COLLEGE OF MEDICINE AND HEALTH SCIENCES

SCHOOL OF PHARMACY

DEPARTMENT OF PHARMACOLOGY

IN VIVO ANTIMALARIAL ACTIVITY OF METHANOLIC EXTRACT OF INNER BARK OF *CORDIA AFRICANA* AGAINST *PLASMODIUM BERGHEI* IN MICE

BY:

HANNA TESHOME

ADVISORS:

ASSEFA BELAY (MSc, B. Pharm)

JIBRIL SEID (MSc, B. Pharm)

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Summary

Background: Malaria is one of the major health problems in Sub-Saharan Africa including Ethiopia. One of the reasons attributed for this is the development and the emergence of multidrug resistant strains of the parasite to the current effective first-choice antimalarial drugs as well as resistant vectors to insecticides. A continued search for other effective, safe and cheap plant-based antimalarial agents thus becomes necessary in the face of these difficulties.

Objective: The present study was aimed to evaluate the in vivo antimalarial activity of *Cordia africana* in mice.

Methods: The inner bark extract of *Cordia africana* was extracted by methanol 80% using maceration method. The resulting extract was evaluated at doses of 100 mg/kg, 200 mg/kg, 400 mg/kg using 4-day suppressive test.

Results: The methanolic crude extract of Cordia africana showed 18.30, 24.84, and 31.37% ($P < 0.05$) chemosuppression at the doses of 100, 200 and 400 mg/kg of extract, respectively as compared to negative control. The extract showed a significant weight loss at 400 mg/kg when compared with negative control but it did not change the body temperature of *P. berghei* infected mice. The extract was safe at the limit dose of 2,000 mg/kg in mice. Phenols, saponins and flavonoids were detected by the preliminary phytochemical screening.

Conclusion: From this study it can be concluded that the inner bark extract of *Cordia africana* has antimalarial activity as seen in its ability to suppress *P.berghei* infection and this can be base for further studies to develop effective antimalarial agents.

Key words*:* *Antimalarial activity, Plasmodium berghei, C. africana, in vivo.*

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List of abrevations and acronyms

ACTs: Artemisinin Based Combination Therapies

ANOVA: Analysis of Variance

ARDS: Adult Respiratory Distress Syndrome

DIC: Disseminated Intravascular Coagulation

FMOH: Federal Ministry of Health

IP: Intra-Peritoneal

IRS: Indoor Residual Spraying

LLINs: Long-lasting Insecticidal Nets

OECD: Organization for Economic Cooperation and Development

RBCs: Red Blood Cells

RDT: Rapid diagnostic tests

SEM: Standard Error of the Mean

SPSS: Statistical Package for the Social Sciences

WHO: World Health Organization

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1. Introduction

1.1. Etiology of malaria and its vectors

Malaria is caused by a parasite called Plasmodium species. There are five *Plasmodium* species that cause human malaria. *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale* are human malaria species. Recently in Asia, *Plasmodium knowlesi* is the fifth major human malaria parasite, which is primarily a pathogen of monkeys (1, 2).

The most prevalent and severe type of malaria is caused by *P. falciparum. Secondly P. vivax* is a common cause of malaria in Latin America, Asia and Oceania. *P. malariae* and *P. ovale* are much less common. In Ethiopia, malaria is contributed by both *P. falciparum* (64%) and *P. vivax* (36%) (3).

Malaria is transmitted to humans through the bites of infected genus Anopheles mosquitoes. Among 400 different species of *Anopheles* mosquitoes thirty are important in malaria transmission. *Anopheles arabiensis, Anopheles funestus, Anopheles gambiae, Anopheles melas, Anopheles merus, Anopheles moucheti* and *Anopheles nili* have been reported as the dominant vector species for the transmission of malaria. Among these, *An. gambiae and An. arabiensis* are the most efficient vectors in malaria transmission especially in Ethiopia (4).

1.2. Epidemiology of malaria

1.2.1. Malaria globally

Malaria is a major public-health problem in the world particularly in sub-Saharan African countries. It causes significant obstacle to socioeconomic development of the society in endemic countries (5). Globally, an estimated 3.2 billion people are at risk of being infected with malaria and developing disease, and 1.2 billion are at high risk according to world malaria report in 2014. According to 2013 malaria report 198 million cases and 584,000 deaths of malaria occurred Worldwide. Malaria mortality rates have decreased by 47% and 54% globally and in Africa, respectively, between 2000 and 2013 (6).

Africa especially SSA are the most affected countries in the world. 163 million cases of malaria have been occurred in Africa and most of them (78%) were in SSA. 528,000 malaria deaths have been estimated in Africa and 83% of these were children under five years in 2013 (6).

