JIMMA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

In vivo anti-plasmodial and cytotoxic activities of *Combretum Molle* (Combretaceae) Seed extract in Swiss albino mice

By: MerkinAnato

June, 2017
Jimma Ethiopia

JIMMA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

In vivo anti-plasmodial and cytotoxic activities of *Combretum Molle* (Combretaceae) Seed extract in Swiss albino mice

By: MerkinAnato

A Thesis submitted to Department of Biology, College of Natural Sciences, Jimma University, in partial fulfillment of the requirement for the degree of Master of Science in Biology (General Biology)

Advisor: Tsige Ketema (PhD)

June, 2017
Jimma Ethiopia

Approval form

Jimma University

School of Graduate Studies

Department of Biology

Title: ***In vivo*** **anti-plasmodial and cytotoxic activities of** ***Combretum Molle*** **(Combretaceae) Seed extract in Swiss albino mice**

By: MerkinAnato

The thesis entitled ***in vivo*** **anti-plasmodial and Cytotoxic activities of** ***Combretum Molle*** **(Combretaceae) [jewoo] Seed extract in Swiss albino mice** has been approved by the Department of Biology for the partial fulfillment of the Degree of Master of science in General Biology.

Approved by the examining board

1. Chairperson,

Name________________________ Signature ______________ Date__________

2. Advisor

Name________________________ Signature______________ Date__________

3. External Examiner

Name________________________ Signature______________ Date__________

4. Internal Examiner

Name________________________ Signature______________ Date__________

Declaration

I, the under signed, declare that this is my bona fida original work, has never been presented in this or other University, and that all the resources and materials used for the thesis have been dully acknowledged.

Name: MerkinAnato

Signature__________________

Date ____________________

Place: Jimma University

Date of submission________________

This thesis has been submitted for examination with my approval as candidate' advisor

Name: Tsige Ketema (PhD)

Signature__________________

Date ____________________

Acknowledgment

I would like to extend my sincere thanks to my adviser Dr. Tsige Ketema for her guidance, assistance, encouragement, supervision, unreserved support in reviewing, commenting and provision of valuable suggestions throughout this research. I would also like to express my appreciation for Departments of Biology and chemistry, College of Natural Sciences, Jimma University for supporting extraction of plant material, laboratory facilities, and overall support. College of Agriculture and veterinary medicine of the same University deserve special gratitude for provision of experimental animals and permit to use their laboratory with all necessary equipment's. I am also grateful to the Ethiopian Public Health Institute (EPHI) for donation of infected (donor) mice for this study.

My special thank also extends to Dr. MeseleBezabih for his professional support (slides reading during histological study); Dr. DachassaTegegne for his support during experimental part of the study in his laboratory; MrGemechuZelekefor supporting anti-plasmodial assay study; Ms Genet Tsegayefor keeping sanitation of mice and laboratory equipment;MrMadie Abdu for his assistance during breeding of mice for the experimental study and MrYohanis supporting for microscopy reading and counting parasites from thin blood smear. Finally, I would like to thank my family for being always beside me during the study period and Gambella regional government educational office for sponsoring this study.

Abbreviations

- 3D7: CQ-sensitive *P. falciparum* strain.
- ACTs: artemisinin-based combination treatments.
- Alb: Albumin;
- ANOVA: Analysis of variance;
- CBC: Complete blood cells count;
- CDC: Centers for Disease Control
- CQ: chloroquine.
- CRP: C-reactive protein
- CRPv: chloroquine resistant *P. vivax* malaria
- DDT: dichlorodiphenyltrichloroethane.
- EPHI: Ethiopia Public Health Institute;
- Hb: Hemoglobin;
- HCT: Hematocrit;
- INDO: CQ-resistant *P. falciparum* strain.
- LD50: 50% lethal dose.
- MCH: Mean corpuscular haemoglobin;
- MCHC: Mean corpuscular haemoglobin concentration;
- MCV: Mean corpuscular volume;
- MST: mean survival time.
- pfcrt: *Plasmodium falciparum* chloroquine resistance transporter.
- RBC: Red blood cells;
- WBC: White blood cells
- WHO: world health organization.

Table of Contents

Acknowledgment i
Abbreviations ii
List of figures v
List of Tables vi
Abstract vii
1. Introduction 1
  1.1. Background and justification 1
  1.2. Significance of the study 2
  1.3. Objective of the Study 3
    1.3.1 General Objective 3
    1.3.2 Specific objectives 3
2. Literature Review 4
  2.1 Epidemiology of malaria 4
  2.2. Transmission of Malaria 5
  2.3. The life cycle of malaria parasite 5
  2.4. Malaria Diagnosis 6
  2.5. Prevention & control 7
  2.6. Treatment 7
  2.7. Antimalarial drug resistance 8
    2.7.1. Drug resistance of *P. falciparum* 8
    2.7.2. Drug resistance of *P. vivax* 9
  2.8. Antiplasmodial activities of Traditional Medicinal Plants 10
    2.8.1 Global perspective 10
    2.8.2. Practice of Anti-plasmodial medicinal plant use in Africa 11

2.8.3. Tradition of use of anti-plasmodial plants in Ethiopia 13
3. Materials and Methods 14
3.1. Plant material Preparation 14
3.2. Experimental Animals and Protocol 14
3.3. Acute toxicity determination 15
3.4. Animal treatment and blood sample collection 15
3.5. Haematological and biochemical assay 15
3.6. Histological study 16
3.7. *In vivo* anti-plasmodial tests 16
3.7.1. Parasite Inoculation and Extract Administration 16
3.7.3. Determination of Parasitemia 17
3.7.4. PCV Determination 17
3.7.5. Determination of Mean Survival Time (MST) 18
3.8. Data analysis 18
3.9. Ethical consideration 18
4. Results 19
4.1. Acute toxicity 19
4.2. Haematological parameters test 19
4.3. Biochemical tests 20
4.4. Effect of *C. molle* seed extract on immune cells 22
4.5. Histological analysis 23
4.6. Anti-plasmodial activities of seed extract of *C. molle* 24
5. Discussion 26

List of figures

Figure 1 Life cycle of Malaria parasite: 5

Figure 2 Levels (Mean ± SEM) of kindney test indicators (urea and creatinine) of Swiss albino mice received *C. molle* seed extract. 19

Figure 3 Level of liver enzymes (mean ± SEM) of Swiss albino mice received single dose of *C. molle* seed extract. 20

Figure 4 Level of albumin (mean ± SEM) of Swiss albino mice received single dose of *C. molle* seed extract. 21

Figure 5 c - reactive protein level (mean ± SEM) in Swiss albino mice received single dose of *C. molle* seed extract. 22

Figure 6 Levels of immune cells (mean ± SEM) in Swiss albino mice received single dose of C. molle seed extract. 23

Figure 7: kidney (a, b) and Liver (c, d) microphotograph of Swiss albino mice received *C. molle* seed extract. 24

Figure 8: Pattern of rectal temperature of *PbA* infected mice treated with crude extract of seed of *C. molle.* 25

List of Tables

Table-1: Some traditional medicinal plants that have moderate to good anti-plasmodial activities in Africa including Ethiopia…………………………………………………………...11

Table 2: Hematological parameters of Swiss albino mice received three different dose of *C. molle* seed extract. 18

Table 3: Effect of seed Crude Extracts of c. molle on rodent malarial 24

Table 4: Temperature, weight and packed cell volume of infected mice treated with seed of *C. molle* in the 4 day suppressive test 25

Abstract

Background: *Combretum molle* (Combretaceae) seed has been traditionally used for treatment of malaria and other aliments in Gambella region, Ethiopia. Thus, aim of this study was to evaluate *in vivo* cytotoxic and anti-plasmodial activities of *C. molle* plant using mice.

Methods: The acute toxicity study was conducted using single dose administration of crude seed extract of the plant at different doses. Also effect of the extract on hematological and biochemical parameters, and tissues of different organs were assessed. Furthermore,anti-plasmodial activity of the seed crude extract, its chemo-suppressive potential, and protection against some clinical symptoms was assessed.

Results: Acute toxicity study didn't show any observable effects in mice under all doses. The crude seed extract of *C. molle* showed 63.5% of parasite suppression in Swiss albino mice infected with *Plasmodium berghei* ANKA (*Pb*A) parasite at dose 125mg/kg. Relatively survival time of mice treated with the same dose showed significant increment compared to the negative control. But, significantly lower than mice treated with standard drug, chloroquine (CQ). On the day-4 post-infection (p.i) the plant extract showed significant ($P<0.05$) protection against body weight reduction, high rectal temperature and haemolysis of RBC at relatively lower doses. However, it was not significantly different from mice group treated with CQ. Findings from haematological study showed that seed extract of *C. molle* did not cause significant effects on most red blood cells indices, while except eosinophil all white blood cells (WBCs) indices showed significant reduction ($P<0.05$) under use of almost all doses. Also, level of liver enzymes such as glutamic oxaloacetate transaminase (sGOT) and glutamic pyruvic transaminase (sGPT), showed significant increment ($P<0.05$) in mice treated with different doses of the plant extract, while level of albumin was significantly reduced ($P<0.05$). Similarly, significant increament ($P<0.05$) in levels of kidney function indicators: urea and creatinine was observed.

Conclusion: The study revealed thatat lower dose (125mg/kg) crude extract of *C. molle*seed has lower toxicity on haematological, biochemical and immunological parameters. Also at this dose, high chemo-suppressive activity against murine malaria parasite (*Pb*A) was documented. Thus further isolation and*in vivo* evaluation of active components of the plant is recommended.

Keywords: anti-plasmodial, *C. molle*, biochemical,hematological, histology, murine malaria.

1. Introduction

1.1. Background and justification

Combretum molle is one of the herbal plants belongs to the family Combretaceae which includes 20 genera and about 600 species (Miaffo*et al*., 2015).C. molle is distributed especially in savannah vegetation that cuts across from Senegal to West Cameroon, but generally exists in tropical and subtropical Africa regions (Burkill, 1985). Documented species distribution of Combretum molle is native to Botswana, Central African Republic, Congo, Democratic Republic of Congo, Djibouti, Eritrea, Ethiopia, Gabon, Kenya, Lesotho, Namibia, Senegal, Somalia, South Africa, Sudan, Swaziland, Tanzania, Uganda and Zambia (Orwa et al, 2009).

This plant is popularly used in South Africa for the treatment of stomach pains, dysentery, gastric ulcers, abdominal disorders, and other illnesses (Eloff *et al*., 2008). Also, roots of *C. molle* seem to have a variety of uses against hookworm, stomach pains, snake bite, leprosy, fever, dysentery, general body swellings, and abortion as well as for swelling of the abdomen, sterility and constipation(Fyhrquist*et al*., 2012).

This plant bark and root in vitro tested in different African countries for treatment of bacteria (Ragusa and Araya, 2012). Also seed of the plant tested against fungi and bacteria, (Masoko*et al*.,2007;Amare and Tadesse, 2016),and leaf againsthelminths(Ademola and Eloff,2010). In addition,*C. molle*bark extract was tested for its *in vitro* antimicrobial activities (Ragusa and Araya, 2012;Amareand Tadesse, 2016).

In fact, most of the plants are used indiscriminately without adequate information on toxicity risks, it has become imperative to assess the safety of plants used for medicinal purposes for possible toxicity(Miaffo et al., 2015).Thus, the only part such as leaf and twigs of this plant toxicologically studied in animal model.

Moreover,different species of *Combretum* plant havebeen reported to have *in vitro*anti-plasmodial activity against chloroquine sensitive *Plasmodium falciparum* strains(Karou*et al.*,2003,Ancolio*et al.*,2002; Salon*et al.*,2013).However,tannin, stem bark, seed and leave extract of *C. molle* plant have revealed in vitro activity against *P. falciparum* (Asres*et al.*, 1998;Asres*et al*, 2001;Gansane*et al.*, 2010).

Almost every part of these plants (roots, leaves, seeds, twigs, and stem barks) have been used in African traditional medicine for the treatment of various ailments and diseases (Miaffo*et al.*, 2015),Similarly, seed of this plant is widely used by traditional practitioners in Ethiopia, mainly among rural residents in Gambella region, for treatment of malaria, HIV and other aliment (personal communication). Also, most of the traditional practitioners in the region claim that *Combretum molle* seed is one of the potential traditional medicinal plants with broad healing property. However, although seed of *C. molle* plant widely used by local residents of Gambella region for treatment of malaria, and shows good *in vitro* antiplasmodial activity, it's*in vivo* cytotoxic and cytotoxic and anti-plasmodial activities of seed of the *C. molle* plant using mice as animal model.

1.2. Significance of the study

The findings of this study will serve as a base line data for researchers working on designing of anti-malarial drugs from traditional medicinal plants (*C.molle*) both nationally and internationally. Moreover, results from the cytotoxic activity study will helps to know dose-effect relation of seed extract of the plant. This knowledge is crucial for the researchers to predict the possible side effects of using seed of the plant and quantify optimum effective dose that would not cause harmful effects.

1.3. Objective of the Study

1.3.1 General Objective

- To evaluate the in vivo cytotoxic and anti-plasmodial activities of *C. molle* seed extractusing Swiss albino mice

1.3.2 Specific objectives

- To investigate cytotoxic effect of seed extracts of *C. molle* in Swiss Albino mice,
- To estimate effect of seed extracts of *C. molle* on hematological and biochemical parameters of Swiss albino mice,
- To assess *in vivo* immunomodulatory activity of seed extracts of *C. molle* using Swiss albino mice
- To assess the *in vivo* antimalarial activity of the seed extracts of *C. molle* against chloroquine sensitive *Plasmodium berghei* ANKA (*Pb*A),
- To determine the effect of seed extracts of *C. molle* on PCV, body weight, rectal temperature and survival time of the *Pb*A infected mice,

2. Literature Review

2.1 Epidemiology of malaria

According to Gilman and Goodman.,2001, Malaria is a disease that is widespread in many parts of the world, mainly in tropical and subtropical regions. It is caused by protozoan parasites belong to the genus Plasmodium and represented by over 120 species and infectious to man, simians, rodents, birds and reptiles (Krettli*et al*.,2001). Malaria in humans is caused by five species of parasites belonging to genus Plasmodium. Four of these (*P. falciparum, P. vivax, P. malaria and P. ovale*) are human malaria species that are spread from one person to another via the bite of female mosquitoes of the genus Anopheles (WHO, 2015). In recent years, human cases of malaria due to *P. knowlesi* have been recorded. This species causes malaria among monkeys in certain forested areas of South-East Asia. Current information suggests that *P. knowlesi* malaria is not spread from person to person, but rather occurs in people when an Anopheles mosquito infected by a monkey then bites and infects humans (zoonotic transmission) (WHO, 2015).

