ANTIMALARIAL ACTIVITY OF *CALPURNIA AUREA* HYDROALCOHOLIC LEAF EXTRACT IN MICE INFECTED WITH *PLASMODIUM BERGHEI*

Birhanu, Z.[1]; Wuhab, M. A.[2]; Abula, T.[2]

[1]Department of Pharmacognosy, School of Pharmacy, University of Gondar, P.O Box 196, Ethiopia
[2]Department of Pharmacology, School of Pharmacy, University of Gondar, P.O Box 196, Ethiopia

[*]zewdu.birhanu@uog.edu.et , zbirhanu@gmail.com

Abstract

The rapid spread of resistance encourages the search for new active compounds. Plants used in traditional medicine have been used as a potential source of new antimalarial drugs. In Ethiopian traditional medicine, the leaves of *Calpurnia aurea* Ait. Benth. are used for the treatment of several diseases including malaria. In an ongoing search for effective, safe and cheap antimalarial agents from plants, the 80% methanol leaf extract of *C. aurea* was evaluated for its in vivo antimalarial activity, in a 4-day suppressive assay against *Plasmodium berghei* infected mice. *C. aurea* extract displayed a significant ($P < 0.05$) antimalarial activity at doses of 400 and 600 mg/kg with chemosuppression values of 38.22±17.87% and 46.51±3.67% respectively. Acute toxicity studies revealed that the crude extract possesses no toxicity in mice at a maximum dose of 2000 mg/kg suggesting the relative safety of the plant when administered orally. The results of the present study indicate that methanolic leaf extract of *C. aurea* possesses antimalarial principles which support the traditional use of the plant in the treatment of malaria in Northwest Ethiopia.

Keywords: Antimalarial activity, *Calpurnia aurea*, *In vivo*, *Plasmodium berghei*.

Introduction

Malaria is a major world health problem due to the emergence and spread of *Plasmodium falciparum* that is resistant to almost all of the currently available antimalarial drugs [1, 2, 3, 4].

Despite the fact that artemisinin combination therapies are the first-line treatments for uncomplicated *P. falciparum* malaria in the majority of malaria endemic countries, in recent times, partial artemisinin-resistant *P. falciparum* malaria has emerged on the borders of Cambodia and Thailand. The limited availability of artemisinin combination therapy together with decreased sensitivity of *P. falciparum* to artemisinin and its derivatives required the discovery and development of new antimalarial drug (s) [4, 5, 6]. On the other hand, costs of antimalarial combination therapies are over ten times costlier than those of the traditional drugs currently used in tropical countries where a single drug therapy is widely used. Thus, implementation of combination therapy would entail higher direct and indirect costs to the health service. This higher cost is out of reach for many developing nations including Ethiopia [3]. Drug resistance is now widely disseminated throughout malaria-endemic regions; no new chemical class of antimalarials has been introduced into clinical practice since the report for the development of resistance *P. falciparum* to the currently available antimalarial agents [7, 8]. As such, due to the burden of the disease, WHO has given due emphasis for the development of new antimalarial drug [8]. The search for new drugs through the evaluation and validation of traditional medicines offers a good opportunity and a highly credible channel for the discovery and development of better medicines [9, 10]. Traditional medicine research can therefore provide information and new clues regarding the effectiveness of medicinal plants in curing malaria [7]. The success story of the antimalarial drugs quinine and artemisinin, both from plant sources, has virtually showed the way to the study of plants as antimalarial agents [11].

The global importance of the disease, current bottleneck in vector control and the capability of *P. falciparum*, the potentially lethal malaria parasite, in developing resistance to virtually all currently available antimalarial medications including the newest artimisinin derivatives coupled with the rapid expansion of these lethal strains [12, 13] urges for the discovery of new antimalarial drugs from traditionally used medicinal plants. Likewise; numerous studies have described the discovery of novel antimalarial drugs through analysis of medicinal plants used by traditional healers. This suggests a call for urgent effort to identify effective, affordable, alternative antimalarials from plants used traditionally for the treatment of malaria. In Ethiopia, traditional healers have been using medicinal plants to treat different human and livestock ailments from time immemorial [14, 15]. In northwest Ethiopia, *C. aurea* is being used for the treatment of malaria by traditional health practitioners [15]. However, the antimalarial activity and safety profile of this medicinal plant is not scientifically validated and authenticated. With this background, the study was conducted to evaluate the acclaimed *antiplasmodial* activity and safety profile of the leaf of *C. aurea* against *plasmodium berghei* infected mice.