1.2.2. Malaria in Ethiopia

Malaria is ranked as the leading communicable disease in Ethiopia. 75% of the country is malarious (7). Approximately 68% of the total population of 84.3 million lives in areas at significant risk of malaria. According to the federal ministry of health (FMOH), from 2010 to 2011, malaria was the leading cause of outpatient visits, accounting for 15% of all visits, and health facility admissions, with 15% of all admissions. It is one of the top ten causes of inpatient deaths among children less than 5 years of age and adults (8). The most risky sections of the population are children under five years of age and pregnant women (9). Generally, areas below 2,000 meters above sea level in altitude are considered as malaria-endemic (10).

1.3. Life cycle and pathogenesis of malaria

The life cycles of all human malaria parasite species are similar. Infection begins when sporozoites, the infective stages, are injected by a mosquito and are carried around the body until they invade liver hepatocytes where they undergo a phase of asexual multiplication (exoerythrocytic schizogony) resulting in the production of many uninucleate merozoites. These merozoites flood out into the blood and invade red blood cells (RBCs) where they cinitiate a second phase of asexual multiplication (erythrocytic schizogony) resulting in the production of about 8 to 16 merozoites which invade new RBCs. This process is repeated almost indefinitely and is responsible for the disease, malaria (1).

The *Plasmodium* parasite completes its life cycle through the mosquito when some of the merozoites that penetrate RBCs do not develop asexually into schizonts, but instead change into male and female sexual forms known as gametocytes. These circulate in the person's bloodstream, awaiting the arrival of a blood-seeking female *Anopheles* mosquito. When a female mosquito bites an infected person, it sucks up gametocytes along with blood. Once in

the mosquito's stomach, the gametocytes develop into sperm-like male gametes or large, egg-like female gametes. Fertilization produces an oocyst filled with infectious sporozoites. When the oocyst matures, it ruptures and the thread-like sporozoites migrate, by the thousands, to the mosquito's salivary glands. The cycle starts over again when the mosquito bites its next victim (11).

Malaria, especially P. falciparum malaria, can cause various pathologic complications involving various systems of the body. Cerebral malaria, acute renal failure, black water fever, hypoglycaemia, adult respiratory distress syndrome, disseminated intravascular coagulation, hypotension and shock are among the common manifestations of complicated malaria. Complicated malaria in pregnant women and in patients with HIV co-infection has increased morbidity and mortality (12).

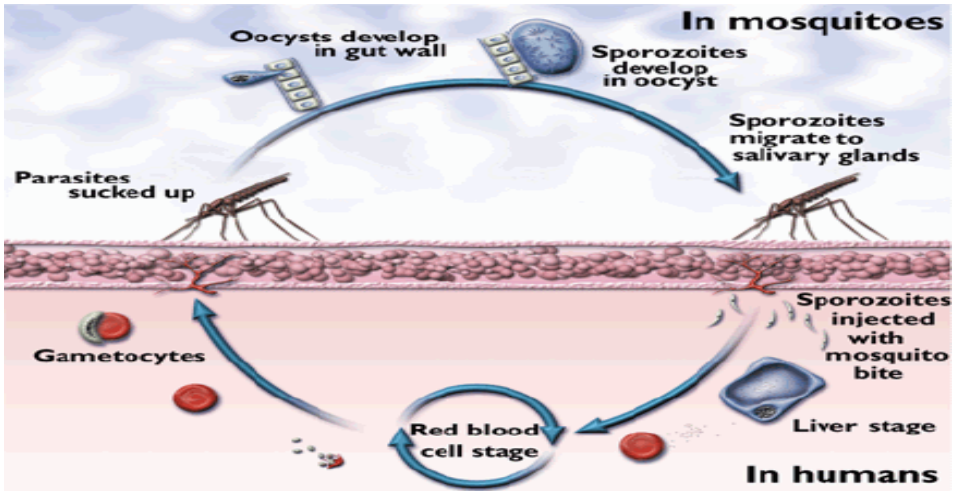

Figure 1. Life cycle of the malaria parasite.

1.4. Clinical manifestations of malaria

The symptoms first appear 10 to 16 days after the infectious mosquito bite (11). The first symptoms of malaria, common to all the different malaria species, are nonspecific and mimic a flu-like syndrome and the presentation may include headache, fever, shivering, arthralgia, vomiting, hemolytic anemia, jaundice, hemoglobinuria, retinal damage and convulsions. Although fever represents the cardinal feature, clinical findings in malaria are extremely

diverse and may range in severity from mild headache to serious complications leading to death, particularly in *falciparum malaria* (13).

1.5. Diagnosis of malaria

Malaria is diagnosed by the microscopic examination of blood films or by antigen-based rapid diagnostic tests (RDT) (14). Microscopy of stained thick and thin blood smears remains the gold standard for confirmation of diagnosis of malaria. High sensitivity, detection of malarial parasites at low densities and quantification of the parasite load are the advantages of microscopic examination. It is possible to distinguish the various species of malaria parasite and their different stages (15).

