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Evaluation of AntimalarialActivity of *Otostegiaintegrefolia*Leaf Extracts against*Plasmodium berghei* in Mice

By

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Acronyms

°C	Degree Celsius
ACT	Artemisinin Combination Therapy
AIDS	Acquired Immunodeficiency Syndrome
AMREF	African Medical and Research Foundation
ANOVA	Analysis of Variance
CDER	Center for Drug Evolution and Research
$CHCl_3$	Chloroform
CQ	Chloroquine Phosphate
CRPv	Chloroquine Resistant *Plasmodium vivax*
DDT	Dichlorodiphenyltrichloroethane
dH_2O	Distilled Water
DHA	Dehydroartemisinin
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECA	Economic Commission for Africa
EHNRI	Ethiopian Health and Nutrition Research Institution
FMOH	Federal Ministry of Health, Ethiopia
GDP	Growth Domestic Product
HIV	Human Immunodeficiency Virus
IC	Inhibition Concentration
IL	Interleukin
IP	Intraperitoneal
IPTP	Intermittent Preventive Treatment for Pregnant women
IRS	Indoor Residual Spraying

LD	Lethal Dose
LLINs	Long Lasting Insecticidal Nets
MDA	Mass Drug Administration
MIC	Minimum Inhibition Concentration
Ml	Maximum Inhibition
MST	Mean Survival Time
MeOH	Methanol
MVI	Malaria Vaccine Initiative
NC	Negative Control
NIAID	National Institute of Allergy and Infectious Diseases
OP	*Otostegia perisca*
PC	Positive Control
PCV	Packed Cell Volume
PfEMP	*Plasmodium falciparum* Erythrocyte Membrane Protein
PQ	Premaquine
SEM	Standard Error Mean
SP	Sulfadoxine-pyrimethamine
SPSS	Stastical Package for Social Sciences
SSA	Sub-Saharan Africa
TCM	Traditional Chinese Medicine
TNF	Tumor Necrosis Factor
WEF	World Economic Forum
WHO	World Health Organization

ABSTRACT

Malaria remains one of the most deadly parasitic diseases in the tropics and subtropics. It is the cause of death of more than 650,000 people every year with the majority affected groups are children under the age of five and pregnant women. The spread of drug resistant malaria parasite is the major challenge in the control of the disease. Therefore, there is an urgent need to develop new and efficient antimalarial drugs that are easily be accessible and affordable by those poor living in the malarious regions. In the attempt to search for new antimalarial drugs, the present study aimed to discover an effective plant based extracts from the leaf of *Otostegia integrefolia* against chloroquine (CQ) sensitive strain of *Plasmodium bergheiin vivo* in Swiss albino mice. The standard 4-day suppressive test was employed to determine percent parasitaemia inhibition caused by extracts of the plant material. Extracts were administered at doses of 200, 400 and 800 mg/kg body weight (b.wt) of mice. Aqueous, methanol and chloroform crude extracts of the leaf of *O. integrefolia* suppressed*P. berghei* parasitaemia significantly ($P<0.05$) in mice with a maximum inhibition in the methanol extract treated mice (56.77%), followed by aqueous (44.45%) and chloroform (39.16%) at thehighestdose of 800mg/kg b.wt. In addition, the extract prolonged the survival time of *P. berghei* infected mice compared to the non-treated controls. However, it did not prevent body weight loss and reduction in PCV values. Safety of the extract was also revealed and mortality, sign of serious acute plus sub-acute toxicity were not observed in those mice that received up to a single dose of 2000mg/kg/day during the follow up period. Therefore, this result showed that crude aqueous, methanol and chloroform extracts of the leaf of *O. integrefolia*are nontoxic and possess potent antimalarial effects which may offer the potential for a safe, effective and easily affordable phytomedicine in the fight against human malaria parasites.

Key words: Antimalarialactivity,*In vivo*, *Otostegia integrefolia*, *Plasmodium berghei*, toxicity, parasitaemia

1 INTRODUCTION

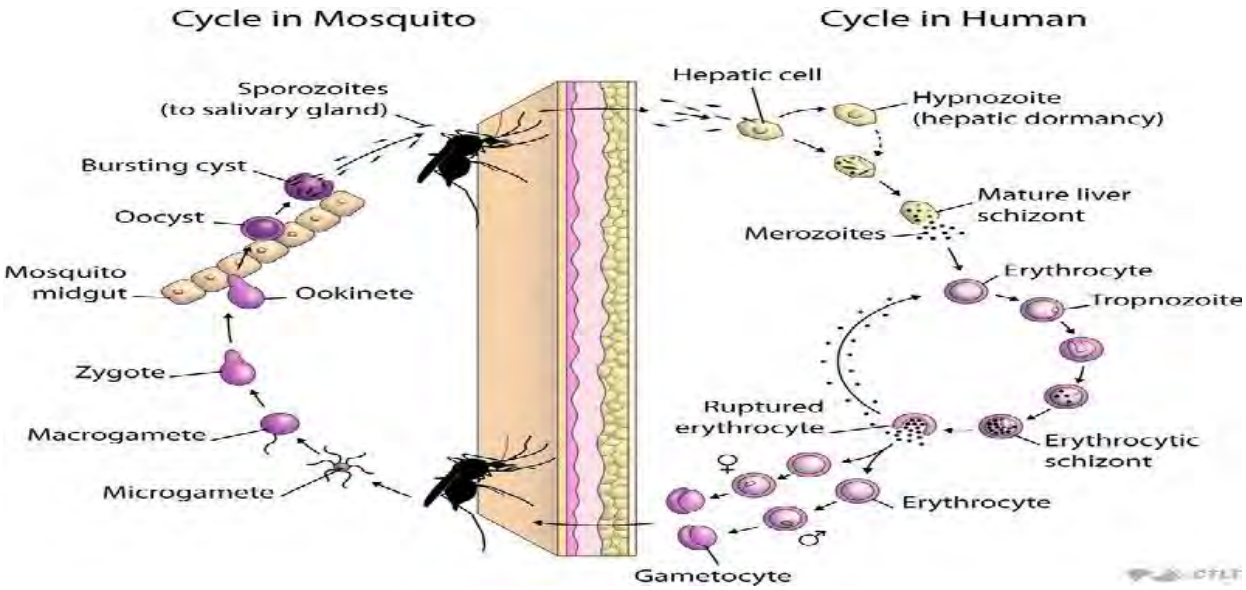
1.1 Malaria: Etiologic agents and transmission