Materials and Methods

Plant material

This study was conducted from January to October 2013. The antimalarial medicinal plant was selected based on ethnomedicinal survey fallowing leads supplied by local healers in Northwest Ethiopia [15].

Leaves of *Calpurnia aurea* (Aiton) Benth. (Fabaceae) was collected from the vicinity of Gondar zuria district, about 35 km west of Gondar town. The plant was authenticated by taxonomists at the National Herbarium, Department of Biology, Addis Ababa University with a Voucher specimen (Herb No. 086789) and was deposited there in the national herbarium room. Specimen nomenclature was based on the flora fauna of Ethiopia and Eritrea. Leaves of *C. aurea* were washed, shade dried under room temperature and pulverized in to coarse powder using sterile mortar and pestle.

Preparation of crude extract

The coarse powder (600gm) *C. aurea* leaf was exhaustively extracted by maceration with 80% methanol for three consecutive days at room temperature using electrical rotary shaker. The methanol extract was prepared by soaking 100g of the dry powdered leaves of *C. aurea* in 1L of methanol and was filtered by using whatman filter paper No. 1 (150mm). The mark was re-macerated three times. The filtrates collected were combined and concentrated in an oven at 40 degree centigrade (yield: 21% w/w). Finally, the dried extract was kept in desiccator until used for antimalarial testing.

Phytochemical Screening

The 80% methanol extract of leaves of *C. aurea* was screened for the presence of bioactive plant chemical constituents as alkaloids, terpenoids, flavonoids, tannins, saponins, phenols and cardiac

glycosides using standard qualitative phytochemical screening test procedures [16].

Experimental Animals

Either sex of Swiss albino adult inbreed mice weighing 20-28 gram were used in the study. The animals were maintained on standard pellet diet and clean drinking water *ad libitum* and caged in groups of five in plastic cages at 22 ± 2^0c on a 12 hour light-dark cycle with proper ventilation. These inbred mice were kept in the Animal House in Pharmacology Department, School of Pharmacy, University of Gondar and were acclimatized to laboratory condition for a week prior to the experiment. All procedures performed were reviewed and approved by the department of Pharmacology review committee and conform to internationally accepted principles.

Parasite Isolate

For *in vivo* antimalarial assays of plant extract, Chloroquine sensitive *Plasmodium berghei* ANKA strains were obtained from Aklilu Lema Institute of Pathobiology, Addis Ababa. The parasites were maintained by serial passage of blood from infected mice to non-infected ones.

Parasite Inoculation

To infect the mice, blood sample was collected from auxiliary vessels of a donor mouse with a rising parasitaemia of about 30-37%. Then, the blood was diluted in normal saline upon addition of few drops of trisodium citrate as anticoagulant so that the final suspension would contain about $1x \ 10^7$ infected RBCs in every 0.2ml suspension. Therefore, each mouse used in the study was infected intraperitoneally with 0.2ml infected blood containing about 1×10^7 *P. berghei* parasitized red blood cells. This was prepared by determining both the percentage parasitaemia and the RBC count of the donor mouse and diluting the blood with isotonic saline [8].

Acute Toxicity Study

Acute oral toxicity test was conducted as per the internationally accepted protocol OECD guidelines 425 [17]. A limit dose of each 2000mg/kg body weight of *C. aurea* was administered on a single test animal orally by gavage. Before the administration of a single dose of the extract, the mice were fasted for 3hours. Fallowing the period of fasting, mice weighed and the extract administered according to their body weight. After administration of the extract, food was withheld for 1hour. The mice

were observed continuously for one hour after administration of the extracts; intermittently for four hours, over a period of 24 hours and for 14 days. Gross behavioral changes such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhoea, mortality and other signs of toxic manifestation were observed.

As no mortality of experimental mouse was observed at the limit dose of 2000mg/Kg body weight for the LD50 study, four additional mice were dosed sequentially so that a total of five mice were tested [17].

Evaluation of Antiplasmodial Activity of the Extract

In screening of the plant extract for its blood schizonticidal activity on an early infection, the standard four-day suppressive test was used. This is the most extensively used initial test, in which the efficacy of a compound is assessed by comparison of blood parasitaemia and mouse survival time in treated and untreated mice. The parasitaemia is determined to find qualitatively the presence and degree of activity at the screening doses. For treated mice the survival-time (in days) recorded and the mean survival time calculated in comparison with that of the negative control group (taken as100% parasite growth) [18, 19, 20].