In Africa, Malaria accounts for 10 % of the total disease burden; Over 90% of deaths occur in sub-Saharan Africa (WHO, 2005). The burden of morbidity and mortality is inclined towards children, not yet immune (Snow *et al*., 2005), and pregnant women where malaria parasites are sequestered in the placenta (Roweand Kyes, 2004). According to, Muregi*et al*.,(2003), the burden of mortality in sub-Saharan Africa is accounted for 89% of the deaths where5% of children die from the disease before reaching 5 years of age. Generally, in the countries of the sub-Saharan tropical zones, this disease kills out of the million peoples per year, and this mortality is very important in the children of less than five years.

It is the most killer infectious disease with an estimated 3.3 billion people at risk in 2010 and the first prioritized tropical disease of the WHO (World Malaria Report, 2011). Vogel, (2010) reported that about half of the world's population is at risk of malaria and one to two million annual deaths, mostly among African children, can be attributed to malaria alone. Malaria has become the main concern of the World Health Organization as a result of its re-emergence and expansion of its distribution to previously non-endemic areas (Alemu*et al.*, 2013).

2.2. Transmission of Malaria

The transmission intensity depends on the prevalence and infectiousness of gametocytes that circulate in peripheral blood and the number of Anopheles mosquitoes in the area was result in a high turnover of parasites and infection pressure (Sauerwein 2007). The causative agent, is transmitted by the female Anopheles mosquito species, which has also developed resistance against insecticides, such as dichlorodiphenyltrichloroethane (DDT), and chemoprophylaxis has not often yielded the expected results (White, 2004). Additionally, the disease causing protozoans have developed resistance against most of the drugs currently used to treat malaria (Kang *et al*, 2014) and spread to previously malaria free areas because of changes in the climate (Deressa *et al*., 2003).

Areas with high rainfall have increased malaria incidence because of an increase in breeding sites; elevation along with cooler temperatures and lower humidity is also a factor as transmission rarely occurs above 2000-2500 meters (Lamar *et al*., 2007). In areas where malaria transmission is seasonal and unstable, the disease burden is confined to a wide age range, and adults as well as children suffer severe morbidity. In areas with intense transmission, however, the burden of disease is confined to the youngest age groups, as adults would have already developed immunity and the highest incidence would be among children under 5 years old (Himeidan*et al*., 2005).

2.3. The life cycle of malaria parasite

The general features of the following parasitic life cycle in (Figure 1) apply to all *Plasmodium* species. During a blood meal, a malaria infected female anopheles mosquito inoculates sporozoites into the human host. Sporozoites infect liver cells and mature into schizonts, which rupture and release merozoites into the blood stream where they infect red blood cells (Mueller, 2007). According to Lamar *et al*., (2007), blood stage parasites are responsible for the clinical manifestations of the disease. In case of infection during a mosquito blood meal; there is an asymptomatic incubation period of approximately 7 to 30 days while the parasites develop in the liver and during the initial multiplication in the blood.

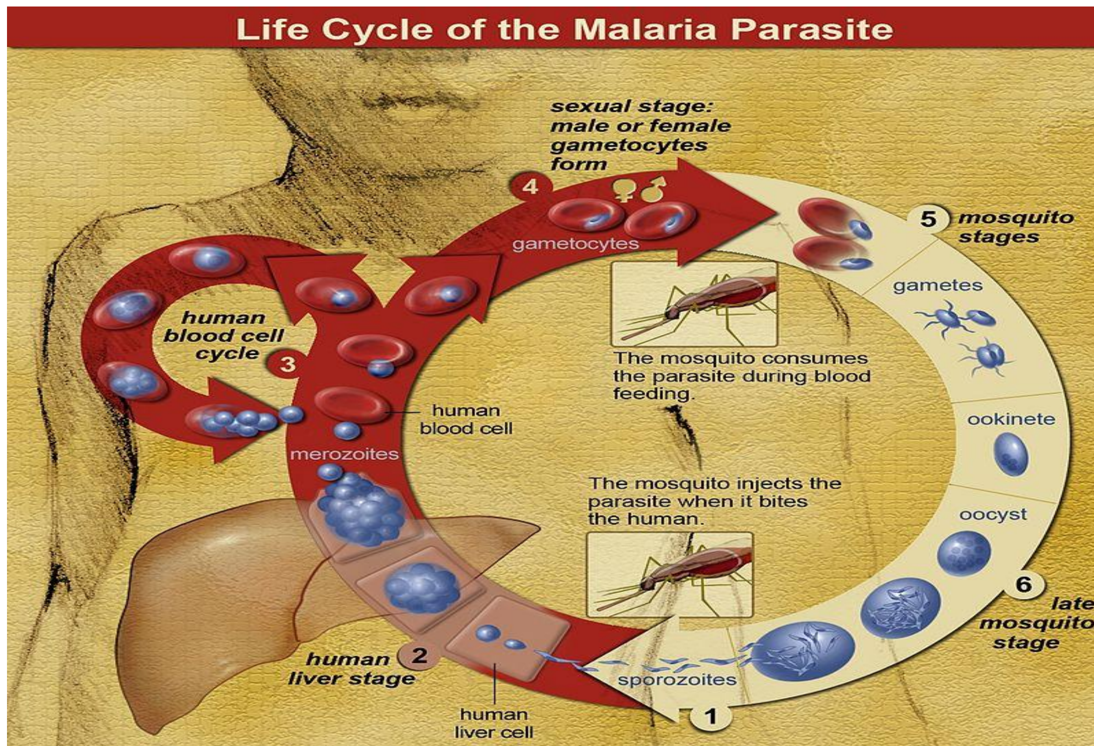

Figure 1 Life cycle of Malaria parasite (NIH, 2013)

2.4. Malaria Diagnosis

The widely used method to diagnose malaria is made on the basis of clinical history, signs, or symptoms. In many endemic areas without adequate diagnostic capacity, patients with a febrile illness are likely to receive the diagnosis of malaria. In most endemic areas, microscopic slide examination of peripheral blood remains the most widely used test as well as the gold standard for detecting malaria parasitemia (Michaeland Wilson, 2013). To prepare a thick blood film for Microscopic diagnosis, a blood spot is stirred in a circular motion with the corner of the slide, and allowed to dry without fixative. A thin blood film is prepared by immediately placing the smooth edge of a spreader slide in the drop of blood, adjusting the angle between slide and spreader to 45°, and then smearing the blood with a swift and steady sweep along the surface and staining blood films Giemsa stain (20-30 min), Leishman stain (45 min), and rapid Field stain method (10 sec) (Chotivanich, 2007)

2.5. Prevention & control

Malaria transmission can be reduced by preventing mosquito bites due to distribution of mosquito nets and insect repellents, or mosquito-control measures such as spraying insecticides and draining standing water (where mosquitoes breed) (Kilama, and Ntoumi, . 2009). Control activities approaches include the reduction in mosquito breeding grounds (environmental modification), target the larva stages with chemical or biological agents, and massive insecticide spraying for the adult mosquitoes. Biological control methods include the introduction of fish which eat the mosquito larvae), and bacteria (eg, *Bacillus thuringiensis*) which excrete larval toxins. Case detection and treatment is another potential control method. Identifying and treating infected persons, especially asymptomatic individuals, will reduce the size of the parasite reservoir within the human population and can lower transmission rates (Makar, 2011).

2.6. Treatment

The first antimalarial drug was quinine, isolated from the bark of Cinchona species (Rubiaceae) in 1820. It is one of the oldest and most important antimalarial drugs, which is still used today. In 1940, another antimalarial drug chloroquine was synthesized and used for the treatment of malaria until recently (Saxena S,*et al*., 2003).Once the diagnosis of malaria has been confirmed, appropriate treatment must be initiated immediately and specific treatment regimen depends on whether the case is diagnosed as complicated or uncomplicated malaria (Lamar *et al.,* 2007).

Treatment should be guided by three main factors: the 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. Knowledge of the geographic area where the infection was acquired provides information on the likelihood of drug resistance of the infecting parasite and enables the treating clinician to choose an appropriate drug or drug combination and treatment course. Chemoprophylaxis includes taking chloroquine when in chloroquine-sensitive areas (Basir*et al*.,2012). In areas with chloroquine-resistant *P. falciparum,* quinine sulphate, mefloquine, or atovaquone /proguanilis suggested World Malaria report.

P. vivax malaria should be treated with chloroquine in areas where this drug is effective; an appropriate ACT (not artesunate plus sulfadoxine-pyrimethamine) should be used in areas where *P. vivax* resistance to chloroquine has been documented World Malaria report (WHO, 2011). Chemoprophylaxis agents do not eliminate *P. vivax* and *P ovale* (forms of the parasite that remains in the liver), primaquine phosphate treatment is recommended when living in endemic areas (Aribodoret al., 2016). Consequently, new drugs or drug combinations are urgently needed today for the treatment of malaria. These drugs should have novel modes of action or be chemically different from the drugs in current use (Ramalhete*et al*., 2008).

2.7. Antimalarial drug resistance

Drug resistance of malaria parasites has been defined as the ability of a parasite strain to multiply or survive in the presence of concentrations of a drug that normally destroy parasites of the same species or prevent their multiplication.Resistance to antimalarial drugs has been described for two of the four species of malaria parasite that naturally infect humans (*P. falciparum* and *P. vivax)*(Aribodoret al., 2016).

2.7.1. Drug resistance of *P. falciparum*

P. falciparum has developed resistance to nearly all antimalarial drugs in current use, although the geographical distribution of resistance to any single antimalarial drug varies greatly. Chloroquine-resistant *P. falciparum* malaria have been described everywhere that is transmitted except for non malarious areas of Central America (north-west of the Panama Canal), limited areas of the Middle East and Central Asia(Teka*et al.*, 2008).

Sulfadoxine pyrimethamine (SP) resistance occurs frequently in South-East Asia and South America. SP resistance is becoming more prevalent in Africa as that drug is increasingly being relied upon as a replacement for chloroquine. Mefloquine resistance is frequent in some areas of South-East Asia and has been reported in the Amazon region of South America and sporadically in Africa (Aribodor*et al*., 2016).

In Ethiopia, the first report on the emergence of chloroquine resistant *P. falciparum* was made in 1986 after isolation of chloroquine resistant *P. falciparum* from patients in areas bordering the neighbouring countries (Sudan, Somalia, and Kenya) (FMOH, 1999). Since then, studies conducted in different parts of the country showed that chloroquine resistant *P. falciparum* has spread to all malarious area of the country(Aribodor*et al*., 2016).

Between 1985 and 1990, the treatment with mefloquine, combined with sulfadoxine /pyrimethamine was started, but by the year 1990 the cure rate had fallen to 71% in adults and 50% in children and could no longer be used due to resistance (Price *et al*., 1997).Faster drop in therapeutic efficacy of SP for the treatment of uncomplicated falciparum malaria enforced the adoption of Artemether-lumefanthrine (Coartem) (AL) in place of SP as a first line treatment in 2004 (FMOH, 2004).

While artemisinin-based combination treatments (ACTs) have played an effective role in controlling the disease in many malaria endemic areas, the appearance of resistant parasites to artemisinin derivatives in wide area of Southeast Asia encompassing south Vietnam to central Myanmar underscores the fragility of malaria treatment measures (Rope, 2011).

2.7.2. Drug resistance of *P. vivax*

P. vivax infection acquired in some areas has been shown to be resistant to chloroquine and/or primaquine (Murphy et al., 1993). Unequivocal evidence exists with high-grade chloroquine resistance on the islands of New Guinea and Indonesia, is evidence of accumulating for declining chloroquine efficacy in many other *P vivax*-endemic areas (Ratcliff, 2007).

Yeshwondim, (2010) was reported that chloroquine resistant *P. Vivax* malaria (CRPv) is coming from different malaria endemic areas of Ethiopia. The treatment failure (13%) associated with drug resistance reported in Ketema*et al.*, (2011) was relatively the highest as compared to earlier few reports from Ethiopia and Africa is a good indicator of the emergence and spread of chloroquine resistant P. vivax strains in malaria endemic area of Ethiopia.

According to Teka*et al*, (2008) malaria surveillance report confirms that *P. vivax* malaria is responsible for 40% of malaria cases in Ethiopia. Getachew*et al*, (2015) study on Chloroquine efficacy for Plasmodium vivax malaria treatment in southern Ethiopia highlights evidence of clinical treatment failure due to CQ resistance emerging in three (Shone, Guba and Batu) out of four sites (Shele, Shone, Guba and Batu)in southern Ethiopia which calls for increased drug resistance monitoring and re-evaluation of malaria treatment guidelines. The chloroquine treatment on *P.vivax* failure reported in Ketema*et al* (2011) study (13%) was relatively the highest as compared to earlier few reports from Ethiopia and Africa as the reporter's concluded and forwarded. This report is a good indicator of the emergence and spread of chloroquine resistant *p. vivax* strains in malaria endemic area of Ethiopia.

2.8. Antiplasmodial activities of Traditional Medicinal Plants

2.8.1 Global perspective

Traditional medicine has been defined by WHO as therapeutic practices that has been in existence before the development and spread of modern scientific medicine and is still in use today (Sofowora.,1996). According to WHO (2011) estimates, up to 80% of the rural population in the developing world still relies on herbal medicine. The long tradition of herbal medicine continues to the present day in China, India, and many countries in Africa and South American (Kong*et al*., 2003).