Commercially available RDTs are often more accurate than blood films at predicting the presence of malaria parasites, but they are unable to tell how many parasites are present (16). However, the specificities, sensitivities, numbers of false positives, numbers of false negatives and temperature tolerances of these tests vary considerably, illustrating the difficulties and challenges facing current RDTs (17).

1.6. Prevention and control of malaria

There are three types of measurements that can prevent malaria. These are personal protective, chemoprophylaxis and unit protective measures. None of the recommended interventions is 100% effective. Even in combination and when efficiently and consistently applied, malaria infection may still occur in high incidence (18).

Over the past decade the use of LLINs, in combination with improved drug therapies, indoor residual spraying (IRS), and better health infrastructure has helped reduce malaria in many African countries. However, insecticide resistance in the vector is an evolving threat to these gains (19).

In 2005, the Ethiopian government launched a massive expansion of the malaria prevention and control programme. The programme was aimed mainly at the reduction of malaria in populations living below 2,000 m above sea level. The massive scale-up of malaria control interventions, including case diagnosis and treatment, distribution of long-lasting insecticidal

nets (LLINs), and indoor residual spraying of households with insecticides have preferentially targeted malaria-endemic areas (10).

1.7. Treatment of malaria

Malaria is easily preventable and treatable if correct measures are taken which can include proper diagnostic tools, the availability of antimalarials and insecticides. Bed nets impregnated with insecticides and indoor residual spraying have eliminated malaria vectors from several settings (20).

Once the diagnosis of malaria has been made, appropriate antimalarial treatment must be initiated immediately.Treatment should be guided by three main factors which include the nature of infecting *Plasmodium* species, the clinical status of the patient and the drug susceptibility of the infecting parasites as determined by the geographic area where the infection was acquired and the previous use of antimalarial medicines (21).

Currently antimalarials are mostly targeting the asexual blood stage of the parasite's life cycle. There are many other attractive targets that need to be investigated. The liver stages and the sexual stages will become more important as malaria control moves towards malaria elimination. Compounds active against the liver schizont would have a prophylactic effect and *P. vivax* elimination requires effectors against the dormant liver hypnozoites (22).

The antimalarials in common use come from the following classes of compounds: the quinolines (chloroquine, quinine, mefloquine, amodiaquine, and primaquine), the antifolates (pyrimethamine, proguanil and sulfadoxine), the artemisinin derivatives (artemisinin, artesunate, artemether, arteether) and hydroxynaphthaquinones (atovaquine) (3).

Quinine remains an important antimalarial drug almost 400 years after its effectiveness was first documented. However, its continued use is challenged by its poor tolerability, poor compliance with complex dosing regimens, and the availability of more efficacious antimalarial drugs (23).

Currently artemisinin based combination therapy (ACT) is used as the first line treatment of uncomplicated falciparum malaria in over 100 countries and is the cornerstone of malaria

control and elimination programs in these areas. However, despite the high potency and rapid parasite killing action of artemisinin derivatives there is a high rate of recrudescence associated with artemisinin monotherapy and recrudescence is common even when ACT is used (24), whereas chloroquine is still commonly used for the treatment of non-falciparum species (25).

1.8. Antimalarial drug resistance

Malaria is a major public health problem mainly due to the development of resistance by the most lethal causative parasitic species, *P. falciparum* to the mainstay drugs like chloroquine (26). Chloroquine-resistant forms of *P. falciparum* malaria first appeared in Thailand in 1957, then spread through South and Southeast Asia and by the 1970s were being se0en in SSA and South America. The rise in chloroquine resistance contributed to a worldwide increase in malaria-related mortality, particularly in SSA (27).

Drug resistance in malaria has been defined as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended, but within the limits of tolerance of the subject" (28).

ACT is used as the first line treatment of uncomplicated falciparum malaria in over 100 countries and is the cornerstone of malaria control and elimination programs in these areas. However, despite the high potency and rapid parasite killing action of artemisinin derivatives there is a high rate of recrudescence associated with artemisinin monotherapy and recrudescence is common even when ACT is used (24).Therefore New drugs with unique structures and mechanism of action are urgently required to treat sensitive and drug-resistant strains of malaria (26).

1.9. Plants as a source of antimalarials

Traditional medicine uses crude extracts. Traditional medicines are used by ~ 80 % of Africans as a first response to ailment. Many of the traditional medicines have demonstrable antiplasmodial activities (29). Recent advances in antimalarial drug discovery from natural sources include plant extracts and compounds isolated from plants, bacteria, fungi and

marine organisms. These compounds offer new and novel scaffolds for development as antimalarials (30).