Swiss albino mice of either sex weighing 20-28g were then infected with 10^7 *P. berghei ANKA* strain and kept in the main chamber before they were divided in groups of five. The infected mice were randomly divided into three test groups and two control groups (each for chloroquine as a standard drug and distilled water as a negative control). Each groups containing five mice per cage. The test extract was prepared in three different doses (200mg/kg, 400mg/kg, and 600mg/kg of body weight) and chloroquine 25mg/kg body weight in a volume of 0.2ml. The extract was administered as a single dose per day. Both the extract and the drug were given through intragastric route by using oral gavage to ensure safe ingestion of the extract and the drug.

Treatment started 3 hours post infection on day 0 and then continued daily for four days (i.e. from day 0 to day 3) (with the same dose and same route). On the fifth day (day 4), 24 h after the last dose, thin blood smears were prepared using blood from the tail vein of each mouse. Every smear was air-dried, fixed in methanol, air-dried again and Giemsa (10%) stained for 10 minutes. Then, each stained slide examined under microscope with an oil immersion objective of 100x magnification power and parasitaemia determined microscopically by counting 4 fields of approximately 100 erythrocytes per field.

The difference between the mean value of the control group (taken as 100%) and those of the experimental groups were calculated and expressed as percent reduction or activity using the following equation [8, 18, 19].

$$Activity = 100 - \frac{\text{Mean parasitaemia treated}}{\text{Mean parasitaemia in negative control}} \times 100$$

% parasitaemia= [Number of parasitized RBC/Total number of RBC counted] x 100.

Statistical Analysis
Data obtained from this work are presented as mean plus or minus standard error of the mean (M±SEM). Statistical significance was determined by One-way ANOVA and Scheffe's post hoc test using SPSS version 16.0. For all the data obtained, results were considered statistically significant at 95% confidence level and P-value<0.05.

Results
Preliminary phytochemical analysis
Phytochemical screening of the hydroalcoholic leaf extract of *C. aurea* revealed the presence of bioactive secondary plant metabolites as alkaloids, cardiac glycosides, diterpenes, flavonoids, phenolic compounds, saponins and tannins while triterpenes were absent.

Acute Toxicity
The acute toxicity study indicated that *C. aurea* leaf extract did not cause mortality of mice at 2000mg/kg. Gross physical and behavioural observation of the experimental mice also revealed no visible signs of acute toxicity indicated by lacrimation, hair erection, and reduction in their motor and feeding activities.

In vivo Antimalarial Activity of the Crude Extract
The result of this study showed that *C. aurea* leaf extract possess antiplasmodial activity against chloroquine sensitive *P. berghei* (ANKA strain) infection in Swiss albino mice. The complete data is presented in Table 1. As evident from the chemosuppressions obtained during the 4-day early infection test, all the mice treated with the extract showed reduced parasite load as compared to the corresponding negative control group even though no complete parasite clearance. Chemosuppression is inversely related to parasitaemia. In this study, plant extracts which have been shown to reduce parasitaemia to low levels demonstrated corresponding high chemosuppresion. Moreover; mice treated with *C. aurea* leaf extract survived

longer compared to mice in the corresponding negative control group. *In vivo* evaluation of the crude leaf extract of *C. aurea* against *P. berghei* in mice exhibited a dose dependent chemossupressive effect at various doses employed in this study. The hydroalcholic leaf extract at 400 and 600 mg/kg body weight per day showed 38.22±17.87 and 46.51±3.67% chemosuppression respectively. Average parasitaemia at these doses was significantly lower (p < 0.05) than that of the untreated control group. The lowest dose, 200 mg/kg, even though not statistically significant, displayed suppressive activity (13.5±4.56 %).

Percent parasitaemia in the negative control group in day 4 after infection with *P. berghei* parasites was 34.82±3.51% (the highest observed). Parasitaemia in the negative control was higher than in all the treatment groups. This illustrates that the extract had effect on the growth of *P. berghei* in mice at all the tested doses. However, none of the observed antimalarial activities of the extract was comparable to that of chloroquine (25mg/kg/day) which exhibited 100% chemosuppression that totally cleared the parasite on day four under identical condition and this value was significantly (*P*<0.001) higher than the chemosuppression values obtained in *C. aurea* extract treated groups.

Mortality of mice were monitored on daily basis and no mouse died in the positive control group which received oral administration of chloroquine on daily basis and all the five mice were healthy up to the 30th day post treatment. On the contrary, in the negative control group, which was receiving distilled water on daily basis, all the five mice died 7th day post infection (Table 2). As shown in the table, methanol extract from leaves of *C. aurea* prolonged mean survival time of the study mice in a dose dependent manner indicating that the extract suppressed *P. berghei* and hence diminished the overall pathologic effect of the parasite on the study mice.