Medicinal plants have been the focus of many drug studies and also considered as alternative sources of antimalarial agents since long ago (Attiso, 1983). WHO addressed the need for research and policy on the prophylactic and therapeutic effects of antimalarial medicinal plants, through formation of linkages between researchers working on traditional antimalarial methods (Bodekerand Willcox, 2000). Previous studies have shown that more than 1200 medicinal plants from 160 families are used worldwide to treat malaria (Willcox and Bodeker, 2004).

According to Kaushik*et al*. (2015), seventeen medicinal plants known for South Indian traditional medicinal usage and pharmacological activities were evaluated for their antiplasmodial activity against CQ-sensitive P. falciparum (3D7) and CQ-resistant *P.*

falciparum(INDO) strains and their toxicity against HeLa cell line. Among the seventeen plants studied, seven have been tested (in vitro) for their antiplasmodial activity against both the 3D7 and INDO strains for the first time. Five of these seven plants showed promising (Pf3D7 IC50 6µg/ml to 20µg/ml) anti-plasmodial activity. Interestingly four of these plant extracts showed greater potency against the CQ-resistant INDO strain than against the CQ-sensitive 3D7 strain.

2.8.2. Practice of Anti-plasmodial medicinal plant use in Africa

In Africa, traditional medicine is part of the culture, and is practiced by a variety of traditional medicine practitioners, including herbalists, bone setters, and birth attendants as it is less expensive, accessible and acceptable (Sofowora, 1996). In Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of children with high fever resulting from malaria is the use of herbal medicines at home. In China, for example, traditional herbal preparations account for 30-50% of the total medicinal consumption (WHO, 2012).

The increasing prevalence and distribution of malaria mainly attributed to the emergence and spread of drug resistant parasites, have recently led to the study of medicinal plants with claimed antimalarial activities compared to those used empirically to treat other diseases (Clarkson*et al*., 2004). Efforts have been directed towards the discovery and development of new chemically diverse antimalarial agents and a number of *in vitro* antimalarial tests have been conducted in many African countries.

Med, (2008), noted as two Mozambique traditionally used plants have promising antimalarial activity with lowest IC_{50} values were found for the ethyl acetate extracts of *Momordicabalsamina*(IC50= 1.0 ±0.1 µg/mL) and *Pittosporumtobira*(IC50= 4.8 ±1.8 µg/mL) for further investigations anti plasmodial drugs. A remarkable number of investigations have also been conducted on medicinal plants traditionally used in African traditional medicine and other traditional health care systems (Banzouzi*et al*., 2004) has been reported that about two hundred thirty-nine Madagascan medicinal plants have been identified as having antimalarial properties.

An in vitro study of seven plants from the Democratic Republic of Congo has indicated antiplasmodial activities, a study of 134 plants from South Africa and that of 18 plant species from Venezuela have also reported the in vitro anti-plasmodial activities (Tona*et al.,* 2004). Other in vitro studies from eight Ivorian medicinal plants and fifty-five extracts from eleven plants in malaria therapy in Kenya were reported to have anti-plasmodial activities both on chloroquine sensitive and chloroquine resistant strains of P. falciparum (Tran *et al*., 2003).

Table-1: Some traditional medicinal plants that have moderate to good anti-plasmodial activities in Africa including Ethiopia.

Plant types	Family	*Genus and species name*	Part of plant used	Type of assay	% of parasite suppression	Reference
Perennial shrub	Liliaceae	*Asparagus africanus Lam*	Root	In vivo (mice model)	27.84%	(Dikasso *et al.,*2006)
Shrub	Meliaanhceae	*Bersama abyssinica Fresen*	Root bark	In vivo (mice model)	30.01%	(Berhane, 2008)
Shrub	Sapindaceae	*Dodona angustfolia L.F*	Seed	In vivo (mice model)	37.79%	(Berhane, 2008)
Tree	Combertaceae	*Terminaliamollis*	Root bark	In vivo (mice model	44%	(Mugangaet *al.,* 2014)
Tree	Rutaceae	*Zanthoxylumchalybeum*	Root bark	In vivo (mice model	37%	(Muganga et al., 2014)
Herb	Phytolaccaceae	*Phytolaccadodocandra*	Leave	In vivo (mice model)	33.60%	(Getinet, 2014)
Herb	Lamentaceae	*Fuerstiaafricana*	Leave	In vivo (mice model)	(>70%)	(Mugangaet *al.,* 2014)
Shrub	Fuphorbiaceae	*Croton zambesicus*	Leave	In vivo (mice model)	80.7%	(Okokonet *al.*, 2005)
Tree	Meliaceae	*Azandirachita indica*	Leave	In vivo (mice model)	52.32%	(Valechet *al.,* 2001)

1.

In Cameroon, a large number of plant species have been identified as antimalarial medicinal plants. Pure products have been isolated from some of these plants amongst which are those whose antimalarial activities are comparable to or more active than chloroquine on sensitive and resistant strains of *P. falciparum* (Builders *et al*., 2011 andTane*et al*., 2005). Several Nigerian medicinal plants have been found to demonstrate an interesting antimalarial potential in the search for a cure against various species of resistant antimalarial agents (Ibrahim *et al*., 2012).

According to criteria used Nite (2014)a pure compound was considered highly active if IC50< 0.06μM, being active with 0.06μM ≤IC50 ≤ 5μM, weakly active when 5μM≤ IC50≤10μM and compounds with IC50>10μM were considered inactive. The inhibition percentages were proposed for in vivo activity of antimalarial extracts at a fixed dose of 250 mg $kg^{-1}day^{-1}$: 100-90% (very good activity); 90-50% (good to moderate); 50-10% (moderate to weak); 0% (inactive).

2.8.3. Tradition of use of anti-plasmodial plants in Ethiopia

Ethno botanical studies reported 8 insecticides and 11species of plants used as anti-malarial agents in one of the districts in West Gojam, Ethiopia. These species of plants are among the widely used plants for medical purposes. Since malaria is a serious disease in Ethiopia and many developing countries, the list of traditionally used plants to control it must be backed by phytochemical studies to develop an appropriate phytomedine (Abiyot*et al*., 2006).A number of studies have also been conducted on the in vitro evaluation of the anti-malarial activity of several Ethiopian traditional medical plants. It has been reported that extracts from plants such as *Hageniaabysinica* and *Berssamaabysinica*(Kassa, *et al*., 1996), *Asparagus africanus* (Dikasso *et al*., 2006) ,*Croton machrostachys, Calpurina aura, Dodniaangustifolia*(Solomon, 1992), *Withania somenifera, Vernonia amygdalina* (Bogale and Petros, 1996), *Artemesiaafra, Artemesiarehanand, Ajugaremota* (Kassa *et al.*, 1998), have significant antimalarial activity against *P. falciparum*. Asmare and Kesara (2015) reviewed on ethnopharmacology study of the commonly used antimalarial herbal agents for traditional medinepractice in Ethiopia. These are *Phytolaccadodecandra*(Getinet, 2014), *JuniperusproceraHochst and Eucalyptus camaldulensis*(Hedberg*et al*., 2005) and *Calpurnia aurea*(Hedberg*et al*., 2005).

3. Materials and Methods

3.1. Plant material Preparation

The seed of *C. molle* was collected from the vicinities of Gambella Region, Agnua zone, Gog woreda (about 886 km south west of Addis Ababa). The Voucher specimen [CM7 (*Combertum molle No.7*)] was identified by Mr. ZewdieAchiso and deposited at the Herbarium of the Department of Biology, Jimma University, Ethiopia.Seed of the plant part was collected and dried in the processing room and then powdered, kept at room temperature in a well-closed and amber bottle until extracted**.**

The dried and powdered plant material (600g) was extracted by maceration (100 g of dried plant seed powder of the *C. molle* in 300 mL of 80% methanol) for three consecutive days (72hr) at room temperature. The extraction process was facilitated by using frequent shaking(Tiwari *et al*., 2011). The mixture was first filtered using cotton wool and then with Whatman filters paper No. 1 (Whatman®, England). The residue was re-macerated for another 72hr twice and filter. The combined filtrate was then dried by rotary evaporator (Buchi Rota vapor, Switzerland) at a temperature of 40°C and 45 rpm. After dried in water bath, a total dry extract was harvested and the dried extract kept at −20°C in a tightly closed bottle in a refrigerator until used for *in vivo* cytotoxicity, and anti-plasmodial testing.

3.2. Experimental Animals and Protocol

A total of 65 Swiss albino mice were used for acute toxicity, cytotoxic and antiplasmodial activity testing of *C. molle* seed extract. Accordingly, both sex mice, age 8 to 10 weeks, and weighed of 30 to 40 g were maintained at temperature of 22±3°C, relative humidity of 40-50% and 12 h light/12 h dark cycle(WHO, 2012). The animals were housed in transparent plastic cage with SS sipper 250 mL water bottle. Wood shaving was used as bedding and it was replaced every morning after the cage was cleaned and disinfected with 70% alcohol. The animals were kept under unlimited access to food and water. The mice were allowed to acclimatizing to the laboratory environment for at least one week before being subjected to the experiments.

3.3. Acute toxicity determination

Mice were randomly arranged into four groups (each group containing 5non-infected)for acute toxicity testing of the crude extract of *C. molle*seedat different dose levels (500 mg/kg, 1000 mg/kg, and 2000 mg/kg), and the last group, considered as control(Ketema T, *et al*., 2015).The acute toxicity was carried out in accordance to OECD guidelines.The mice were acclimatized and then fast forabout three hours. The extract was administered to the animals orally after it dissolved in 20% of Tween 80 in saline. The control groupreceived 0.5mL of 20% of Tween 80 in saline through the same route. Any acute sign of toxicity and mortality of each group within 24 hours after administration of the extract was recorded. Toxicity signs such as death, changes in physical appearance, and behaviour including loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, and diarrhoea, and other signs of overt toxicity were observed for 72 hr. The feeding condition (consumption of pellet and water) was recorded for 14 days.

3.4. Animal treatment and blood sample collection

Animals were randomized into four groups (I-IV) each containing four mice grouped for haematological and biochemical study. Doses of the extract used for this assay was 100mg/kg (group I), 250mg/kg (group II) and 500mg/kg (group III) and 0.5 mL 20% of Tween 80 in saline (group IV or negative control). Animals were sacrificed 24 hours after the last doses were administered (orally) lasted seven days. The mice at the time of sacrifice were weighed and then terminally anaesthetized. Blood sample was collected through cardiac puncture and collected in EDTA coated tubes. The abdominal cavity was opened through a midline abdominal incision and theliver and kidney were immediately removed and placed in fixative and processed for histological study.

3.5. Haematological and biochemical assay

About 0.2mL blood sample collected in EDTA tubewas used for quantification of total WBC, lymphocytes, monocytes, basophils eosinophil, neutrophils, RBCs, Haemoglobin (Hb), Haematocrit(HCT), platelets, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular haemoglobin (MCH) using CBC machine. About

1mL of the remaining blood sample was centrifuged at 10,000 rpm for 10 min. The supernatant (plasma) was transferred into new eppendorf tube and followed by measurement of liver enzymes, serum glutamic oxaloacetate transaminase (sGOT) and serum glutamic pyruvic transaminase (sGPT), albumin (Alb); indicators of kidney function such as creatinine (Cr) and urea; inflammation test, C-reactive protein (CRP) using LAMBDA 750 UV/Vis/NIR Spectrophotometer.

3.6. Histological study

For histological analysis, liver, and kidney of the sacrificed mice were collected in 10% buffered neutral formaldehyde; paraffin-embedded liver, and kidney tissues were labelled and stained with hematoxylin and eosin. Slides were coded and scored for histological evidence of liver and kidney damages.

3.7. *In vivo* anti-plasmodial tests

For *in vivo* anti-plasmodial activity tests a 4-day suppressive methods was used in accordance to Peters(Peters W.,1967). Hence, rodent malaria parasite *Plasmodium berghei* ANKA (*Pb*A), chloroquine sensitive strain, was used to infect Swiss albino mice for the four day suppressive test. The parasite was obtained from Ethiopian Public Health Institute (EPHI). For this study, a donor mouse with a rising parasitemia level of 20%-30% was sacrificed by decapitation and its blood was collected in a slightly heparinized syringe from the auxiliary vessels containing 0.5% trisodium citrate. The blood was then diluted with physiological saline solution (0.9%) into 5×10^7 infected RBCs (Nardos A and MakonnenE.,2017).

3.7.1.Parasite Inoculation and Extract Administration

The infected blood was diluted with normal saline with the intention that each 0.2 mL contained approximately 1×10^7*Pb*A parasitized erythrocytes. 15 female and 10 male Swiss albino mice (n=25) weighing 30-40g in each group were inoculated on the first day (D0), intraperitoneally, with 0.2 mL of infected blood. The mice were then divided randomly into five groups of five [3 female and 2 male, (n=5)] mice for each group.Three groups of the animals were assigned as the test groups and the other two groups were used as control (positive and negative). Three hours

after infection with *Pb*A, the three test groups were orally administered with 125, 250 and 500mg/kg/day doses of the *C. molle* seed crude extract. Chloroquine at the dose of 10mg base/kg/day (orally) and an equivalent volume of vehicle (0.5 ml of 0.9% of saline) was administered to the positive and negative control groups respectively, for four consecutive days (D0 to D3).

3.7.2. Monitoring of clinical and physical changes

The body weight of each mice in all the groups was measured before infection (day 0) and on day 4 using a sensitive digital weighing balance. Also, the rectal temperature of the mice in all the groups was measured using a digital thermometer before infection and then daily up to day 6.

