22	0.076
Chloroform 25	24.08 ± 0.30	18.8 ± 0.37	0.000
Choloroform 50	26.38 ± 0.87	26.6 ± 0.81	0.514
Chloroform 100	20 ± 1.21	17.8 ± 1.2	0.009
EA 25	22.5 ± 0.51	19.2 ± 0.49	0.014
EA 50	22.4 ± 1.20	20.2 ± 0.37	0.054
EA 100	22.56 ± 1.22	25.04 ± 1.08	0.742
Tween	23.68 ± 1.72	21.94 ± 1.68	0.252
Chloroquine	23 ± 1.08	22.94 ± 1.66	0.939

years, and an impressive number of new drugs have been isolated from natural sources, many which are based on their use in traditional medicine. Various therapeutic plants have been used for years in daily life, especially in those areas are short of modern medicine to treat a variety of diseases. Natural products cover a diversity space not yet available from synthetic libraries, with an unrivalled success rate as drug leads. Bioactive plant compounds have served as templates for several synthetic drugs and as precursors for the production of semi synthetic drugs (Wessjohann, 2000; Newman et al., 2003).

Z. scabra Sond is a perennial herb, climber or trailing to 6 m, widespread in tropical and southern Africa. It is mentioned in Ethiopian folk medicine for treatment of a variety of infectious diseases. In an ethnomedicinal study conducted in Ankober district of Ethiopia, highest fidelity level values were recorded for *Z. scabra* (95%) and *Hagenia abyssinica* (93.75 %) showing conformity of knowledge on the species of best healing potential (Lulekal et al., 2013). Some prominent uses of the plant include treatment of skin diseases, gonorrhea, syphilis, cleansing uterus before a child is delivered and malaria. It has also been reported that 80% methanol extracts of *Z. scabra* exhibits antimicrobial activity against most common bacterial pathogens like *S. aureus* and *E. coli* (Bruck, 2004). Studies on the plant use have also shown that the leaves are prepared by boiling, and then the decoction taken in the form of a drink. Sometimes, the plant is mixed with several other plants for treatment of malaria. The ethanol extract of the plant has exhibited antibacterial activity against some gram negative bacteria (Desta, 1993; Giday et al., 2007).

Parasitemia and chemo-suppression

The plant was extracted using various solvents; 80% methanol was used for the preparation of the crude extract followed by successive extraction using chloroform, ethyl acetate and hexane. The chloroform and ethyl acetate fractions were checked for antimalarial activity, while the hexane fraction was devoid of enough yields for evaluation of its activity. Thus, it is plausible to assume the active constituents for this plant would lie in either of the two extracts. The crude as well as the fractions of *Z. scabra* resulted in chemo suppression of various percentages. The 80% methanolic extracts provided 62.5, 72.85 and 76.01% suppression with increasing doses of 100, 200 and 400 mg/kg, respectively. The ethyl acetate fraction was found to be the most active with suppression of 71.88% for 25 mg/kg, 62.47% for 50 mg/kg and 77.62% for the 100 mg/kg. Similarly, the 25, 50 and 100 mg/kg of chloroform fractions also showed 53.57, 73.95 and 61.31% suppression, respectively.

In the case of survival of the animals, after seven days of treatment, the ethyl acetate groups had shown better outcome, i.e. 100% for both 50 and 100 mg/kg doses. In another study about the *in vitro* antibacterial activity of the different solvent fractions of the plant, it has been revealed that ethanolic and methanolic extracts showed the highest growth inhibition while ethyl acetate and chloroform extracts showed moderate inhibition. It has been shown in the study that ethanolic extracts of the plant effectively inhibit growth of both gram-positive and gram-negative bacteria while the aqueous extract showed no activity (Anad, 2012). This contrasts with our findings where the ethyl acetate fraction was found to be more potent. The difference might be attributed to differences in sensitivity between the protozoa and bacteria, and study models applied. Assuming the results obtained, it is suggested that the most potent antimalarial principles present in *Z. scabra* could be concentrated in the ethyl acetate fraction and organic solvent extraction method could be better for the isolation of antimalarial compounds from this particular medicinal plant.

Drugs like chloroquine lead to decreased parasitemia and resultant recovery of severe malaria. They also reduce parasitemia through various ways like reducing parasite nutrient intake, interfering with parasite metabolic

Table 3. Phytochemical screening test of crude extracts of *Zehneria scabra*.

Phytochemical	Test	Test result
Alkaloid	Wanger's test	+
Carbohydrates	Molisch's test	-
Amino acids	Ninhydrin test	-
Glycosides	Borntarger's test	+
Tannins	Ferric chloride test	+
Saponins	Foam test	-
Flavonoids	Lead acetate test	+
Phenols	Ferric chloride test	+
Diterpenes	Copper acetate test	+

pathways like heme metabolic pathway which is involved in the metabolism of iron. Drugs also negatively affect parasite reproduction and growth (Ziegler et al., 2002; de Villiers and Egan, 2009). The plant extracts reduced the level of parasitemia and prolonged survival up to the 10th day. Chloroquine had a good chemo-suppression of 100% as determined on the fourth day post-infection and a 100% survival rate by 10th day post-infection.

The activities as well as the safety studies of this plant confirm the ethnopharmacological usefulness as antimalarials, thus its claim by the folkloric medicine. The plant extracts with chemo-uppression like the ethyl acetate fraction offer potential for isolation of lead antimalarial compounds. It can also be used as a base for the characterization of some active compounds that could be used as markers for standardization of the extracts for use as traditional antimalarial and thus contribute to the development of potential antimalarial medicine from the biodiversity of Ethiopia. The extracts prolonged the mean survival time of the mice indicating that the extracts suppressed *P. berghei* and reduced the overall pathologic effect of the parasite on the mice. However, neither the extracts nor the standard drug cured the infection. This could be due to recrudescence of *P. berghei* parasites after apparent cure (Bhat and Surolia, 2001).

As shown in Table 3, preliminary phytochemical tests reveal the presence of a variety of secondary and primary metabolites in the crude extract. At the same time, the ethyl acetate fractions were found to be more active. This fraction also minimized body weight reduction. The survival time percentage of mice treated with this fraction was also longer than the mice treated with the other extracts. This can also suggest the potential for isolating pure compounds with much higher antimalarial activity. However, this is not always the case as the anti-plasmodial activity of plants could also emerge as a result of the synergistic interactions of the different bioactive components. Some examples include alkaloids, flavonoids, triterpenoids, phenols and other compounds (Mengiste, 2012).

Conclusion

The activities as well as the safety studies of this plant confirm the ethno-pharmacological usefulness as antimalarial medicine, thus its highest utilization by the folkloric medicine. The ethyl acetate fraction with chemo suppression offer potential for isolation of lead anti-malarial compounds. It can also be used as a base for the characterization of some active compounds that could be used as markers for standardization of the extracts for use as traditional antimalarial and thus contribute to the development of potential antimalarial medicine from the biodiversity of Ethiopia.

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Conflict of Interest

Authors have not declared any conflict of interest.

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