Historically, compounds containing novel structure from natural origin represent a major source for the discovery and development of new drugs for several diseases (30). Natural compounds, mostly from plants, have been the mainstay of traditional medicine for thousands of years. They have also been the source of lead compounds for modern medicine. The advantage of natural compounds for the development of drugs derives from their innate affinity for biological receptors. Natural compounds have provided the best antimalarials known to date (29). Medicinal plants are a rich source of bioactive phytochemicals. These phytochemicals have an important role in preventing and treating various diseases including malaria (31).

The success of the antimalarial drug quinine and the discovery of artemisinin, the most potent antimalarial drug, both from plant sources, has led to the study of plants as antimalarial agents. The ethnobotanical approach for the search of new and effective antimalarial agents and candidates from plant sources has proved to be more predictive and promising. Therefore, nature particularly plants used in traditional medicine are a potential source of new antimalarial drugs as they contain molecules with a great variety of structures and pharmacological activities (32).

Resistance of malaria parasites to existing drugs complicates treatment, but an antimalarial vaccine that could protect against this disease is not yet available. It is therefore necessary to find new effective and affordable medicines. Medicinal plants could be a potential source of antimalarial agents (33).

1.10. The experimental plant

Cordia africana is grouped under the family of *Boraginaceae* and the genus of *Cordia L*. *Boraginaceae* Is a large family with more than 2,000 species in 100 to 145 genera. 19 genera and 62 species are found in Ethiopian (34).

Cordia (L.) includes trees or shrubs. It has about 250 species in the tropical and subtropical regions of all continents. Around 10 species are found in Ethiopia (34).

Cordia africana (L.) is a small to medium sized tree, up to 18 m tall. Its bark is dark to pale brown, rough, fibrous, slash yellow to white turning green, grey or brown (34).

Figure 2. Picture of *Cordia africana*.

It is widespread in Sudan, South Africa, Angola, tropical Arabia, India and elsewhere in tropical botanic gardens. It is one of the most important timber trees in Ethiopia. It grows from 700 to 2,550 m altitude and It is common in Tigray, Gondar, Gojjam, Wello, Shewa, Arsi, Wollega, Illubabur, Keffa, Gamo Gofa, Sidamo, Bale and Harerge regions in Ethiopia (34).

Traditionally, *Cordia africana* can be used for the treatment of different ailments such as respiratory diseases (35), abdominal pain (36), migraine, broken body, wounds, gastritis, constipation (37), malaria, diarrhea and dental problems (38).

Besides being a source of medicine, *Cordia africana* has been utilized for various other purposes such as used for high quality furniture, doors, windows, cabinet making beehives, interior construction, mortars, and veneering (37).

1.11. Statement of the problem

Malaria is easily preventable and treatable if correct measures are taken (20). However, malaria recrudescence, increasing antimalarial drug and insecticide resistance, and the challenge of having effective vaccines are the major challenge for the success of treatment (5). Additionally, many antimalarial drugs in use today have high toxicity and low

therapeutic margin of indices that exposes patients' additional harm and health expenditure (39).

Malaria is still one of the greatest global public health problems especially in sub-Saharan Africa. It is estimated that it is directly responsible for the deaths of more than 1 million people each year the majority of which are due to cerebral malaria and other complications following infection with *P. falciparum* which is transmitted by female Anopheles mosquitoes and most of the deaths occur in Africa and in children under the age of 5 years (40).

Many plant species are used in traditional medicines of malarious countries and a relatively few number of these have been investigated for evaluation of their antimalarial effect. Still lower is the number of those that have had the active natural compounds isolated and the toxicity determined (41).

Most people living in developing countries including ethiopia are unable to afford the pharmaceutical drugs and those which are affordable are declining in their effectiveness due to the problem of drug resistance. So they heavily rely on traditional herbal medicinal plants which are not proven for their safety and efficacy with reliable scientific data. Therefore, there is a high demand to undertake anti-malarial activity evaluation and toxicological assessment on traditionally claimed medicinal plants and prepare standardized herbal preparations with a reasonable quality and affordable price (5).

1.12. Significance of the study

The emerging of drug resistance to conventional antimalarials, empirical treatment without proper laboratory investigation, challenge of having effective vaccines and adverse effects of the existing antimalarial drugs leads to urgent need of more effective, tolerable and affordable antimalarial drugs.

Plants and their extracts have immense potential for treatment of malaria. However, there is a need for scientific validation, standardization and safety evaluation of traditionally used medicinal plants before they are recommended for treatment of malaria (42). Therefore, the list of traditionally used plants to control malaria must be backed by photochemical studies and scientific authentication to develop an appropriate phytomedicine.

Until recent times, the antimalarial activity and acute toxicity of *Cordia africana*, commonly used plant for the traditional treatment of malaria in Ethiopia, are not found in the literature. The present study, hence, provides information about its safety and efficacy in mice.