3.7.3. Determination of Parasitemia

On the fifth day (D4), thin smears were made from the tail nip blood of each mouse, fixed with methanol and stained with 10% Geimsa pH 7.2 for 15min. The stained slides were then washed gently using distilled water and air dried at room temperature. The duplicate stained slides for each mouse was examined under microscope with an oil immersion objective of 100X magnification. The parasitemia level was determined by counting the number of parasitized erythrocytes out of 100 erythrocytes in random fields of the microscope. Average percentage parasitemia was calculated using the formula (Hilou *et al*.,2006):

$$\%\ \text{Parasitemia} = \frac{\text{Number of infected RBCs x 100}}{\text{Number of non-infected RBCs}}$$

Mean percentage of parasite suppression was calculatedper the following formula (Kalra*et al*., 2006):

$$\%\ \text{Suppression} = \frac{\text{Parasitemia in Negative Control - Parasitemia in Test Group}}{\text{Parasitemia in Negative Control}}$$

3.7.4. PCV Determination

Heparinized capillary tubes were used for collection of blood from tail of each mouse. The capillary tubes were filled with blood up to ¾th of their volume and seals at the dry end with sealing clay. The tubes were then placed in a micro-haematocrit centrifuge with the sealed end outwards and centrifuge for 5min at 11,000 rpm. The tubes were then taken out of the centrifuge

and PCV was determined using a standard Micro-Haematocrit Reader. PCV was measured before inoculating the parasite and after infection on the 4th day post infection, using the following formula:

$$PCV = \frac{\text{Volume of erythrocytes in a given volume of blood}}{\text{Total blood volume}}$$

3.7.5. Determination of Mean Survival Time (MST)

Mean survival time for each group was determined arithmetically by calculating the average survival time (days) of mice starting from date of infection over a period of 30 days (D0-D29). Mortality was monitored daily and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the follow up period. Then mean survival time (MST) for each group was calculated asKetema *et al.,*(Ketema *et al*., 2015):

$$MST = \frac{\text{Sum of survival time of all mice in a group (days)}}{\text{Total number of mice in that group}}$$

3.8. Data analysis

All data was analysed using SPSS software (version 20.0) and the results were expressed as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was used to compare results among and within groups for differences followed by Turkey's HSD post-hoc test. Sample analysis was repeated at least three times to get solid statistical data.

3.9. Ethical consideration

The study was ethically approved by the Ethical Review Committee of the College of Natural Sciences of Jimma University, Ethiopia. Mice were handled in humane way and extreme precautions were taken to avoid stress induced by poor handling techniques.

4. Results

4.1. Acute toxicity

Physical characteristics such as body weight change, sign of any toxicity, regular measurement of water and food consumed, and any behavioral changes among the mice were assessed every day. Accordingly, significant body weight differences between treated and control groups over the observed period was not observed. Or adverse effects that require modification of the procedure were not encountered. Likewise, differences were not seen in amount of pellet and volume of water consumed between treated and control groups.

4.2. Haematological parameters test

Outcomes of hematological parameter showed that, use of higher doses of crude extract *C. molle*seed for seven days cause significant ($P<0.05$) reductionof count of RBCs, Hb and HCT levels compared to the control group. On the other hand relatively lower doses of the seed extract (100 and 250mg/kg) caused significantly ($P<0.05$) increased count of platelet. However, as the dose increased to 500mg/kg, platelet count reduced, but not significantly different from the control group. Other hematological parameters such as MCV, MCH, MCHC and RDW-CV were not affected in mice received the treatment (Table 2).

Table 2 Hematological parameters of Swiss albino mice received three different doses of *C.molle* seed extract.

Groups	Hematological parameters								
	RBC ($10^3/\mu L$)	Hb (g/dL)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dL)	RDW-SD (fl)	RDW-CV (%)	Platelet
Control	10.44± 1.05	15.36± 1.48	50.9± 0.51	48.7± 0.72	14.7± 1.33	30.26± 0.39	35.4± 1.2	25.06± 2.27	687.33± 57.2
100mg/kg	9.25± 1.12	14.27± 1.32	45.07± 0.203	48.7± 0.05	15.45± 1.37	31.75± 0.32	36.42± 1.28	24.3± 2.43	734.2± 36.8*
250mg/kg	9.28± 0.98	14.1± 1.35	45.53± 0.11	48.40± 0.79	15.13± 1.42	31.29± 0.42	35.98± 1.17	24.26± 2.18	746.5± 18.3*
500mg/kg	8.98± 1.23*	13.54± 1.08*	43.52± 0.22*	48.83± 0.51	15.2± 1.29	31.23± 0.43	38.1± 1.3*	25.13± 2.38	714± 45.7

NB: *Values with asterisk are indicating significantly different from values of controls*

RBC- red blood cells,Hb- Haemoglobin, HCT- Haematocrit platelets, MCV-mean corpuscular volume, MCHC-mean corpuscular haemoglobin concentration and MCH-mean corpuscular haemoglobin

4.3. Biochemical tests

The two kidney function indicators; urea and creatinine were significantly affected under use of higher doses of the seed extract (250 and 500mg/kg) (Figure 2). Likewise, level of liver enzymes were affected in mice recived relatively high dose of the seed extract. At highest dose (500mg/kg) level of GPT enzyme was significantly (P<0.05) elevated.

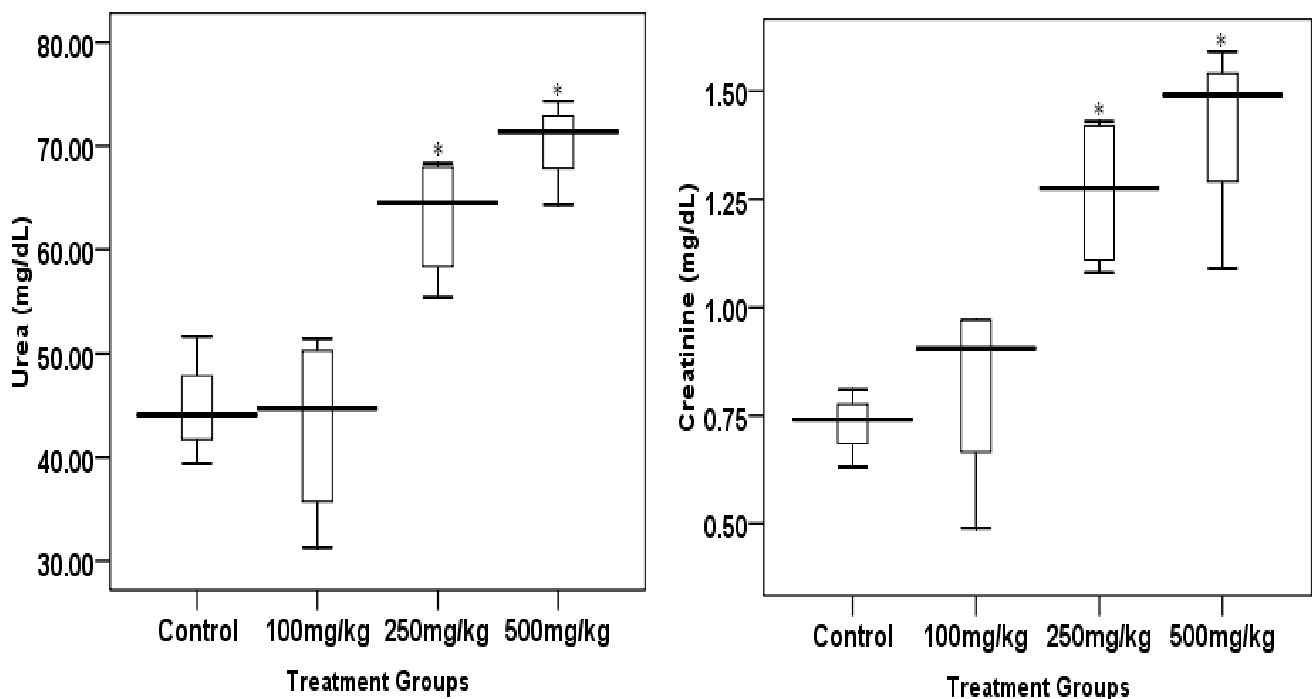

Figure 2 Levels (Mean ± SEM) of kindney test indicators (urea and creatinine) of Swiss albino mice received *C. molle* seed extract for seven days. *Values with asterisk are significantly different (ANOVA, Tukey's HSD post-hoc test) from values of the controls*

While level of the other enzyme, GOT, was significantly increased in mice treated with all doses of the extract (100, 250, and 500mg/kg) (Figure 3). On the other hand level of albumin showed significant reduction in mice treated with the two higher doses (250 and 500mg/kg) (Figure 4).

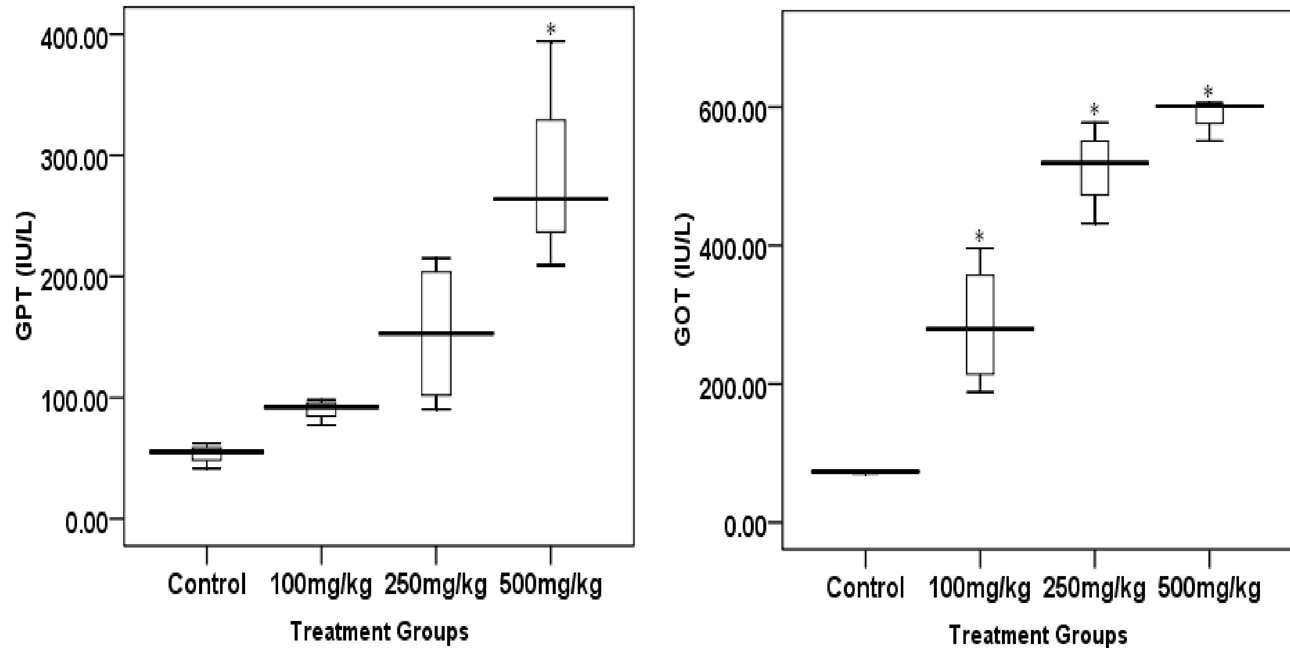

Figure 3 Level of liver enzymes (mean ± SEM) of Swiss albino mice received *C. molle* seed extract for seven days. *Values with asterisk are significantly different (ANOVA, Tukey's HSD post-hoc test) from values of the controls.*

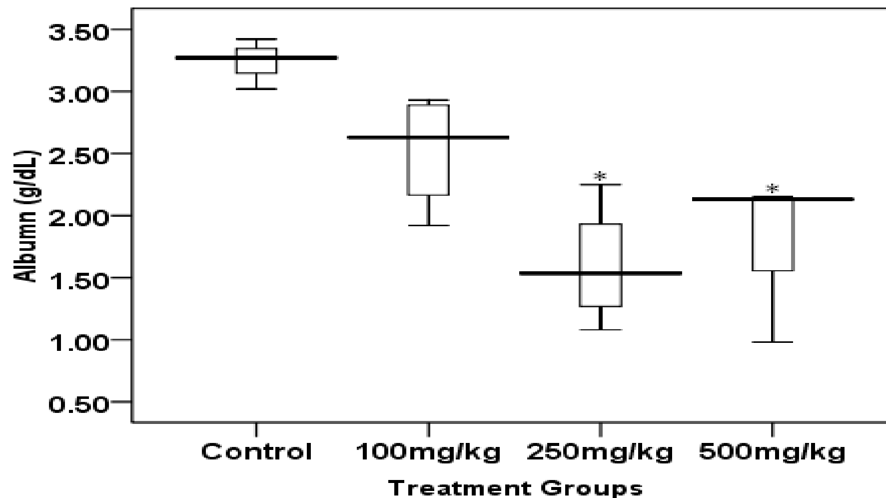

Figure 4 Level of albumin (mean ± SEM)of Swiss albino mice received C. molle seed extract for seven days. Values with asterisk are significantly different (ANOVA, Tukey'sHSD*post-hoc* test) from values of the controls

On the other hand, the plant caused substantial production of one of the inflammation reaction indicators. Under all doses (100, 250 and 500mg/kg) level of C-reactive protein showed significant (P<0.05) increment (Figure 5).

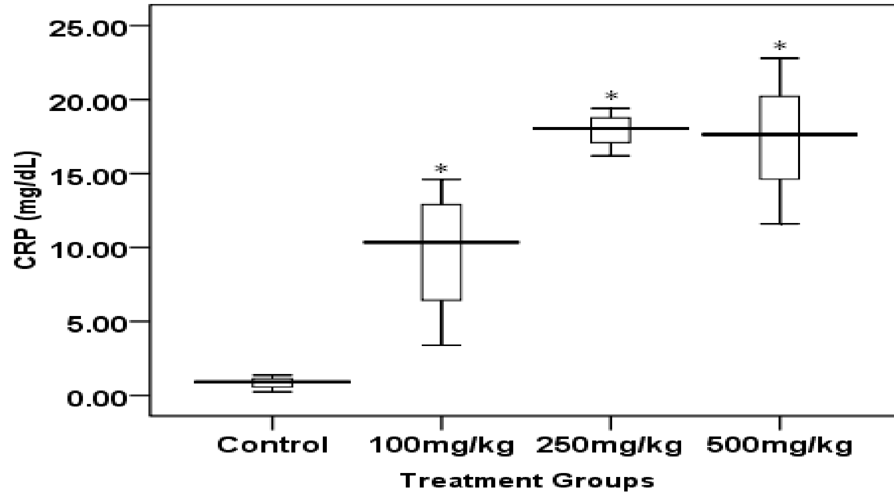

Figure 5 C-reactive protein (CRP) level (mean ± SEM)in Swiss albino mice received C. molle seed extract for seven days. Values with asterisk are significantly different (ANOVA, Tukey'sHSD*post-hoc* test) from values of the controls.

