

In vivo antiplasmodial activity evaluation of the leaves of *Ruta chalepensis* L. (Rutaceae) against *Plasmodium berghei*



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Dessalegn Asmelashe Gelayee,^{1*} Mohammedbrhan Abdelwuhab,² Assefa Belay Asrie³

ABSTRACT

Ruta chalepensis has been used in the Eastern Africa for management of malaria despite lack of scientific evaluations. This study was carried out to evaluate the *in vivo* antiplasmodial activity of the 80% methanol extract of the leaves of the plant. The screening was done using female Swiss albino mice weighing 20–25g and aged 6–8 weeks against chloroquine sensitive rodent malaria parasite, *Plasmodium berghei*, following the standard 4-day suppressive test procedure. The mice were divided into five groups each with five animals: a negative control, a positive control and three test groups. Three extract doses (100, 200 and 400 mg/kg/day) were given orally. Parasitemia level, survival time as well as weight variation were measured for evaluating

antiplasmodial efficacy. Moreover, preliminary phytochemical and acute toxicity studies were carried out. The presence of alkaloids, flavonoids, polyphenols, terpenoids, cardiac glycosides as well as saponins was confirmed. The oral LD₅₀ was determined to be higher than 2000 mg/kg. Antiplasmodial activity of the extract was inversely related with the dose so that the 100 mg/kg dose was the most effective (mean parasitemia of 29.92 ± 0.94 on day 4, $P < 0.001$). *Ruta chalepensis* therefore has antimalarial activity and no significant acute toxicity when taken orally. The study validates the traditional use of the plant in managing malaria. We suggest further investigation on this potential plant to address the call for a novel antimalarial drug.

Key words: antiplasmodial, antimalaria, *in vivo*, malaria, *Plasmodium berghei*, *Ruta chalepensis*

*Corresponding Author: Gelayee Dessalegn Asmelashe Department of Pharmacology, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia. desefikir@gmail.com

INTRODUCTION

Globally, an estimated 3.3 billion people in 97 countries are at risk of being infected with malaria of which 1.2 billion face a high risk. The disease caused 584 000 deaths in 2013 alone. The burden is high particularly in the African region where 90% of all malaria deaths occur and in the children aged less than 5 years who account for 78% of all deaths.¹ The disease has been estimated to cost the continent more than US\$12 billion every year in lost Gross Domestic Product and an average loss of 1.3% of economic growth per year. Up to 40% of the continent's health budget is spent on this disease every year. On average, malaria stricken family loses a quarter of its income through loss of earnings and the cost of treating and preventing the disease.² In Ethiopia, *P. falciparum* and *P. vivax* contribute to malaria morbidity in the nation in relative proportions of 60% and 40% respectively. Approximately 52 million people (68%) live in malaria risk areas³ and over 5 million episodes of malaria occur each year in the nation.⁴

Management of malaria has been challenged by several factors including resistance development against the drugs. *P. falciparum* has already become

less sensitive to antimalarial drugs such as chloroquine, and sulphadoxine pyrimethamine and there are also reports on resistance to currently existing first line drug regimen ACT in parts of Cambodia, Thailand as well as reduced sensitivity in parts of Africa.⁵ Therefore, new knowledge, products and measures and in particular new chemotherapeutic tools are urgently needed. Particularly plants have been an important source of drugs. As high as 25% of the drugs prescribed worldwide were derived from plants and of the drugs considered as basic and essential by the World Health Organization (WHO), 11% are exclusively of plant origin or synthetic drugs obtained from natural precursors.⁶

Majority of antimalarial drugs have been derived from medicinal plants or from structures modeled on plant derived compounds. These include the quinoline-based antimalarials as well as artemisinin and its derivatives.⁷ Worldwide, over 1,200 plant species are reportedly used for the treatment of malaria and fever and thus are potential sources of new anti-malarial treatments.⁸

Ruta chalepensis L. (Family: Rutaceae), locally known as tenadam, is an erect perennial herb,