The mean survival time of the mice received *C. aurea* leaf extract was found to be 7.8±0.58, 8±0.45 and 9.8±1.07 at doses of 200, 400 and 600mg/kg/day respectively, while the corresponding value of the untreated control group was 6.8±0.37 days. Although statistically not significant, the mean survival time of mice receiving 200 and 400mg/kg/day doses of the extract were relatively longer than mice in the negative control group (survived for 6.8±0.37 days only). Furthermore; mice receiving the highest dose of *C. aurea* (600 mg/kg/day) lived longer with a mean survival time of 9.8±1.07 days post-infection, significantly (*P*<0.05) beyond the mean survival period of untreated mice.

Mean survival time of 30±0.00 days was observed for chloroquine treated control groups which is significantly (*P*<0.001) and (*P*<0.05) different from mean survival period of vehicle and extract treated groups respectively.

Discussion

Despite the fact that Ethiopian traditional medicine practitioners use cold water to extract bioactive compounds from *C. aurea* leaves for the treatment of malaria, hydroalcoholic (80% methanol) was used as a solvent for this study since hydroalcoholic extracts of plant materials contain a wide variety of polar and moderately polar compounds than water extracts alone [16]. Although several classes of natural products are responsible for the antiplasmodial activity of many plant species used in traditional medicine to treat malaria, the most important and diverse biopotency has been observed in alkaloids, quassinoids, and sesquiterpene lactones [8, 21, 22].

Alkaloids are the major class of compounds possessing antimalarial activity as exemplified by quinine, one of the oldest antimalarial agents from a plant *Cinchona* and is still in use. There are also a number of antiplasmodial secondary plant metabolites that have shown antimalarial activities belonging to the classes of terpenes, limonides, flavonoids, chromones, xanthones, anthraquinones, phenolic compounds, quassinoids, sesquiterpene lactones (artemisinin) and other related compounds [8, 22, 23]. Accordingly, the observed antimalarial activity of *C. aurea* could be attributed to the presence of plant secondary metabolites like alkaloids, terpenoids, flavonoids, phenolic compounds contained in the leaf of the plant. These may have exerted their antimalarial activity in synergy or any one of them alone. On the other hand the plant may contain an undiscovered antimalarial active chemical compound that could serve as a template for the production of relatively inexpensive or alternative antimalarial drugs.

Even though medicinal plants are assumed to be safe, many of them are potentially toxic. Therefore, evaluating the safety level of the herbal medicine is necessary for the determination of the safe dose that can be used for treatment [17].

All the experimental mice were physically active up to the 14th day post administration of *C. aurea* leaf extract. Therefore, observations that led no death with an oral dose of 2000mg/kg could indicate that the test extract is safe to mice at this dose. As such, the oral LD50 was found to be greater than 2000

mg/kg body weight. Similar study has confirmed the safety of *C. aurea* hydroalcoholic leaf extract even at doses as high as 5000mg/kg in mice [24] and thus may be classified as practically non toxic to mice acutely. This could also explain the safe use of the plant by the local people in traditional treatment of malaria, in N1orthwest Ethiopia [15]. However, long-term traditional use is indicative of lack of immediate and acute toxicity, this does not rule out unforeseeable long-term or latent toxicity.

Although primate models provide a better prediction of efficacy in human than the rodent malaria model, the later have also been validated through the identification of several conventional antimalarial drugs like chloroquine, halofantrine, mefloquine, and artemisinins. In view of their proven use in the prediction of treatment outcomes for human infections, the rodent malaria model remains a standard part of the drug discovery and development process [18, 19]. Primate models like *Aotus* and *Saimiri* monkeys, in which infection with some strains of *P. falciparum* is well characterized, have important role in pharmaceutical development by providing the final confirmation of the choice of a drug candidate for it provide a clear prediction of human efficacy and pharmacokinetics than the rodent models, offering a logical transition to clinical studies [18, 19]. Therefore*, in vivo* evaluation of antimalarial compounds typically begins with the use of rodent malaria parasites such as *P. berghei* due to the fact that *plasmodium* species that cause human disease are essentially unable to infect non primate animal models with the exception of a complex immunocompromised mouse model that has been developed to sustain *P. falciparum* parasitized human RBCs *in vivo* [18, 19].

Based on the above justifications, in this study, the four day standard rodent malaria model [17, 18] was used to evaluate blood schizontocidal activity of hydroalchoholic leaf extract of *C. aurea*, against early *P. berghei* infected mice.