2. Objectives

2.1. General objective

- To evaluate the antimalarial effect of the crude methanol extract of inner bark of *Cordial africana* against *P. berghei* in mice.

2.2. Specific objectives

- To determine the antimalarial effects of crude methanol extract of inner bark of *Cordial africana* in mice infected with *P. berghei using 4-day suppressive test.*
- To assess the acute toxicity of crude methanol extract of inner bark of *Cordia africana* in mice.
- To perform preliminary phytochemical screening on crude methanol extract of inner bark of *Cordial africana.*
- To assess the effect of the extract on the body weight and temperature of *P. berghei* infected mice.

3. Materials and methods

3.1. Chemicals and instruments

Chloroquine (Addis Pharmaceuticals Factory PLC. Ethiopia), methanol (Okhla Industrial, India), 10% giemsa(Sciencelab.com,inc, USA), trisodium citrate (Deluxe Scientific surgico Pvt. Ltd, India), 0.9% normal saline (Addis Pharmaceuticals Factory PLC, Ethiopia), Whatman filter paper (Whatman Ltd, England), microscopic slide frosted (Citoplus, China), electronic balance (ADP 720L, Adam Equipment Co. Ltd), dry oven (250±10% volts, France), microscope, were used. All chemicals and reagents were analytically graded and procured from certified suppliers.

3.2. Collection, preparation, and identification of plant materials

The fresh inner bark of *Cordia africana* was collected from Gondar town in February 2015. Then the plant material was air-dried at room temperature under shade and the size of the dried barks was reduced. After this it was packed in an air tight plastic bag until extraction done. A taxonomist from Addis Ababa University Addis Ababa Ethiopia identified the plant as *Cordia africana* lams. A specimen of the plant was deposited (with voucher no HT01) at the National Herbarium in the College of Natural Sciences at Addis Ababa University for future reference.

3.3. Extraction of plant materials

The dried plant material was weighed by electronic balance. A total of 350g dried barks were undergone metanolic extraction using maceration technique for 3 days with occasional stirring. The mixture was filtered with what man paper number one. The residue was re-macerated twice for the same duration of days and then was filtered (43).

The filtrates were collected and combined together. They were concentrated by oven (<40 ^{o}C.) .Finally, the dried extract was transferred to vial and was kept in desiccator until required for experiment.

3.4. Experimental animals

Thirty Swiss albino mice, weighing 21-32 g and 6 to 8 weeks old, of either sex of inbred at the Animal House of the Department of Pharmacology, University of Gondar, were used. The animals were maintained under standard condition and were fed with a commercial rodent pellet diet and water *ad libitum*. All mice were acclimatized to the working environment one week prior to the experiments (44, 45).

3.5. Parasites

Chloroquine sensitive *P. berghei* was used for induction of malaria in experimental mice. Mice previously infected with *P. berghei* were used as donor. The donor *P. berghei* infected mice purchased from Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Ethiopia. The parasites were subsequently maintained in the laboratory by serial passage via IP route on weekly basis (46, 47).

3.6. Inoculum preparation

Blood from a donor mouse was collected via cardiac puncture with a rising parasitaemia of about 30 to 40%. Zero point five percent (0.5%) trisodium citrate was added as anticoagulant and it was diluted with 0.9% normal saline. Each mouse was inoculated IP on day 0, with 0.2 ml of this diluted blood containing approximately 1×10^7 *P. berghei* infected erythrocytes (46).

3.7. Grouping and dosing of animals

The mice were randomly divided into five groups of five mice each. The negative controls were treated with the vehicle (distilled water) used for reconstitution, whereas positive controls were treated with chloroquine 25mg/kg (the standard antimalarial drug). Three different doses of (100, 200, and 400) mg/kg of crude extract was administered to other groups (45). The standard drug (the positive control), the vehicle (the negative control) and the extracts were administered by oral route with the aid of an oral gavage.

3.8. Pharmacological tests

3.8.1. Acute toxicity test (Limit test)

Female Swiss albino mice were used for acute oral toxicity study. Nulliparous and non-pregnant female mice of 8 to 12 weeks of age were used. It was conducted as per the internationally accepted protocol drawn under OECD guidelines 425 (48).

The mice were fasted for three hours and were weighed. The first animal was given a limit dose of 2,000 mg/kg of extract by oral gavage. The mouse was observed continuously for one hour after administration of the extract; intermittently for 4 hours, over a period of 24 hours and for 14 days. The animal was observed for any gross behavioral changes such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhea, mortality and other signs of toxicity manifestation. As the mouse has survived at limit dose of 2,000 mg/Kg, four other mice were sequentially treated so that a total of five animals were tested (49).