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¹Department of Pharmacology, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia, desefikir@gmail.com, P.O.Box 196, Phone.no=251937377895; ²Department of Pharmacology, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia, mohammedb18@gmail.com; ³Department of Pharmacology, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia, assefabelay@gmail.com

becoming woody at base, and grows up to 1.5 m height. Stems branched at all heights with light-green to blue-green color.⁹ It is one of the most frequently used plants for medicinal purposes. It has been used by the local community as traditional remedy against fever, headache, and common cold and as an insect repellent.^{10,12} It is also used as food flavoring, against rheumatism, mental disorders, dropsy, neuralgia, menstrual problems, convulsions, and other bleeding and nervous disorders in different parts of the world.¹³ An ethnobotanic investigation conducted in Yemeni in 2009 documented an antimalarial use of this plant in such a way that 8-10 fresh leaves are chewed without swallowing twice a day for 5 days.¹⁴ There is also undocumented use of the plant in malaria management by the local people in Ethiopia. Several studies reported that *R.chalepensis* is a rich source of important secondary metabolites such as alkaloids, flavonoids, phenols, amino acids, furanocoumarins, saponins, volatile oil, glycosides, sterols and triterpenes.^{15,16} Analgesic, antipyretic, antioxidant, antiinflammatory, anti-convulsant, and anxiolytic effects are confirmed experimentally.^{16,17}

In Ethiopia, medicinal plants have been commonly used for treatment of several diseases including malaria. It has been frequently described that more than 80% of the population have been using traditional medicine.¹⁸ Therefore efficacy and safety of traditionally used remedies need to be evaluated and this study was carried out as a move to accomplish this objective.

RESEARCH DESIGN AND METHODS

Plant material

Fresh aerial parts of *R.chalepensis*, locally called tenadam, were collected in April 2013 from Gondar, Northwest Ethiopia. The plant specimen was identified and authenticated at the National Herbarium, Department of Biology, Addis Ababa University where a specimen was deposited with a voucher number of A001.

Preparation of plant extract

The leaves were collected, cleaned, shade dried at room temperature for about three weeks, crushed into coarse powder using mortar and pestle, weighed using electronic balance and placed in plastic bags until extraction. Four hundred and thirty gram of the powdered plant was macerated using 80% methanol for 72 hr with occasional stirring. Then, the filtrate was separated from the mark using Whatman filter paper (Grade 1) and the mark was re-macerated further two times. The filtrates were

combined, dried in an oven (Leaders Engineering, UK) at a temperature not exceeding 40°C, and stored at 4°C using air-tight container until further use. For the various experiments conducted, the extract was reconstituted in distilled water at appropriate concentrations.

Experimental animals

Adult Swiss albino mice of female sex weighing 20-25g and aged 6-8 weeks were obtained from Ethiopian Health and Nutritional Research Institute (EHNRI). The animals were housed in a standard polyethylene glycol cage. They were fed with standard diet and water *ad libitum*, and allowed a one-week acclimatization period prior to the study. The animals were maintained under standard conditions of humidity, temperature and 12h light/12h darkness cycle. This experiment was conducted in accordance with the internationally accepted laboratory animal care and use guideline.¹⁹

Parasite: Chloroquine sensitive *Plasmodium berghei* ANKA strain was obtained from EHNRI and was subsequently maintained in the laboratory by serial blood passage from mouse to mouse on weekly bases.

Phytochemical tests: The 80% methanol leaf extract of *R.chalepensis* was subjected to qualitative phytochemical screening (for the presence of alkaloid, flavonoid, polyphenols, terpenoids, cardiac glycosides as well as saponins) according to standard methods.^{20,21}

Test for alkaloids: Thoroughly ground material (2 g) was treated in a test tube with 10 ml of 1% HCl for 30 min in a water bath. The suspension was filtered through cotton into test tube and was divided into two parts and to one part of the solution five drops of Dragendorff's reagent and to the other part five drops of Mayer's reagent were added. If the alkaloids are present the test with Dragendorff's reagent should form a yellowish orange precipitate or a whitish opalescence with Mayer's reagent.

Test for saponins: An aliquot of the extract in a 15 ml test tube was vigorously shaken for 2 min. The frothing which persists for 15 min was inspected for indication of the presence of saponins in the extracts.

Test for Phenols: A mixture of one ml 1% FeCl₃ and one ml of 1% K₃Fe(CN)₆ was prepared immediately before this test. Then, to two ml of filtered solution of the aqueous macerate of plant material,

three drops of a mixture of 1% FeCl₃ and 1% K₃Fe(CN)₆ were added. The final solution should form a green blue colour if it contains phenolic compounds.

Test for flavonoids: The dried 80% methanolic extract (100 mg) was dissolved in a mixture of methanol and water. To 2 ml of the extract solution, three to five drops of 2% lead acetate solution were added. Then, it was observed whether it develops yellow or orange colour which indicates the presence of flavonoids.