For the reason that *P. berghei* is sensitive to chloroquine, it was an appropriate parasite used in the prediction of treatment outcomes for this study. Likewise, chloroquine was used as a standard drug for the parasite *P. berghei* is sensitive to this drug.

Result of this study on Chloroquine-sensitive strain of *P. berghei* in Swiss albino mice showed that the crude methanolic extract of the plant material possess malaria parasite suppressive effect which is an indication that the plant extract is possibly effective against human malaria parasites as well. Furthermore, the mean survival time of mice treated with the extract of this plant was longer

compared to the respective negative control group, confirming that the crude extract of the study plant suppressed *P. berghei* and probably reduced the overall pathogenic effect of the parasite on the study mice. Therefore, this plant may be used to suppresses the *Plasmodium* parasites until the patient get modern medical attention in rural areas where health institutions are far away. When a standard antimalarial drug is used in mice infected with *P. berghei,* it suppresses parasitaemia to none detectable levels [25] which is in agreement with the effect of chloroquine in this study.

The effect on parasitaemia in this study is in agreement with previous studies conducted on different plant species [25, 26, 27, 28]. However, some of the extracts in previous studies possess higher parasite suppression activity compared to the present finding. Therefore, the present study shows that traditional herbal medicine of Ethiopia is promising for the developments of new and easily affordable plant based antimalarial drugs.

In general, if the lethal dose of the test substance is three times higher than the minimum effective dose, the substance is considered a good candidate for further study [25]. Furthermore; Compounds reducing parasitaemia by 30% or more are considered active [29] which is consistent with this study.

Even though not completely known, chloroquine and other related antimalarials like quinine, mefloquine, lumefantrine appear to accumulate in the acidic vesicles of the parasite thereby interfering with degradation of haemoglobin by parasitic lysosomes. Polymerization of toxic haeme to nontoxic parasite pigment hemozoin is inhibited by formation of drug-heme complex. Apart from inhibition of hemozoin polymerization in the parasite food vacuole, intercalation of DNA of the parasite or inhibition of protein synthesis or inhibition of other important enzymes for the parasite are some possible mechanisms of action for the antiplasmdial activity of antimalarial compounds [29, 30].

Therefore, the extract of *C. aurea* could have elicited its antiplasmodial action through any of the aforementioned mechanisms or by some other means yet to be elucidated.

Conclusion

The result of this study indicated that extract of *C. aurea* possess antimalarial activity. This provides a clue for the folkloric efficacy of this plant reported from Ethiopian ethnomedicine. Lack of toxicity

coupled with possessing antiplasmodial activity of *C. aurea* leaf extract suggests its ethnomedicinal usefulness as antimalarial remedy in Northwest Ethiopia. Therefore, it would be worth conducting a bioassay-guided fractionation and isolation of the active compound(s) and a detailed study of their activities *in vivo*.

Authors' contribution

ZB: collected the plant material, prepared the extract, and carried out the antimalarial assay, and drafted the manuscript. TA: Coordinated the work, helped in drafting the manuscript. MB: Performed toxicity study and phytochemical screening and assisted in drafting the manuscript. All authors read and approved the final manuscript.

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Table1. Results of average percentage parasitaemia and percent chemosuppression of hydroalcoholic leaf extract of *C. aurea* against *P. berghei* infected mice.

Drug/extract/ vehicle	Dose (concentration) mg/kg/day	% Parasitaemia ±SEM	% Suppression ±SEM
Vehicle	0.2 ml	34.82±3.51	–
C.aurea	200	26.20±1.36[##]	13.50±4.56[##]
C.aurea	400	18.71±5.41[*#]	38.22±17.87[*#]
C.aurea	600	16.20±1.11[*#]	46.51±3.67[*#]
Chloroquine	25	0.00[**]	100.00±0.00[**]

Key: Values are means ± SEM, n=5, [*] =P< 0.05, [**] = P< 0.001 compared to vehicle treated group.
[#] =P< 0.05, [##] = P<0.001 compared to chloroquine treated group.

Table 2. Mean survival time of *P. berghei* infected mice receiving the various doses of methanolic leaf extract of *C. aurea* and the standard drug.

Drug/extract/vehicle	Dose (concentration) mg/kg/day	Mean survival time ± SEM (days)
Vehicle	0.2 ml	6.8±0.37
C. aurea	200	7.8±0.58[#]
C. aurea	400	8±0.45[#]
C. aurea	600	9.8±1.07[*#]
Chloroquine	25	30±0.00**

Data are expressed as means ±SEM, n=5, [*]=P< 0.05, [**] P<0.001 compared to vehicle treated group.
[#] =P<0.001 compared to chloroquine treated group.