3.8.2. Four-day suppressive test

The chemo suppressive test was done using standard four-day suppressive test against *P. berghei* infection in mice. The mice were randomly divided into five groups with five mice each. The three treatment groups were received at doses of 100, 200, and 400 mg/kg of the crude extracts. The control group was received 25 mg/kg of chloroquine (positive control group) and 0.5 ml/mice of distilled water (negative control group). Treatment was started three hours after infection and then continued for four consecutive days (46, 47).

On the fifth day, thin blood smears from the tail vein of each mouse were prepared on microscopic slides, fixed with absolute methanol and stained with 10% Geimsa solution at pH 7.2 for 15 minutes. The slides were taken out and dried with the room temperature. The number of parasitized RBC was examined under the microscope by a laboratory technician. The percentage suppression of parasitaemia was calculated for each test concentration by comparing the parasitaemia in infected controls with those received different concentrations of the test extract. Average percent parasitemia and percent parasitemia suppression were calculated by using the following formula (47, 50).

$$\% \text{ Parasitaemia} = \frac{\text{Number of parasite RBC}}{\text{Total number of RBC count}} \times 100$$

$$\% \text{ Suppression} = \frac{(X-Y)}{X} \times 100$$

Where X is mean parasitemia of negative control group and Y is mean parasitemia of treated groups.

3.8.3. Determination of body weight

Body weight of each mouse was measured before infection (day 0) and on (day 4) using a sensitive digital weighing balance (49).

3.8.4. Determination of temperature

The rectal temperature of the mice was measured with a digital thermometer before infection, three hours after infection and then daily up to day 4 to see the effect of the extracts on body temperature (49).

3.9. Preliminary phytochemical screening

The methanolic extract of the inner bark of *Cordia africana* was subjected to preliminary phytochemical screening tests for various plant constituents using standard procedures. Identification was done by characteristic colour changes (43, 48, 52). The procedures followed to detect the phytochemicals were described in the annex part.

3.10. Statistical analysis

Results were expressed as means ± SEM and were analyzed using SPSS version 16. Comparisons were made between negative control, positive control and treatment groups of various doses using ANOVA following by post hoc test. At 95% confidence interval, P-values less than 0.05 were considered as statistically significant.

3.11. Ethical consideration

The experimental animals were handled and cared in the experimental procedures according to the local ethical and internationally accepted laboratory animal use, care and welfare guidelines (44).

4. Results

4.1. Percentage yield of extraction

The nature of the extract and the actual and percentage yield of the methanolic extract of inner bark of *Cordia africana* were summarized in Table 1.

Table 1: Percentage yield and physical description of the methanolic extract of the inner bark of *Cordia africana.*

Extracting solvent	Nature of the extract	Color of the extract	Actual Yield (g)	Percentage Yield (%w/w)
Methanol (80%)	Hygroscopic powder	Yellow	30	8.57

4.2. Phytochemical screening

Preliminary phytochemical screening for secondary metabolites was carried out on the crude methanolic extract of inner bark of *Cordia africana.* Flavonoids, phenols and saponins possibly present in this crude extract, shown in Table 2. Since the phytochemical screening tests used were preliminary, specific phytochemical tests are necessary for further confirmation of these results.

Table 2: Phytochemical screening test result of the crude methanolic extract of the inner bark of *Cordia africana.*

Phytochemicals	Remarks
Anthraquinones	_
Terpinoids	_
Saponins	+
Phenols	+
Flavonoides	+
Alkaloids	_
Tannins	_
Cardiac glycosides	_
Sterols	_

4.3. *In vivo* acute toxicity test

The acute toxicity of the crude extract of the inner bark of *Cordia africana* showed that it did not cause any mortality within 24 hrs up to the limit dose of 2,000 mg/kg body weight of mice.

4.4. Four-day suppressive test

Four-day suppressive test of crude methanolic extract of the inner bark of *Cordia africana* significantly suppressed parasitemia in *P. berghei* infected mice. At 100, 200, and 400 mg/kg body of the crude extract, chemosuppressions were 18.30%, 24.84%, and 31.37%, respectively as shown in Table 3.

Table 3: Suppressive activity of crude methanolic extract of inner bark of *Cordia africana* against *P. berghei* in mice (Results are expressed as: Mean ± SEM; n = 5).

Treatments	Doses	% Parasitemia	% Suppression
Negative control	0.5 ml	30.60±2.04	0.00^{e3}
CAIBE	100 mg/kg	25.00±2.83	18.30^{e3}
	200 mg/kg	23.00±2.57	24.84^{e3}
	400 mg/kg	21.00±1.95	31.37^{e3a1}
Positive control	25 mg/kg	3.00±1.00	$90.20^{a3b3c3d3}$

Where CAIBE=*Cordia africana* inner bark extract; a=as compared to negative control; b=as compared to 100 mg/kg; c=as compared to 200 mg/kg; d=as compared to 400 mg/kg; e=as compared to positive control; 1=$p<0.05$; 2=$p<0.01$ and 3=$p<0.001$.