Test for Terpenoids: Five milliliter of plant extract was mixed in 2 ml of chloroform followed by the careful addition of 3 ml concentrated (H₂SO₄). A layer of the eddish brown colouration formed at the interface indicates a positive result for the presence of terpenoids.

Test for glycosides: Small amount of extract was dissolved in 1 ml water and sodium hydroxide solution was added. A yellow colour indicates the presence of glycosides.

Preliminary Acute Toxicity Test

The acute toxicity median lethal dose (LD₅₀) of the extract was estimated p.o. in Swiss albino mice following the 2008 Organization for Economic Co-operation and Development (OECD) guideline.²² Accordingly, a limit test was done at 2000 mg/kg using a total of three mice with each mouse given the extract sequentially in 48 hr. The mice were carefully observed for a total of 15 days for any change in body weight (weekly), changes in skin and fur, lacrimation, salivation, diarrhea, lethargy, sleep, coma and death.

Grouping and Dosing of Animals

The mice were divided into negative control, positive control and three test groups of five animals each. The negative control received distilled water and the positive control received chloroquine (25 mg/kg) and the test groups received orally different doses of the 80% methanol leaf extract of *R. chalepensis* (100 mg/kg, 200 mg/kg and 400 mg/kg body weight). These doses were selected according to the result obtained from the acute toxicity test study.

Parasite Inoculation: The parasitized erythrocytes for the test were collected from an infected donor mouse with rising parasitaemia of 30%. The mice were sacrificed by head blow, and blood was collected in a Petri dish with an anticoagulant (0.5% trisodium citrate) by severing the jugular vein. The blood was then diluted with physiological

saline (0.9%) in proportion of 1:4. Each mouse was then inoculated with 0.2 mL of blood containing about 107 *P. berghei* infected erythrocytes on day 0 through intra peritoneal route.²³

In vivo antiplasmodial test: Antimalarial activity screening of the crude extract of *R. chalepensis* was carried out using a 4-day suppressive standard test according to the method of Fidock. 20 After three hours of parasite inoculation, three test groups of mice were administered with 100, 200 and 400 mg/kg of the crude extract. The negative control group mice were treated with 2 ml/100g of the vehicle (distilled water) and the positive control groups were treated with 25 mg/kg of chloroquine.²³

Determination of parasitaemia: On the fifth day (96 hrs post infection), a drop of blood was taken from tail snip of each mouse on frosted slide and smears were prepared, fixed with methanol and stained with 10% Giemsa solution at pH 7.2 for 15 min. Then, five fields were randomly selected on each stained slide and examined under microscope with an oil immersion objective (×100 magnification power). The parasitaemia level was determined by counting the number of parasitized erythrocytes on randomly selected fields of the slide. Parasite suppression was calculated by using the following formula.

$$\% \text{ parasite suppression} = \frac{\text{mean parasitemia in negative control} - \text{mean parasitemia in study group}}{\text{mean parasitemia in negative control}} \times 100$$

Where by: % Parasitaemia = (Number of parasitized RBC/ Total number of RBC count) × 100

Determination of survival time and mean body weight

The survival-time (in days) was recorded for all mice and the mean survival time of the extract treated groups was compared to the vehicle treated group. Bodyweight of each mouse prior to treatment initiation and at the end of treatment (96 hrs post infection) were taken to see if there was bodyweight change as an adverse effect of the extract treatment.

Statistical analysis: Data were analyzed using Windows SPSS Version 20. One-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test was used to compare parasitemia among the groups. Paired t-test was also used to compare initial and final results of the bodyweight following extract treatment. Results obtained from the study were presented as mean plus or minus standard error of the mean (M ± SEM). A P value < 0.05 was considered as statistically significant.

Ethical clearance: The animals were handled according to the international animal care and welfare guideline¹⁹ and an ethical clearance was obtained from the ethical review committee of the department of Pharmacology, University of Gondar.

RESULTS

This study explored the phytochemical contents, the acute toxicity as well as blood schizonticidal activity against *P.berghei* of *R.chalepensis*.

Percentage yield: The 80% methanol leaf extract of *Ruta chalepensis* was a dark green, hygroscopic semi-solid at room temperature with a percentage yield of 20.93% w/w.