4.4. Effect of *C. molle* seed extract on immune cells

Effect of the plant seed extract on innate immune cells such as basophils showed variability. Under lowest dose (100mg/kg), percentage of basophils showed significant increment (P<0.05), while as dose increased to 250 and then 500mg/kg, the level of this immune cell significantly decrease (P<0.05) compared to the control mice. On the other hand, eosinophils count showed consistent significant increment (P<0.05) at relatively high doses (250 and 500mg/kg). Except, at the lowest dose (100mg/kg) for lymphocytes count, under all doses, differences were not observed between the control and treated groups. Moreover, almost all immune cells showed significant (P<0.05) reduction under use of the highest dose (500 mg/kg) of the extract (Figure 6).

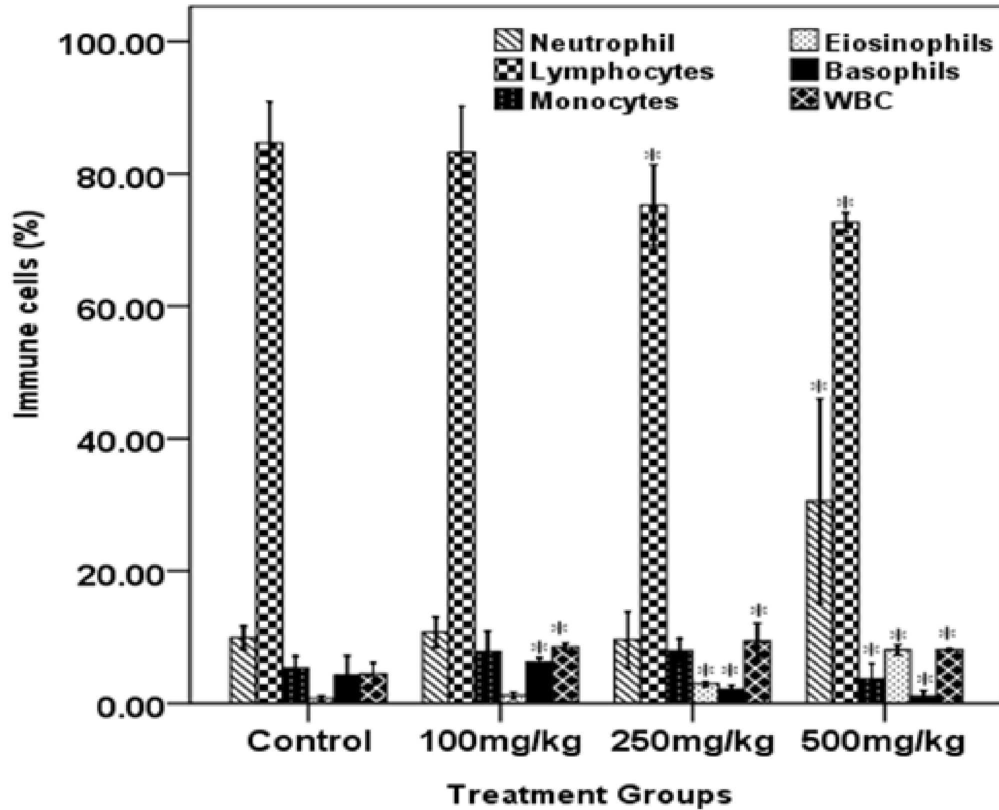

Figure 6Levels of immune cells (mean ± SEM)in Swiss albino mice received C. molle seed extract for seven days. Values with asterisk are significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of the controls

4.5. Histological analysis

Higher dose of seed extract of *C. molle* (500mg/kg) caused necrosis of cells of kidney and liver (Figure 7). While, at relatively lowest doses use (100 and 250mg/kg), kidney cells were normal, but liver cells showed slight changes such as fatty and water charges.

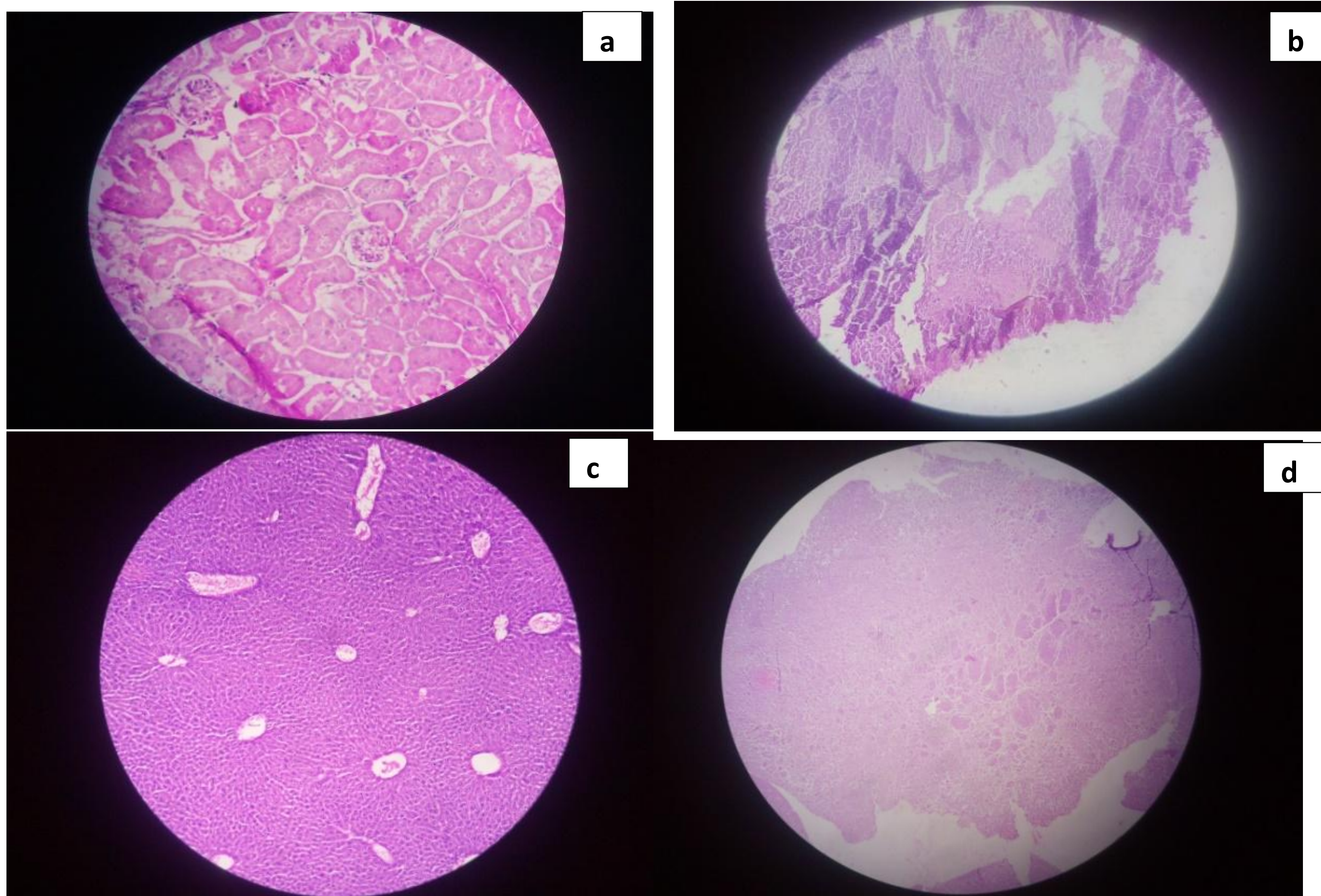

Figure 7 Tissues of kidney (a= control, b= treated with 500mg/kg seed extract of C. molle and Liver (c= control, d= treated with 500mg/kg seed extract of C. molle) microphotographof Swiss albino mice received C. molle seed extract. Formalin-fixed, paraffin-embedded tissue sections stained with hematoxylin and Eosin (HE). The sections were photographed with X40 objective (bars are 100μm).

4.6. Anti-plasmodial activities of seed extract of *C. molle*

The mean percentage of parasitemia of mice infected with *Pb*A and subsequently treated with (125 and 250mg/kg) crude seed extract of *C.molle* showed significant (P<0.05) reduction. However, as the dose increased, to 500mg/kg, parasite count was almost the same with the negative control (untreated but infected control mice). On the 4 post infection day, the methanol extracted seed of the *C. molle* at 125 and 250mg/kg exhibited an inhibition of 63.5% and 36.6%, respectively. Percentage parasite inhibition/suppressionof the seed extract was significantly (P<0.05) higher at relatively lower doses (125 and 250mg/kg), but significantly (p<0.05) lower than the standard drug (chloroquine 10 mg/kg/day), which showed 85.23% chemo-suppression. As the dose of seed extract of the plant increased, its parasite suppression potential was decreased. Although, the survival time of the seed extract treated group (125mg/kg) post infection was significantly (P<0.05) higher (12 days) than the negative control (9 days), it was much shorter than that of the standard drug, chloroquine treated group (Table 3).

Table 3 Percent parasite suppression and mean survival time of mice (n=5) infected with *Pb*A and treated with crude seed extracts of *C. molle*

Groups	Anti-malarial activities of the extract on day 4 post-infection (p.i.)		Survival time (Day)
	% Parasitemia (Mean ± SEM)	% parasite suppression	
Control	35.7± 1.51	0.0	9.5
125	15.5 ± 1.9 ^{a2}	63.5 ± 3.49^{a1}	12 ^{a}
250	26.9 ± 1.88 ^{a1}	36.6 ± 2.72 ^{a2}	10.5
500	36.9 ± 1.4	14.47 ± 5.09	9.5
CQ	6.28 ±0.27^{b3}	85.23 ± 0.04 ^{b2}	>30

Data are expressed as mean ± SEM; a = compared to control, b = to CQ (10mg/kg); ^{1}p< 0.05; ^{2}p<0.01, ^{3}p<0.001, p.i. = post-infection, Numbers in the first column refer to dose in mg/kg, CQ= chloroquine.

The comparison analysis on Packed Cell Volume (PCV) of mice treated with the seed extracts of *C. molle* (under all doses) was significantly (P<0.05) reduced on day 4 post-infection than mice group treated with standard drug (CQ) on the same day. But, significantly (P<0.05) increased than the negative control. Similarly, protection of the seed extract from body weight reduction was showed significant improvement in mice groups treated with different doses of seed extract

of *C. molle* than negative control. But did not showed significant differences from CQ treated mice. On 4-day post-infection of *Pb*A infection increment of rectal temperature was recorded in all mice treated with different doses of the extract, while it was reduced in CQ treated mice group. However, mice groups treated with 125 and 250mg/kg seed extract of the plant showed significantly (P<0.05) lower rectal temperature than the negative control (Table 4).

Table 4 Temperature, weight and packed cell volume of infected mice (n=5) treated with crude extract of seed of *C. molle* in the 4 day suppressive test

Groups	Packed cell volume			Body weight			Temperature		
	PCV-D0	PCV-D4	% Change	BW0	BW4	% change	T0	T4	% Change
Control	63.2±0.97	49.6±0.19	13.6	33.8±0.63	28.6±0.2	5.2	35.84±0.54	36.7±0.77	-0.86
125	62.8±0.09	51.2±0.68	11.6^{b2}	33.8±0.96	32.9±0.33^{a2}	1.8	36.54±0.16	36.62±0.52	-0.08^{a2}
250	61.2±0.78	51.6±0.67	9.6^{b2}	36.4±0.47	33.6±0.4^{a1}	2.6	36.88±0.19	37.02±0.13	-0.14^{a2}
500	59.6±0.49	52±0.26	7.6^{b1}	36.1±0.91	32±0.24^{a1}	4	36.18±0.19	36.68±0.11	-0.5
CQ	57±0.68	53.5±0.65	3.5	34.5±0.41	32±0.16	2.5	37.6±0.34	36.45±0.45	1.15

Data are expressed as mean ± SEM; a = compared to control, b = to CQ (10 mg/kg); $^{1}p<0.05$; $^{2}p<0.01$, D0 = pre-treatment value on day 0, D4 = post-treatment value on day four, CQ = chloroquine. Numbers in the first column refer to dose in mg/kg.

Analysis of rectal temperature over five days post-infection revealed that there was a declining pattern of temperature after day 4-post-infection. Accordingly, as the disease progress, Day 4 to 5, rectal temperature showed dropping, although significant differences were not observed (Figure 8).

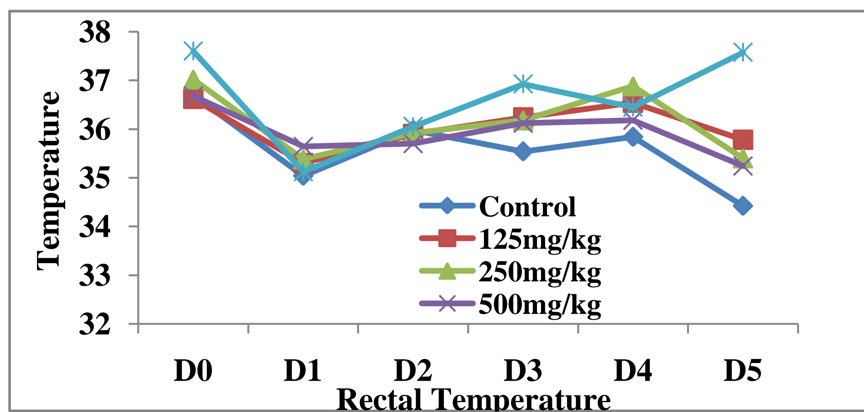

Figure 8 Pattern of rectal temperature of *PbA* infected mice treated with crude seed extract of *C. molle*

5. Discussion

The majority of traditional medicines used in the developing countries have not been evaluated for quality, safety, and efficiency to the same standard as those in developed countries (Alebachew, 2013). Recently, due to increasing emergence of drug resistant trait in plasmodium parasite, globally there is urgent need of novel anti-plasmodial medicine (Clarkson *et al*., 2004).Despite wide use of *C. molle* as medicinal plant for treatment of various ailment including helmnthic, protozoal, bacterial and other infectious diseases in different parts of Africa (McGaw*et al*., 2001, Fyrquist*et al* .,2002; Bussmann*et al* .,2006; Gronhaug*et al* ., 2008), studies conducted on it's *in vivo* toxicity and animal model anti-malarial test is scanty. The agenda of current study is also part of the attempts on discovery of safety profiles and alternative anti-malarial drug focused on the evaluation of oneof traditional medicinal plant which has been widely used by local community of Gambella region for treatment of malaria, skin problems and HIV infections (personal observation). Unlike the other parts of this plant used in other regions of Africa, seed is widely used for all medication purposes in Ethiopia, Gambella region.