Unlike 100 and 200 mg/kg extract treated mice; 400 mg/kg extract treated mice showed a significant ($P < 0.05$) suppression when compared to negative control group. However, there were no significant ($P > 0.05$) parasitemia suppression differences among extract treated groups. The chemo-suppression induced by the standard drug, chloroquine, was 90.20% which was significant ($P < 0.001$) as compared to other treatment groups.

4.5. Effects of the extract on body weights of mice

The crude methanolic extract of inner bark of *Cordia africana* brought -0.38, -1.79 and -2.41% weight loss activities at 100, 200 and 400 mg/kg while distilled water treated mice showed -0.85% as shown in Table 4.

Table 4: Effect of crude methanolic extract of the inner bark of *Cordia africana* on body weight of *P. berghei* infected mice (Results are expressed as: Mean ± SEM; n = 5).

Treatments	Doses	Body Weight (gm)		
		Pre-treatment	Post-treatment	% Wt Change
Negative control	0.5 ml	29.32±1.30	29.07±1.34	$-0.85^{d1,e2}$
CAIBE	100 mg/kg	26.40±1.51	26.30±1.46	-0.38^{d2}
	200 mg/kg	28.44±1.12	27.93±1.06	-1.79^{e3}
	400 mg/kg	27.82±0.49	27.15±0.49	$-2.41^{a1,b2,e3}$
Positive control	25 mg/kg	27.62±0.79	27.95±0.84	$1.19^{a2,b1,c3,d3}$

Where CAIBE=Cordia africana Inner Bark Extract; a=as compared to negative control; b=as compared to 100 mg/kg; c=as compared to 200 mg/kg; d=as compared to 400 mg/kg; e=as compared to positive control; 1=p<0.05; 2=p<0.01; and 3=p<0.001.

Unlike 100 and 200 mg/kg treated mice, 400 mg/kg of extract treated mice showed a significant ($P < 0.05$) body weight loss as compared to distilled water treated ones. There were clear differences ($P < 0.05$) in weight loss among extract treated groups. However, chloroquine treated mice showed a significant ($P < 0.01$) weight gain when compared to the distilled water treated ones.

4.6. Effect of extract on body temperatures of mice

The crude methanolic extract of inner bark of *Cordia africana* brought -0.30, 0.14, and 0.17% change in body temperature of mice at 100, 200 and 400 mg/kg while the distilled water treated mice showed -0.52% change in body temperature. Even though the extract treated mice showed an increase in body temperature, they were insignificant ($P > 0.05$) compared to the negative and positive controls. But chloroquine treated mice showed significant ($P < 0.05$) body temperature increases as compared to negative controls as shown in Table 5.

Table 5: Effect of crude methanolic extract of the inner bark of *Cordia africana* on body temperature of *P. berghei* mice (Results are expressed as: Mean ± SEM; n = 5).

Treatments	Doses	Body Temperature (C^o)		
		D_0-Temp	D_4-Temp	% Temp Change
Negative control	0.5 ml	36.88±0.26	36.69±0.25	-0.52^{e2}
CAIBE	100 mg/kg	37.08±0.17	36.97±0.18	-0.30^{e1}
	200 mg/kg	36.58±0.23	36.63±0.22	0.14
	400 mg/kg	35.28±0.98	35.34±1.02	0.17
Positive control	25 mg/kg	36.54±0.30	37.12±0.31	$1.59^{a2,b1}$

Where CAIBE=Cordia africana Inner Bark Extract; D_0-Temp is Body Temperature on day 0; D_4-Tmp is Body Temperature on day 4, *a=as compared to negative control; b=as compared to 100 mg/kg; e=as compared to positive control; 1=p<0.05; and 2=p<0.01.*

5. Discussion

Plasmodium species that cause human disease are essentially unable to infect non-primate animal models. So, *in vivo* evaluation of antimalarial compounds typically begins with the use of rodent malaria parasites such as *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei.* From these, *P. berghei is* used in predicting treatment outcomes of any suspected antimalarial agent due to its high sensitivity to chloroquine making it the appropriate parasite for the study of antimalarial activity of certain medicinal plants (46).

Preliminary phytochemical screening tests of crude methanolic extract of the inner bark of *Cordia africana* possibly showed that the presence of flavonoids, phenols and saponins. These compounds are known to show antimalarial activity (52, 53), and therefore the current extract probably showed antimalarial activity due to the presence of such bioactive secondary metabolites.

Phenols are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants. Plant polyphenols have drawn increasing attention due to their potent antioxidant properties and their marked effects in the prevention of various oxidative stress associated diseases such as malaria (54). Similar results had been found on *Parkia biglobosa* at which phenolic compounds had major antimalarial activities (55). Hence, the parasitaemia suppression effect of the crude methanolic extract of the inner bark of *Cordia africana* may be attributed to the presence of these phenolic compounds.