Phytochemical screening of *R.chalepensis*: Preliminary phytochemical screening of the 80%

methanol leaf extract of *R.chalepensis* revealed the presence of alkaloids, phenolic compounds, saponins, flavonoids, terpenoids and cardiac glycosides. [Table 1.](#)

Acute oral toxicity of *R.chalepensis*: Only lethargy that lasts 45 minutes was observed in the mice after oral administration of 2000 mg/kg extract of *R.chalepensis*. The oral LD50 of the extract was thus greater than 2000 mg/kg. The mice were physically active and fed and drunk as that of the control groups administered with the vehicle (distilled water) within the 15 days observation period. All the mice used in the acute toxicity test gained a weekly bodyweight increment in the 15 days observation period.

In vivo antimalarial activity of *R.chalepensis* Outcome of the four-day suppressive test was expressed as percent of reduction of parasitaemia level relative to the negative control mice and is summarized in [Table 2.](#) The extract demonstrated antiplasmodial activity at all test doses but the reduction in parasitaemia level was inversely related to the dose. Thus the highest effect observed was for the 100 mg/kg extract dose (46.01%) and it reduced parasitaemia to significant level (p<0.01). the parasite suppression effect progressively decreased as the dose increased to 200 mg/kg and 400 mg/kg.

Effect of *R. chalepensis* on survival time of mice

As shown in [Table 3,](#) the mean survival time (days) of the 100 mg/kg extract treated group was 9.83 ± 0.33 and this significantly (p=0.032) differ from the vehicle treated group. However despite an increase in survival, the observed effect is not significantly different for the two higher doses.

Effect of *R. chalepensis* on body weight of mice

As can be seen in [Table 4,](#) all extract treated groups as well as control groups have lost body weight following their treatment. This was highest (8.2%) for the 400 mg/kg extract treated group. Pair wise comparison of the bodyweight before and after completion of the treatment revealed that the 200 mg/kg and 400 mg/kg dose extract treated groups lost their body weight significantly (P<0.05) compared to the vehicle treated group.

DISCUSSION

The present study was driven by an ethnobotanic study that reported antimalarial and anti fever uses of *R.chalepensis*,¹⁴ experimental study which reported anti-oxidant as well as anti-inflammatory activities,¹⁷ and undocumented claims by the local

Table 1 Results of phytochemical screening of methanolic seed extract of *R. chalepensis*

Serial number	Tests	Extract
1	phenols	+
2	Saponins	+
3	Alkaloids	+
4	Flavonoids	+
6	Terpenoids	+
7	Glycosides	+

(+) = Present

Table 2 Effect of 80% methanol crude extract of *R. chalepensis* on parasitaemia level of *P. berghei* infected Swiss albino mice

Treatment	% parasitaemia at D4	% parasite Suppression
Distilled water 2 ml/100g	55.42 ± 0.72	0.00
RC 100 mg/kg	29.92 ± 0.94 (P= 0.000)	46.01
RC 200 mg/kg	40.75 ± 1.49 (p= 0.023)	26.47
RC 400 mg/kg	50.25 ± 1.42 (p= 0.081)	9.33
Chloroquine 25 mg/kg	1.47 ± 0.66 (p=0.000)	97.35

Values are expressed as Mean ± SEM; n = 5; RC= *Ruta chalepensis*; D4= day four;

Table 3 Mean survival time of infected mice after treatment with 80% methanol crude extract of *R. chalepensis*

Treatment	mean Survival time ±SEM (day)
Distilled water 2 ml/100g	7.67 + 0.33
RC 100 mg/kg	9.83 + 0.33 (p=0.032)
RC 200 mg/kg	9.07 + 0.29 (p=0.081)
RC 400 mg/kg	8.33 + 0.33 (p=0.552)
Chloroquine 25 mg/kg	28.00 + 0.00 (p=0.000)

Values are expressed as Mean ± SEM; n = 5; RC= *Ruta chalepensis*; D4= day four

Table 4 Body weight changes of the mice after administration of the hydroalcoholic extract of *R. chalepensis*

Mice group	Mean Bodyweight(g) at D0 ±SEM	Mean Bodyweight(g) at D4 ±SEM	% change	P value
Distilled water 2 ml/100g	24.01 + 0.58	23.90 + 0.44	-0.46	0.36
RC 100 mg/kg	23.68 + 0.90	25.47 + 0.97	7.03	0.04
RC 200 mg/kg	22.53 + 1.33	24.01 + 0.62	6.16	0.083
RC 400 mg/kg	21.37 + 1.11	22.47 + 0.64	4.9	0.13
Chloroquine 25 mg/kg	20.93 + 0.27	22.80 + 0.49	8.2	0.014