Traditional practitioners and indigenous people of the region collect seed of this plant, clean it, roast traditionally and grindthe roasted *C. molle* seed by traditional mortar with pistil. Finally boil the grinded powder of *C. molle* seed with small amount of water to harvest concentrated oily juice of the seed extract for treatment of different ailments. This local traditional extraction supported with the finding of Okello*et al.*,(2010) as reported as ‘’decoctions were used to prepare herbal teas from the hard parts of the plants such as root, rhizome, seeds and stem barks’’.Unlike, the traditional practice of the local community, seed of the *C. molle* extraction for this study carried out the infusion (maceration) of raw *C. molle* seed powder as finding forwarded George and Pamplona, (2000), to preserve almost all active particles and chemical structure with their properties. In the same manner, following the traditional practice, to mimic local community, after single oral doseof acute toxicity testing, seed crude extract of the plant was administered for seven consecutive days and cytotoxic effects determined through quantifying hematological parameters such as RBC indices, WBC indices and total platelet count; Bio-chemical assay determined the toxic effect of *C. molle* seed extract over kidney and liver function indicators and their histology.

The relative safety of the crude extract of C. *molle* seed was tested at the graded dose of up to 2,000 mg/kg.As effect of *C. molle* seed crude extract significant body weight, rectal temperature, consumption of standard experimental mice food and volume of water consumption differences between treated and control groups over the experimental follow up period (14 days) was not observed. Accordingly, the crude extract of *C. molle* seed showed less acute toxicity(but not significant) even at highest dose (2000 mg/kg) compared with control group.

However, *C. molle* seed methanol crude extract single administration showed less acute toxicity, it's longer use (seven days treatment cytotoxicity assay) even at low doses (100mg/kg) had negative impact on proper functioning of kidney and liver. This was observed by excess secretion of liver enzymes (sGPT and sGOT), and reduction in production of protein (albumin) by liver cells as well as excess creatinine and ureaobserved in plasma of treated mice.It is belived that rise in level of plasma enzymes (GPT and GOT) are the pertinent indicators for liver toxicity (hepatoxicity)(Singh*et al*.,2011).The estimation of this enzyme is a more specific test for detecting any liver abnormalities since it is primarily created in the liver(Amacher*et al*.,2002). Albumin is the main protein in blood and is made by the liver(Thapa*et al*.,2007), and an out come of hepatotoxicity leads to decrease albumin production. So that significant reduction in level of albumin is direct indication for failure of liver to produce adequate level of this protein.

Likewise, excess levels of creatinine and urea in plasma of mice treated with the plant extract was detected. Usually, kidneys maintain optimum chemical composition of the body fluids by acidification of urine and removal of metabolite wastes as creatinine, urea, uric acid, and ions (Biyani*et al*.,2003).In case of deterioration of kidney function, urea and creatinin levels always increases in combination (Hismiogullari1 *et al.,* 2011).Thus the observed excess kidney function indicators: urea and creatinine in combination has considered to be associated due to implication of the toxic effect of the *C. molle* seed crude extract on kidney. This revealed that a continuous use of the plant seed extract has toxic effects on vital organs. Furthermore, this was more revealed on histological study of tissues of different organs, where at highest dose (500mg/kg) intense necrosis of cells of the organs (liver and kidney) documented.

The seed crude extract of *C. molle* plant caused excess secretion of CRP, an inflammation indicator in mice treated with even at low doses. This protein is a special type of protein (plasma

protein) produced by the liver cells only during episodes of acute inflammation, in response to pro-inflammatory cytokines called interleukin-(IL-1β), IL-6 and tumor necrosis factor alpha (TNF-α) (PCOSN.,2003). As a consequence, the level of CRP in plasma highly increases during acute phase response to tissue injury, infection, or other inflammatory stimuli (Gruys*et al*.,2005). Different *in vitro* studies suggested that mollic acid glucoside isolated from *C. molle* leaves has anti-inflammatory and anti-asthmatic activities (Ojewole, 2008, Shah *et al*.,2011 and Yeo*et al*.,2012). However, in the current *in vivo* study the observed elevated level of CRP, an indicator of systemic inflammation, could be due to the damaged liver due to the toxic effect of *C. molle* seed crude extract, since elevated liver enzymes are associated with higher CRP concentrations and liver is the main production site of CRP (Kerner*et al*., 2005). Population of Gambella region, Ethiopia (where the plant is collected) has a practice of using this plant for some days (seven days are common) continuously for treatment of different ailments (personal observation). This practice might prone the users for unexpected inflammation reaction even at low doses and increases vulnerability to various diseases, such as rheumatoid arthritis, atherosclerosis and asthma (Mueller*et al*., 2010).

The most common hematology findings in toxicology studies are mild decreases in RBC count, hemoglobinconcentration and hematocrit (Ahmad*et al*.,2011). Accordingly some hematological parameters such as RBC count, Hb and HCT levels were significantly reduced in mice treated with the highest dose (500mg/kg) of the*C. molle* seed crude extract. It implied that at high dose for the long period use, seed extract of the *C. molle* plant could have also toxic effect on someRBC indices and increase susceptibility to anemic condition. Some another hematological parameters such as MCV, MCH, MCHC and RDW-CV were not affected in mice received the treatment. As an evidence it has been reported that the RBC counts, hemoglobin concentration and hematocrit is decreased in chronic renal failures (Suresh M *et al* 2012), and decrease in hematocrit is apparent even among patients with mild to moderate renal insufficiency. Primary cause of decrease RBC count in chronic renal failure is impaired erythropoietin production and other factors which suppress marrow erythropoiesis and shortened red cell survival(Suresh *et al* 2012).

On the other hand, low doses (100 mg/kg and 250mg/kg) of the plant seed extract associated with excess production of platelets count. The observed elevated count of platelet in mice treated with 250mg/kg dose might be related to the organs damage. It appears that platelet homeostasis is controlled by thrombopoietin, a glycoprotein hormone produced by the liver and kidney which regulates the production of platelets. Damaged liver and kidney might increase release of this hormone to the blood and increases production of platelets. This was evidenced by the renal or liver failure conditions associated with the increased platelet production in patients with kidney and liver problems (Stockelberg, *et al*., 1999; Linthorst*et al*.,2002). This is because of high level of thrombopoietin in blood of patients which causes abnormal production of platelets (Makar*et al*., 2013).

Different substances used for medication or stimulation could have immune-suppressive or immune-stimulation property. Hence, both immune-stimulating and immunosuppressing agents have their own standing and search for better agents, exerting these activities is becoming a field of major interest all over the world (Shula*et al*. 2012). Moreover, stimulatory or suppressive agents have been shown to possess activity to normalize or modulate pathophysiological processes(Nagarathn*et al*., 2013).In addition to this, the basic function of immune system is to detect and destroy the non-self and thus a defense mechanism. These system works throughout the body via an intricate regulation of cellular and humoral factors (Savant *et al*., 2014).

In the current assessment, although many differences were not observed in number of immune cells at use of lower dose (100mg/kg), in mice treated with highest doses (250mg/kg and 500mg/kg) of the crude extract, significant reduction in counts of most of the immune cells such as lymphocytes, neutrophils, monocytes and basophils was observed seven days administration of *C. molle* seed crude methanol extract. This implied that at the use of relatively high doses of the *C. molle* seed crude extract for longer duration can cause immune-toxicity as the same as the report of Gqaleni*et al*., (2012)on *in vitro* testing of African traditional medicines for cytotoxic, immune-modulatory and anti-HIV activities. Similarly, these adverse events reported have been associated with high doses and long-term usage comparable to (Das *et al*., 2014) side effects and toxicity of *Panax ginseng* belongs to Araliaceae.

In broad sense, immunity implies resistance to injury particularly by poisons, foreign proteins and invading pathogens (Alan and Todd, 2001) common for both adaptive and innate immunity. By its working capacity, adaptive or acquired immunity differs from the innate response as noted by Nagarathn*et al*., (2013), it is specific, has an element of memory (which provides long term immunity), and is unique to vertebrates.Under detail condition the physiology for humoral component of adaptive immunityis involves the proliferation of antigen-stimulated B lymphocytes into antibody-secreting plasma cells. On the other hand cellular component is mediated by T lymphocytes and it is the predominant cell types being helper T cells (Th) and cytotoxic T cells (Nagarathn*et al*., 2013).These cells of the immune system can engulf bacteria, kill parasites or tumors cells, or kill viral infected cells. Often, these cells depend on the T helper subset for activation signals in the form of secretions formally known as cytokines, lymphokines, or more specifically interleukins (Meera., *et al* 2008) Mainly the observed significant reduction of counts of lymphocytes at high dose (500mg/kg) of *C.molle* seed crude extract has direct implication on possible suppression of adaptive immunity which is critical in controlling different diseases. Therefore,the result of depleted immunity specially lymphocyte components, increases susceptibility to the infectious and non-infectious diseases (Das *et al*., 2014), may afforded from taking high amount of *C. molle* seed for long period of time.

In fact,several scholars has been reported about efficacy of *C.molle* plant against different microbes (Sahlu,2013;Amare and Tadesse, 2016;Masoko*et al*.,2007), to the best of our knowledge *in vivo* anti-plasmodial and cytotoxic study of seed extract of the plant is the first report in its kind. Hence, the preliminary evaluation and screening of the crude extract of *C. molle* seed showed that the plant had a good degree of anti-plasmodial activities against murine malaria in mice mainly under lower dose. Finding of this study supports the earlier reports about the in vitro antiplasmodial activity of *C. molle* plant. The plant was reported as it has Saponins, Triterpenes, glycosides, phenols, flavonoids, phenolic compounds, saponin, and alkaloids and anthraquinones(Ayuba*et al*.,2015). Thus, the observed anti-plasmodial activity could be attributed to the contribution of these chemical components(Oyedemi*et al*.,2010;Koevi*et al*.,2015). Some of the studies conducted so far have reported that anti-plasmodial activities of plant extracts are mainly linked to the range of compounds including terpenes, steroids, coumarins, flavonoids, phenolic acids, lignans, xanthones, anthraquinones, berberine, limonoids, naphthquinones, sesquiterpenes, quassinoids, indole and quinoline alkaloids

(Caraballo*et al*.,2004). Furthermore, secondary metabolites such asalkaloids and flavonoids have been known to elicit anti-plasmodial activity by blocking protein synthesis in *Plasmodium falciparum* and chelate with nucleic acid base pairing of the parasite respectively (Lui*et al*., 1992, Okokon*et al*., 2011, Inbaneson*et al*., 2011). Moreover, saponin, flavonoids and tannins have antioxidant property they can cause oxidative damage to the *Pb*A parasite (Davies,2000).

Different observation encountered in the current study was, at lowest dose (125mg/kg), the plant seed extract showed better anti-plasmodial activity. Thus, the range in which the extract can suppress the parasite effectively without causing toxic side effects could be lower dose (125mg/kg) than at relatively higher doses (250 and 500mg/kg) similar with (Ritter *et al*., 2015) Pharmacokinetics range.However at lower dose chemo-suppressive activity of the plant extract showed significant increment with reduction of parasite load compared to the higher doses, at higher doses different physiological parameters were affected, and cells of vital organs necrotized. This phenomenon was implicated in the analysis of hematological and biochemical parameters. Lowest dose (100mg/kg) of seed extract of *C. molle* did not cause change on function of vital organs such as liver and kidney, count of immune cells, status of hematological parameters and histology of different organs.

The test extracts of *C. molle* seed crude extract prevented a loss of body weight in infected mice with increasing parasitemia. The comparison analysis indicated that the extracts significantly prevented weight loss at all dose levels compared to the controls. *Plasmodium* infection is correlated with the incidence of high destruction of red blood cells andanemia that may result life threatening (Ajaiyeoba*et al*., 2006, and Jigam*et al*., 2011). During the four-day suppressive test, the effect of the extract of *C. molle*seed crude extracton the PCV was tested to assess the reversing effect of the extract from infection induced anemia. The 125, 250 and 500 mg/kg body weight of extract showed significant prevention against PCV reductionrespectively increased compared to control group. This may be due to the effect of the extract in preventing PCV on early infection. Chloroquine showed a strong protective effect in preventing PCV reduction compared to extract-treated groups and negative control.Increment of rectal temperature was observed in the present study with *C. molle* seed crude extract, significant preventive effects were observed at all dose levels.

Theparasite suppression of *C. molle* methanol seed crude extract (63.5 ± 3.4%) at lowest dose (125 mg/kg) is also comparable to the reported antimalarial activity of *Cassia occidentalis* root bark and *Phyllanthusniruri,* which produced 60% suppression of parasitemia in vivo (Fennell *et al*., 2004), and that of the essential oil of *Cymbopogoncitratus*, which produced 62.1% suppression at 200 mg/kg in vivo (Tchoumbougnang*et al.,* 2005). 60.12% *Bosciaangustifolia* (Muthaura*et al*., 2007), 55.88% *Nigella sativa* (Abdulelah and Zainal-Abidin, 2007), 52.32%*Azadirachta indica* (Valecha*et al*., 2001) In this study, the effect *C. molle* seed crude extract of medium dose (250mg/kg) on parasitemia suppression (36.6 ± 2.72%),is more effective than the ones reported by previous studies such as on *Asparagus africanus* (Dikasso *et al*., 2006), *Amarantusspinosus* (Hilou *et al*., 2006), *Withania somnifera* (Dikasso *et al*., 2006) and *Clerodendrummyricoides* (Muregi*et al*., 2007) with percentage inhibitions of 27.84%, 30.94%, 33.75%, and 31.7% respectively. However, the present result at lower dose (125mg/kg)relatively less antiplasmodial activities than the ones reported on *Croton zambesicus*(Okokon*et al*., 2005), *Anonnasenegalensis* (Ajaiyeoba*et al.,* 2006), and with percentage inhibitions of, 80.7% and 76.3% respectively.