Methanol extract of the inner bark of *Cordia africana* was tested for its acute toxicity in mice. None of the test mouse died or showed signs of acute toxicity within 24 hours of treatment with the test extract. The extract was nontoxic to test mice, as they did not show signs of acute toxicity within 24 hours at the doses of 2000 mg/kg.

The four-day suppressive test is a standard test commonly used for antimalarial screening, and the determination of percent inhibition of parasitaemia is the most reliable parameter (56). When a standard antimalarial drug is used in mice infected with *P. berghei*, it suppresses parasitemia significantly ($p<0.05$) (57). This is in agreement with the effects of chloroquine in this study which achieved 90.20% suppression of parasitaemia on a 4-day

suppressive test. The chemosuppressive effect of *Cordia africana* inner bark extract has shown the highest suppressive effect (31.37%) occurred at 400mg/kg/d which is comparable to the suppressive effect of other studies reported on *Azadrachata indica* (58) and *Nauclea natifolia* (59). The percentage of parasitaemia measured at 400mg/kg changed significantly ($P<0.05$) from those in the negative control group indicating that the extract has antiplasmodial activity and supports the traditional claim as antimalarial herbal remedy in Ethiopia.

Body weight loss and fever are features of rodent malaria infections (60).The extract did not prevented significantly ($p<0.05$) body weight loss at tested doses when compared with the infected untreated control, this may be due to the extract may contain an agent which suppressed appetite. The result is similar to from reported on seed extract of *Dodonaea angustifolia*, which fails to prevent body weight loss (61).

A decrease in the metabolic rate of infected mice occurs before death and is accompanied by a corresponding decrease in internal body temperature (62). Ideally, the rectal temperature decreases as parasite level escalates. Active compounds should prevent the rapid dropping of rectal temperature. The crude extract increased body temperature of mice, which might be constituents responsible for this effect were likely found in the extract. However, the increase in body temperature by extract is not significant when compared to distilled water treated mice.

6. Conclusion

From this study it can be concluded that the methanol extract of inner bark of *Cordia africana* has antimalarial activity as seen in its ability to suppress *p.berghei* infection and this can be a base for further studies to develop effective antimalarial agents.

7. Suggestions for further work

From this study, it can be suggested that:

- The crude extract of the inner bark of *Cordia africana* should be fractionated to isolate, identify and elucidate the possible antiplasmodial compounds.
- The subacute, chronic and cytotoxic potential of the plant should be studied to know better about the safety of the plant for traditional usage.
- It would appropriate to study other biological activities of the plant claimed by traditional medicine.
- It would also better to the study the effect of the plant on human malaria parasites.

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9. Annexes

The phytochemicals found in the crude methanol extract of inner bark of *Cordial africana* will be detected by the following standard preliminary phytochemical screening tests.

Test for phenols

1ml aqueous solution of the crude extract will be treated with few drops of 10% ethanolic ferric chloride and a color change to dark blue is affirmative of the positive test.

Test for saponins

0.5 g of a hydro-alcoholic extract will be dissolved in 10 ml of distilled water in a test tube. The test tube will be stoppered and shaken vigorously for 30 sec and allowed to stand in a vertical position and observed over 30 min. The formation of "honey comb" froth that persisted for half an hour indicates the presence of saponins.

Test for alkaloids

Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

Test for flavonoids

Five ml of dilute NH_3 solution will be added to 3 ml aqueous filtrate of the sample followed by addition of 2 ml of concentrated H_2SO_4, and formation of yellow color indicates presence of flavonoids.

Test for anthraquinones

5.0 g of dried extract will be shaken with 10.0 ml of benzene. This will be filtered and 5.0 ml of 10% ammonia solution will be added to the filtrate. The mixture will be shaken and the presence of violet colour in the ammoniacal (lower) phase indicated the presence of free hydroxy anthraquinones.

Test for tannins

Two ml of water diluted sample will be treated with 3 drops of 10% ferric chloride and formation of bluish-black color indicates presence of tannins.

Test for terpenoids

2ml of extract was added to 2ml of acetic anhydride and concentrated H2SO4. Formations of blue green rings indicate the presence of terpenoids.

Test for triterpenes

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

Test for diterpenes

Extracts will be dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes

Test for cardiac glycosides

To 0.5 g of extract diluted to 5 ml in water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. Then 1ml of concentrated sulphuric acid will be added. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer

Test for Steroids

1 ml of the extract will be dissolved in 10 ml of chloroform and equal volume of concentrated H_2SO_4 will be added by sides of the test tube. The appearance of upper layer turning to red and sulphuric acid layer showing yellow with green fluorescence indicated the presence of steroids