Values are expressed as Mean ± SEM; n = 5; RC= *Ruta chalepensis*; D0 (the first 24 hr post infection); D4= day four

people for the use of the plant against malaria. It was done to evaluate the antiplasmodial activity, to determine phytochemicals present and the acute oral toxicity of the plant. Such studies are generally supported by the WHO. This organization has encouraged the use of validated, low-cost traditional antimalarial preparations to treat fever and malaria. Research priorities include the assessment of efficacy and safety of new herbal antimalarials and standardization of extracts.²⁴

In this study, a hydroalcoholic (80% methanol) solvent was used for extraction as such solvent is good for extracting a wide variety of polar and moderately polar compounds.²⁵ The study confirmed the presence of alkaloids, polyphenols, flavonoids, saponins, cardiac glycosides and terpenoids in the extract of *R. chalepensis* which is consistent with previous studies.^{15,16} The oral LD50 was determined to be greater than 2000 mg/kg and is in line with a previous study where the oral LD50 is reported to be greater than 5000 mg/kg.¹⁶ The only observed toxicity was rapid and brief duration of lethargy which strengthens the CNS depressant activity of the plant reported so far.¹⁶ Thus, *R. chalepensis* lacks significant acute toxicity in mice at the dose levels used in this experiment. However further studies such as sub-acute toxicity needs to be done. Rodent model with *P. berghei* was used for this study because the model is widely accepted. The 4-day suppressive test is a standard test commonly used for antimalarial activity screening and the determination of percent inhibition of parasitaemia is the most reliable parameter. This method has become popular during scientific evaluation of potential phytomedicines for early investigations of the *in vivo* efficacy of an antimalarial activity.²³ In the four day suppressive test, treatment with 100 mg/kg and 200 mg/kg doses of *R. chalepensis* extract reduced the parasite load in the mice suggesting activity against the blood stage of the parasite. Thus the plant is considered active in the screening test and can be candidate for doing further studies. A higher than 10% parasite suppression (i.e. a mean group parasitemia level of less than or equal to 90% of that of the vehicle

treated control group) usually indicates that the test compound is active in standard screening studies.²⁶

Antiplasmodial activity was demonstrated at all test doses but magnitude of effect was inversely related to the dose. The observed highest effect at the smallest dose seems consistent with the traditional claim that low dose (8-10 fresh leaves) has been chewed without swallowing twice a day for 5 days in the treatment of malaria.¹⁴ The progressive decrease in parasite suppression activity of the extract with an increase in the dose may be due to its crude nature so that with higher doses there may be negative interactions among the phytochemicals present in the extract. The same reason (i.e. crude nature) may explain why the maximal effect of the extract was still very much lower compared to the standard drug, chloroquine.

As the follow up on survival of the mice revealed, the symptoms of the disease on the extract treated groups were becoming worse and eventually death occurred due to a rise in parasitemia while chloroquine treated groups were alive by the end of the month following the treatment. The rise in parasitemia of extract treated mice might suggest that the actions of the extract were small (insufficient) and/or were short-lived due to rapid metabolism or elimination.²⁷

The potential for antimalarial drug development of several phytochemicals such as alkaloids, terpenoids, flavonoids, coumarins, phenolics, polyacetylenes, xanthenes, quinones, steroids and lignans was documented in previous studies.^{28,29} Thus one or more of the phytochemicals demonstrated in the present study may be responsible for the observed antiplasmodial activity of *R. chalepensis*.

A significant increase in survival time (days) was demonstrated only with the 100 mg/kg extract treatment and this parallels its highest antiplasmodial activity in the study. Therefore at this dose, the disease progression was delayed better than the two higher doses.

Moreover, extract treatment protected the mice from losing weight which is parallel to the effect observed in antimalarial activity i.e. highest and

significant ($p=0.04$) weight gain was noted for the 100 mg/kg dose. This protection from weight loss is explained by the antimalarial effect of the plant since body weight loss is a feature expected in rodents infected with malaria parasite.³⁰ The protection from weight loss, added to the decrease in mean parasitemia, is also suggestive for antimalarial effect of the extract.

CONCLUSIONS

Ruta chalepensis therefore has antimalarial activity and no significant acute toxic effect when taken orally. The study validates its traditional use in malaria treatment. We suggest further investigation on this potential plant in the search for a novel anti-malaria drug.

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CONFLICT OF INTEREST

We declare there is no conflict of interest.

AUTHORS CONTRIBUTION

DAG developed the proposal and DAG and MA finalized it. Experiment was carried by, DAG, ABA. Data analysis, manuscript development and final approval was done by DAG, MB, ABA.

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