According to Nite*et al* (2014)the following inhibition percentages were proposed for *in vivo* activity of antimalarial extracts at a fixed dose: 100-90% (very good activity); 90-50% (good to moderate); 50-10% (moderate to weak); 0% (inactive). Therefore, the observed parasite inhibition of the crude seed extract of *C. molle* fall in the range of good to modern (90-50%) when used relatively at lower dose. Thus, further fractionation of the crude extracts and re-evaluation of each isolate compound could leads for discovery of novel antimalarial drug with unique mechanism of action.

6. Conclusion

At relatively lower dose,*C. molle* seed extract didn't cause effects on haematological and biochemical parameters. However, it has good chemo-suppressive activities on murine malaria (*Pb*A). As dose increased, its anti-plasmodial activity declined while, its cytotoxic effect increased. Thus, further isolation and evaluation of active components of the seed extracts for their toxicity and anti-plasmodial activity should be a priority area of investigation.

7. Recommendation

This significant suppression of parasitemia by the methanol extract of *C.molle* seed on day 4 was documented. Further fractionation of the crude extracts and re-evaluation of *in vivo* cytotoxic and antiplasmodial effect of each isolate compound could leads for discovery of novel antimalarial drug with unique mechanism of action.

7. References

Abdulelah, HAA., and Zainal-Abidin, BAH. (2007). In Vivo Anti-malarial Tests of Nigella sativa (Black Seed) Different Extracts.*Am. J. Pharmacol. andToxicol*. **2**; 46-50.

Abiyot, B. (2006). Ethno botany of plants used as insecticides, repellents and antimalarial agents in Jabitehnan district, West Gojjam_ *SINET, Ethiopian J Sci.* **29**; 87-92.

Ademola, O., and Eloff, N. (2010).In vitro anthelmintic activity of Combretum molle (Combretaceae) against Haemonchuscontortusova and larvae.*Veterinary Parasitol.***169**; 198–203

Ahmad S ,Aliakbar H , Alireza S and Abdolali M (2011). Characterization of Blood Cells and Hematological Parameters of Yellowfin Sea Bream (Acanthopagruslatus) in Some Creeks of Persian Gulf.*World Journal of Zoology*.**6**; 26-32.

Ajaiyeoba E, Falade M, Ogbole O, Okpako L, Akinboye D(2006). In vivo antimalarial and cytotoxic properties of *Annonasenegalensis* extract. *Far J Tradit Complement Altern Med.***3**; 137–41.

Alebachew M, Kinfu Y, Makonnen E, Bekuretsion Y and Urga K.,92013). Toxic Effects of Aqueous Leaf Extract of Vernonia bipontiniVatke on Blood, Liver and Kidney Tissues of Mice. *Momona Ethiopian Journal of Science (MEJS),***5** ; 15-31.

Alemu, K., Worku, A., Berhane, Y. (2013). Malaria infection has spatial, temporal, and spatiotemporal heterogeneity in unstable malaria transmission areas in northwest Ethiopia. *PLoS One*.**8**; 79 -96.

Amacher, D.E. (2002). A toxicologist's guide to biomarkers of hepatic response.*Hum Exp Toxically*, **21**; 253-262.

Amare, T. and Tadesse, E. (2016). In vitro Antibacterial Effect of Combretum molle and Fr1 against Staphylococcus aurous and E. coli Isolated from Bovine Mastitis. *World J Biol Med Sci.* **3**; 115-131.

Ancolio, C.; Azas, N., Mahiou, V., Ollivier, E., di Giorgio, C., Keita, A., Timon-David, P., Balansard, G. (2002). Antimalarial activity of extracts and alkaloids isolated from six plants used in traditional medicine in Mali and Sao Tome. *Phytother Res.***16**; 466–469.

Aribodor D. N., Ugwuanyi I. K., Aribodor O. B (2016).Challenges to Achieving Malaria Elimination in Nigeria.*American Journal of Public Health Research*.**4**; 38-41.

Asmare, A. and Kesara, N.B. (2015). A review of ethno pharmacology of the commonly used antimalarial herbal agents for traditional medine practice in Ethiopia: *African J Pharmacy and Pharmacl*. **9**; 615-627

Asnake, S., Teklehaymanot, T., Hymete, A., Erko, B. and Giday.M (2015).Evaluation of the antiplasmodial properties of selected plants in southern Ethiopia.*BMC Complementary and Alternative Med.***15**; 1-12

Asres, K. and Balcha, F. (1998). Phytochemical screening and in vitro antimalarial activity of the stem bark of Combretum molle. *JPharm Ethiopian.***16**; 25–33.

Asres, K., Bucar, F., Knauder, E., Yardley, V., Kendrick, H., Croft, S.L. (2001). In vitro antiprotozoal activity of extract and compounds from the stem bark of Combretum molle.*Phytother Res.***15**; 613-617.

Attiso MA. (1983). Phyto pharmacology and phytotherapy: In Traditional Medicine and Health Care Coverage, *WHO, Geneva*. **4**; 194-206

Banzouzi, J.T., Prado, R., Menan, H., Valentin, A. (2004). Studies on medical plants of Ivory Coast: investigation of Sidaacuta for in vitro antiplasmodial activities and identification of an active constituent. *Phytomed.***11**; 338-341.

Basir R, Rahiman SF, Hasballah K, Chong W, Talib H, Yam M, et al (2012). plasmodium berghei ANKA infection in ICR mice as a model of cerebral malaria. *Iran J Parasitol*.7; 62-74

Biyani, M.K, Banavalikar, M.M., Suthar, A.C., Shahani, S., Sivakami, S., Vidri, J. (2003). Antihyperglycemic Effects of Three extracts from Momordicacharantia. *J. of Ethnopharmacol.***88**; 107-111

Bodeker, G., Willcox, M. (2000).The first international meeting of the Research Initiative on Traditional Antimalarial methods.*J Altern Compliment Med*. **6**; 195-207.

Bogale, M., Petros, B. (1996).Evaluation of the anti-malarial activity of some Ethiopian traditional medical plants against *P. falciparumin vitro*. *SINET: Ethiop J Sci*.**19**; 233-243.

Builders M,Wannang N,Aguiyi J., (2011). Antiplasmodial activities of Parkiabiglobosa leaves: In vivo and In vitro studies. *Annals of Biological Research.***2**:8-20.

Bussmann, R.W., Gilbreath, G.G., Soilo, J.(2006). Plant Use of the Massai of Sekenani Valley, Massai Mara, Kenya.*J EthnobiolEthnomed*.**4;** 24-30.

Caraballo, A., Caraballo, B., Rodriguez-Acosta, A. (2004). Preliminary assessment of medical plants used as antimalarial in the south eastern Venezuelan Amazon. *Rev Soc Bras Med Trop*. **37**; 186-188.

Chotivanich, K., Silamut, K., Day, N., (2007). Laboratory diagnosis of malaria infection – A short review of methods.*NZ J Med Lab Sci.* **61**; 4-7

Clarkson, C., Maharaj, V., Crouch, N., Grace, O., Pillay, P, .Matsabisa, M., Bhagwandin, N., Smith, P., Folb, P. (2004). In vitro antiplasmodial activity of medicinal plants native to or naturalised in South Africa. *J Ethno pharmacol.* **92**; 177-191

Das, S., Bordoloi, R., Newar, N. (2014). A Review on Immune Modulatory Effect of Some Traditional Medicinal Herbs.*J PharmaceutChemBiol Sci*.**2**; 33-42.

Davies,.K.J.A. (2000).“Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems,” *IUBMB Life*.**50** ; 279–289.

Deresse W., Ali A., and Enqusellase, F., (2003). Self-treatment of malaria in rural communities, Butagira, South Ethiopia.*BWHO*.**81**; 261-268

Dikasso D., Makonnen E., Debela A., Abebe D., Urga K., (2006). In vivo antimalarial activity of hydroalcholic extracts from Asparagus africanus Liliaceae. In mice infected with Plasmodium berghei. *Ethiop. J. Health Dev*. **20**; 112-118.

Dikasso D., Makonnen E., Debella A., Abebe A., Urga K., Makonnen W, Melaku D, Kassa M, and Guta M (2006). Anti-malarial activity of Withania somnifera L. Dunal in mice. Ethiop.*Med.J*. **44**; 279-285.

Eloff, J.N., Katerere, D.R., Mcgaw, L.J. (2008). The biological activity and chemistry of the southern African Combretaceae.*J Ethnopharmacol*.**119**; 686-699.

Fennell, C., Lindsey, K., McGaw, L.l (2004). Assessing African medical plants for efficacy and safety: pharmacological screening and toxicology.*J Ethnopharmacol.***94**; 205–217.

FMOH, (2004). Malaria Diagnosis and Treatment Guidelines for Health Workers, Addis Ababa, Ethiopia; **2nd ed**; 1-40

Fyhrquist, P., Mwasumbi, L., Haeggstro, CA.,Vuorela, H., Hiltunen, R., Vuorela, P .(2012). Ethnobotanical and antimicrobial investigation on some species of Terminalia and Combretum (Combretaceae) growing in Tanzania.*J. Ethnopharmacol.***79**; 169-177.

Gansane, A., Salon, S., Tuatara, L.P., Traore, A., Hutter, S. (2010). Antiplasmodial activity and toxicity of crude extracts from alternatives parts of plants widely used for the treatment of malaria in Burkina Faso: Contribution for their preservation. *Parasitol. Res.***106**; 335–340.

Getachew, S., Thriemer, K., Auburn, S., Abera, A., Gadisa, E., Abraham, P., Aseffa, N. and Petros, B. (2015). Chloroquine efficacy for Plasmodium Vivax malaria treatment in southern Ethiopia. *Malar J*.**14**; 1-8

Goodman and Gilman's.,(2001). The pharmacological bases of Therapeutics.*McGraw-Hill. USA*. **10th ed**; PP. 1069.

Gqalenia, N., Ngcoboa, M., Parboosingb, R., and Naidoob, A. (2012). In vitro testing of african traditional medines for cytotoxic, immunemodulatory and anti-HIV activities. *Far J Tradit Complement Altern Med*. **9**; 2-11.

Grønhaug, T.E., Glæserud, S., Skogsrud, M., Ballo, N., Bah, S., Diallo, D., Pualsen, B.S. (2008). Ethnopharmacological survey of six medical plants from Mali, west Africa. *J ethnobiolEthnomed*.**4;** 21**-** 26

Gruys, .E, Toussaint, M.J.M.,Niewold, T.A., and Koopmans, S.J. (2005).Acute phase reaction and acute phase proteins.*J Zhejiang Univ Sic* B. **6**; 1045–1056.

Hilou, A., Nacoulma, G., Guiguemde, T.R. (2006). In vivo antimalarial activities of extracts from Amaranthusspinosus and Boerhaaviaerecta in mice.*J Ethnopharmacol.***103**; 236–240.

Himeidan, Y.E., Elbashir, M.I., Rayah, El-A., and Adam, I. (2005).Epidemiology of malaria in New Halfa.*Eastern Mediterranean Health J*.**11**; 499-504

Hismiogullari A A ,Hismiogullari SE, DincEssiz OY, and Rahman K(2011). Evaluation of biochemical findings in mice exposed to thiamphenicol treatment. *African Journal of Pharmacy and Pharmacology* **5**; 428-431.

Ibrahim, H.A. Imam, I. A., Bello, A. M., Umar, U., Muhammad, S., Abdullahi, S.A. (2012). The Potential of Nigerian Medinal Plants as Antimalarial Agent: *A Review. Inter J Sci Technol*. **2**; 600-605.

Inbaneson, S.J., Ravikumar, S., Suganthi, P. (2011). In vitro antiplasmodial effect of ethanolic extracts of coastal medical plants along Palk Strait against Plasmodium falciparum. *Asian Pac J Trop Biomed.* **2**; 364-367.

Jigam AA, Akanya HO, Dauda BE, Ogbadoyi EO (2011). Antiplasmodial, analgesic and anti-inflammatory effects of crude *Guierasenegalensis* (Combretaceae) leaf extracts in mice infected with *Plasmodium berghei*. *J PharmacognPhytother.* **3**;150–154.

Kalra, B.S., Chawla, S., Gupta, P., Valecha, N. (2006). Screening of antimalarial drugs.*Ind J Pharmacol.***38**; 5–12.

Kang, F.P.A., Onguéné ,Lifongo L, J C NdomSippl, W and Mbaze M(2014). The potential of anti-malarial compounds derived from African medinal plants, part II: apharmacological evaluation of non-alkaloids and non-terpenoids. *Malaria J*.**13**; 76-81.

Karou, D., Dicko, M.H., Sano, S., Simpore, J., Traore, A.S. (2003). Antimalarial activity of SidaacutaBurm.F. (Malvaceae) and PterocarpuserinaceusPoir.(Fabaceae).*J.Ethnopharmacol*. **89**; 291–294.

Kassa, M., Mshana, R., Ragusa, A., Assefa, G. (1998).In vitro test of five Ethiopian medical plants for anti-malarial activity against P. falciparum.*Ethiop. J. Sci.* **2**; 81-89.

Kassa.M, Kassa M, Mohana R, and Hunde A (1996).Antimalarial activity of Bersama abyssinica against Plasmodium falciparum in vitro.*Eth. phar. J.* **14**; 16-21.

Kaushik N, Bagavan A, Rahuman A A , Kamaraj Ch., Elango G, JayaseelanCh., Kirthi AV, SanthoshkumarTh (2015).Evaluation of antiplasmodial activity of medicinal plants from North Indian Buchpora and South Indian Eastern Ghats.*BioMed Central*.**4** ; 1-8

Kerner, A., Avizohar. O., Sella, R. Bartha, P., Zinder, O., Markiewicz, W., Levy, Y., Gerald, J. (2005). Brook, Doron Aronson.*AssAm J Hematol*.**88**; 1041-1044.

Ketema T., Yohannes M., Alemayehu E., Ambelu A., (2015). Evaluation of immunomodulatory activities of methanolic extract of khat (Catha edulis,Forsk) and cathinone in Swiss albino mice. *BMC Immunology*.**16**; 1-11

Ketema, T., Getahun, K., Bacha, K. (2011): Therapeutic efficacy of chloroquine for treatment of Plasmodium vivax malaria cases in Halaba district, South Ethiopia. *Parasites & Vectors*.**4**;1-7

Kilama W., Ntoumi F. (2009). Malaria: a research agenda for the eradication era. *Lancet*.374; 1480–1482.

Koevi K.A., Millogo V., Fokou J.B.H., Sarr A., Ouedraogo G.A., Bassene E., (2015). Phytochemical analysis and antioxidant activities of Combretum molle and Pericopsislaxiflora.*Inter J BiologChem Sci*. **9**; 2423-2431.

Kong JM., Goh NK. , Chia LS. , Chia TF.,(2003). Recent advances in traditional plant drugs and orchids. *ActaPharmacol.Sin.* **24**; 7-21

Krettli, A.U., Neto, V.F.A., Brandao, M.G.L., Wanessa, M.S., Ferrari, W.M.S. and Cruz, M.I.O. (2001). The search for new antimalarial drugs from plants used to treat fever and malaria or plants randomly selected: a review. *Mem.InstOswaldo Cruz, Rio de Janeir*.**96**; 1033-1042.

Lamar, J., Martschinske, R., Tetreault, G., Doud, C. (2007). Navy Medal Department Pocket Guide to Malaria Prevention and Control.**1**; 1-12.

Linthorst, G.E., Folman, C.C., van Olden, R.W., von dem Borne, A.E. (2002). Plasma thrombopoietin levels in patients with chronic renal failure.*Hematol J.* **3**; 38-42.

Lui, K.C., Yang, S.C., Roberts, M.F. (1992). Antimalarial activity of Artemisia annua flavonoids from whole plants and cell cultures. *Plants Cell.***11**; 637-640.

Makar, R.S., Zhukov, O.S., Sahud, M.A., Kuter, D.J. (2015). Thrombopoietin levels in patients with disorders of platelet production: diagnostic potential and utility in predicting response to TPO receptor agonists. *Inter J Scientific Engineering Res*. **6**; 716-772.

Masoko, P., Picard, J., Eloff, J.N. (2007). The antifungal activity of twenty-four Southern African Combretum species (Combretaceae).*South Far J Bot*.**73**; 173–183

McGaw LJ, Rabe T, Sparg SG, Jager AK, Eloff JN, van Staden J (2001). An investigation on the biological activity of Combretum species. *J Ethnopharmacol*.**Vol. 75**; pp.45-50.

Meera S, Gupta A, Kumar NS2008. Immunomodulatory and antioxidant activity of a polyherbal formulation.*Int. J. Pharmacol.* **4**; 287-91

Miaffo, D, Wansi SL, MbiantchaM ,Poualeu SLK, Guessom OK (2015). Toxicological Evaluation of Aqueous and Acetone Extracts of Combretum molle Twigs in Wistar Rats. *Electronic J Biol.* **11**; 33-45

Michael, L., Wilson, M..D, (2013). Laboratory Diagnofosis of Malaria: Conventional and Rapid Diagnostic Methods. *Arch Pathol Lab Med*.**137**; 805-811.

Mueller, I., Zimmerman, P.A., Reeder, J.C. (2007). Plasmodium malariae and Plasmodium ovale the "bashful " malaria parasites. *Trends Parasito*.**23**; 278–83.

Mueller, M., Hobiger, S., Jungbauer, A. (2010).Anti-inflammatory activity of extracts from fruits, herbs and spices.*Food Chem.* **122**; 987-996.

Muregi, F.W., Ishih, A., Miyase, T., Suzuki, T., Kino, H., Amano, T., Mkoji, G.M., Terada, M. (2007). Antimalarial activity of methanolic extracts from plants used in Kenyan ethnomedine and their interactions with CQ against a CQ-tolerant rodent parasite, in mice. *J. Ethnopharmacol*. **111**; 190-195.

Murphy, G.S., Basri, H., Purnomo, D. (1993). Vivax malaria resistant to treatment and prophylaxis with chloroquine.*Lancet.***341;** 96 -100.

NagarathnaP.K.M ,Reena K , Sriram R , Johnson W (2013). Review on Immunomodulation and Immunomodulatory Activity of Some Herbal Plants. *Int. J. Pharm. Sci. Rev. Res.* **22**; 223-230

Nardos ,A., Makonnen, E. (2017). In vivo antiplasmodial activity and toxicological assessment of hydroethanolic crude extract of Ajugaremota. *Malaria J.***16**; 1-8.

National Institutes of Health (NIH, 2013).Life Cycle of the Malaria Parasite.*Wikimedia Commons, the free media repository*. **19**; 1-3.

Nite KF, Pascal AO, Lydian LL, Jeon N, Wolfangang S, Luc MM.,(2014). The potential of antimalarial compounds drived from Ethiopia medicinal plants. *Malaria journal*.13; 2-10

Ojewole, J.A. (2008). Analgesic and anti-inflammatory effects of mollic acid glucoside, a 1alpha-hydroxycycloartenoid saponin extractive from Combretum molle (Combretaceae) leaf.*Phytother Res.* **22**; 30-35.

Okello*sv.,* Nyunjaro.,Netondo GW., Anyngo JC.,(2010). Ethnobotanical study of medicinal plants usedbysabaot of MT Elonon Kenya.*Af, J Trad CAM.* **7**; 1 -10

Okokon, J.E., Antia, B.S., Igboasoiyi, A.C., Essien, E.E., Mbagwu, H.O.C. (2007). Evaluation of anti-plasmodial activity of ethanolic seed extract of Picralimanitida. *J.Ethnopharmacol.* **111**; 464-467.

Okokon, J., Ofodum, K.C., Ajibesin, K.K., Danlandi, B,, and Gamaneil, K.S. (2005). Pharmacological screening and evaluation of antiplasmodial activity of Croton zambesicus against P. berghei infection in mice.*Indian J. Pharmacol.* **37**; 243-246.

Okokon, J.E., Etebong, E.O., Udobang, J.A., Obot, J. (2011). Antiplasmodial and antiulcer activities of Melantherascadens.*Asian Pac J Trop Biomed*.**2**; 16-20.

Orwa, C., Mutua, A., Kindt, R., ‚Jamnadass, R., Anthony, S. (2009). Agroforestry Database: a tree reference and selection guide. *World Agroforestry Centre, Kenya*. **4;** 1-8

Oyedemi, S.O., Bradley, G., Afolayan, A.J. (2010). In vitro and in vivo antioxidant activities of aqueous extract of Strychonoshenningsii Gilg. *Far J Pharm Pharmacol*. **4**;70–78.

Peters, W. (1967).Rational methods in the search for antimalarial drugs.*Trans R Soc Trop Med Hyg*. **61**; 400-410

Price, R.N., Nosten, F., Luxemburger, C., van Vugt, M., Phaipun, L., Chongsuphajaisiddhi, T., White, N.J. (1997). Artesunate/mefloquine treatment of multi-drug resistant falciparum malaria.*Trans R Soc Trop Med Hyg.* **91**; 574-577

Ragusa, F., and Araya, M.(2012). In vitro antimicrobial activity of Combretum molle against Staphylococcus aurous and Streptococcus agalactiae isolated from crossbred dairy cows with clinical mastitis.*Trop Amin Health Prod*. **44**; 1169-73.

Ramalhete, C., Lopes, D., Mulhovo, S., Rosário, V.E., José, M., Ferreira, U (2008.). Antimalarial activity of some plants traditionally used in Mozambique. *Workshop Plants Medicines*.**31**; 1-9

Ritter, J., Flower, R., Henderson, G., Rang, H. (2015). Pharmacokinetics. Rang & Dale's Pharmacology .*Churchill Livingstone*. **8th ed**.; 125.

Rope, P. (2011).Pact-mediated drug transport in malarial parasites.*Biochemistry*.**50**; 163-171.

Rowe, J.A. and Keys, S.A. (2004). The role of Plasmodium falciparum vary genes in malaria in pregnancy. *Mol. Microbial.* **53**; 1011-1019.

Salon, S., Banana, A., Tuatara, L.P. (2013). In vitro antiplasmodial and cytotoxic properties of some medicinal plants from western Burkina Faso. *Far J Lab Med.* **2**; 81-92

Sauerwein, R.W. (2007). Malaria transmission-blocking vaccines: the bonus of effective malaria control. *Microbes and Infect.***9**; 792-795.

Savant Ch., Joshi N, Reddy S, Abdul-Aziz BM, Joshi H (2014). Immunomodulatory medicinal plants of India: a review. *International Journal of Pharmacology & Toxicology.***4;** 109-115

Saxena, S., Pant N., Jain, D.C., Bakunin, R.S. (2003).Antimalarial agents from plant sources.*Current Sci.***85**; 1314-1329.

Shah, B., Shah, N., Seth, A.K., Maheshwari, K.M. (2011).A review on medical plants as a source of anti-inflammatory agents.*Res J Med Plants.* **5**; 101-115.

Shula, S., Baja, K., Kim, M. (2014). Plants as potential sources of natural immunomodulatory; *Rev Environ Sic Biotechnology* **13**; 17–33.

Singh, A., Bhatt, T.K., Sharma, O.P. (2011). Clinical Biochemistry of Hepatotoxicity.Clinical Toxicology.*J Clinic Toxically.* **5***;* 1-19.

Singh, A., R.A.Vishwakarma and A. Husain, (1988). Evaluation of Artemisia annua strain for higher artemisinin production. *Plantae.Med.* **7**; 475-476.

Snow, R.W., Guerra, C.A., Noor, A.M., Mint, H.Y., and Hay, S.I. (2005).The global distribution of clinical episodes of Plasmodium falciparum malaria.*Nature*.**Vol. 434**; pp.214-217.

Sofowora, A. (1996). Research on Medicinal Plants and Traditional Medina in *Africa. J. CAM*. **2**; 365-372.

Solomon, A., Ban tie, L., Delahunt, T. and Ephraim, E. (2014). In vivo antimalarial activity of the crude leaf extract and solvent fractions of Croton macrostachyusHocsht. (Euphorbiaceous) against Plasmodium berghei in mice.*BMC ComplemplementaryAltern.Med.***14**;14-79.

Stockelberg, D., Andersson, P., Björnsson, E., Björk, S., Wadenvik, H. (1999). Plasma thrombopoietin levels in liver cirrhosis and kidney failure.*J Intern Med*.**246**; 471-475.

Suresh M , Mallikarjuna RN , Sharan B, Singh M , Hari K Shravya B, keerthi G, Chandrasekhar M (*2012*).Hematological Changes in Chronic Renal Failure.*International Journal of Scientific and Research Publications.* **2**; 1-4

Teka, H., Petros, B., Yamuah, L., Tesfaye, G., Elhassan, I., Muchohi, S., Kokwaro, G., Aseffa, A. and Engers, H. (2008). Chloroquine-resistant *Plasmodium vivax* malaria in DebreZeit, Ethiopia.*Malaria J.* **7**; 1-8

Thapa, B.R. and Walia, A. (2007).Liver function tests and their interpretation.*Indian J Pediatr*, **74**; 663-671.

Tiwari, R.K.S, Chandravanshi, S.S Ojha, B.M. (2005). Efficacy of extracts of medicinal plants species on growth of Sclerotiumrolfii root in tomato.*J. Mycol. Plant Pathol*.**10**; 1124-1126.

Tran, Q.L., Tezuka, Y., Ueda, J.Y. (2003). In vitro antiplasmodial activity of antimalarial medical plants used in Vietnamese traditional medine. *J. Ethno pharmacol.* **86**; 249-252.

Valecha, N., Atul, P.K., and Pillai, C.R. (2001). Antiplasmodial effect of three medicinal plants: A preliminary Study. *Current Sc.* **80**; 917-919.

Vogel, G. (2010). Infectious disease new map illustrates risk from the ‘other’malaria. *J. Sci*. **329**; 618–618.

Waako, P., Katuura, E., Smith, P., Folb, P. (2007). East African medinal plants as a source of lead compounds for development of new antimalarial drugs.*Afr J Ecol.***Vol. 45**; pp. 1–5.

White, N.J. (2004). Antimalarial drug resistance.*J Clin Invest.* **113**; 1084–1092.

WHO.(2005). Malaria control Today.Roll Back Malaria Department World Health Organization, *Geneva, Switzerland*.**6**; 1-75.

WHO. (2012). From malaria control to malaria elimination: a manual for elimination scenario planning. **20**; 1-68

WHO.(2015). World malaria report 2013. Geneva, Switzerland: World Health Organization. **14**; 2-6

Willcox, M., Bodeker, G., Rasoanaivo, P. (2004).Traditional Medinal Plants and Malaria.Traditional Herbal Medines for Modern Times. CRC Press, LLC. New York. **10**; 1-11.

Yeo, M., Han, S.U., Nam, K.T., Kim, D.Y., Cho, S.W., Hahm, K.B. (2012). Acute and sub-acute toxic study of aqueous leaf extract of Combretum molle. *Trop J Pharm Res.***11**;217-223.

Yeshwondim, A.K., Tekle, A.H., Dengela, D.O., Yohannes, A.M. (2010). Therapeutic efficacy of chloroquine and chloroquine plus primaquine for the treatment of Plasmodium vivax in Ethiopia.*Acta Trop*. **113**; 105-113.