Malaria is one of the most important vector borne tropical diseases caused by protozoan parasites belonging to the genus *Plasmodium.* There are more than 100 species of *Plasmodium*parasite that can infect many animal species such as reptiles, birds, rodents, monkeys and humans (NIAID, 2007). Four species of genus *Plasmodium; P. falciparum, P. vivax, P. ovale and P. malarae* have long been recognized to infect humans in nature. In addition, there is one species; *P. knowelesi* that naturally infects macaquemonkeys has recently been recognized as a cause of zoonotic malaria in humans (Eede *et al*., 2009; Su, 2010; Hobbs and Duffy, 2011).

Human malaria caused by the five species of genus *Plasmodium* is mainly transmitted through the bite of infected female *Anopheles* mosquitoes during blood meal (Cox, 2010; Echhoff, 2011) and other rare mechanisms including congenitally acquired, blood transfusion, sharing of contaminated needles and organ transplantation (Guerra *et al*., 2008). *Anopheles gambiae* is the most efficient malaria vector in malaria endemic areas of sub-Sahara African followed by *Anophelesfunestus* (Gillies and Coetzee, 1987; Fontenille and Lochouam, 1999). The majority of malaria cases are caused by *P. falciparum*, which causes severe morbidity and most of malaria related deaths worldwide especially in Africa (Nchinda, 1998).

1.2 Life cycle of the malaria parasites

Understanding the biology of *Plasmodium* parasite is the cornerstone for designing malaria prevention and control strategies. The life cycle of the parasite starts during blood meal of malaria infected female *Anopheles* mosquito which inoculates sporozoites into the human host. The sporozoites remain in the circulation for a short period before they enter the liver of the host; and these sporozoites infect liver cells (both the Kuppfer cells and hepatocytes) (Lopez-Antunano and Shmunis, 1980). Those sporozoites which invade the Kuppfer cells may be engulfed and no longer survive. The rest sporozoites that invade the hepatocytes start asexual exoerythrocytic schizogonic cycle and mature into schizonts (the multinucleated stage of the parasite) which rupture and release merozoites into the blood stream and invade the healthy eryth


Cycle in Mosquito
Cycle in Human
Sporozoites
(to salivary gland)
Bursting cyst
Oocyst
Mosquito
midgut
Ookinete
Zygote
Macrogamete
Microgamete
Hepatic cell
Hypnozoite
(hepatic dormancy)
Mature liver
schizont
Merozoites
Erythrocyte
Tropnozoite
Ruptured
erythrocyle
Erythrocytic
schizont
Erythrocyte
Gametocyte

1.3 Pathophysiology and clinical features of malaria

Sequestration and cytoadherence are important elements of the pathophysiology of all the different manifestations of *P. falciparum* infection (Patel*et al*., 2003; Dondorp, 2005). Even though, the nature of clinical manifestation depends greatly on the background level of acquired protective immunity, the first line symptoms are non-specific and similar to symptoms of a minor systemic viral illness (WHO, 2010_b). The most severe form is caused by *P. falciparum;* within 48 hours, asexual blood stage cycle, change the surface properties of infected red blood cells, causing them to stick to blood vessels (a process called cytoadherence which is mediated by parasite derived protein called *Plasmodium falciparum* Erythrocyte Membrane Protein I (PfEMP-I); Producing humps or knobs on the surface of infected erythrocytes that hinders microcirculation blood flow (Dondorp, 2005; Ochola, and Kanyikela, 2005). This phenomenon results in variable clinical features including fever, chills, headache, muscular aching, weakness, vomiting, cough, diarrhea, abdominal pain and other symptoms related to organ failure such as: acute renal failure, generalized convulsions, circulatory collapse, followed by coma and death. In children, the symptoms are present differently and may display: convulsions, confusion and neurological impairment progressing to coma, hypoglycaemia, metabolic acidosis and severe anaemia which are less frequently observed in adults. In addition, the release of malaria antigens, toxins and production cytokines, particularly tumor necrosis factor (TNFd), induced by the release of parasite products during schizont rupture, complemented by the effects of other circulating "endogenous pyrogens" such as interlukin-1 (IL-1) and IL-6 appears to play a central role in the clinical features of malaria particularly heavy fever (Attwooda, 2011).

1.4 The global disease burden and economic impact of malaria

Malaria remains one of the most significant causes of morbidity and mortality in resource poor countries especially in sub-Sahara African children under the age of five years and pregnant women (Kabanywanyi *et al*., 2008; Roca-Feltrer *et al*., 2008). According to World Health Organization (WHO, 2010) malaria is prevalent in 106 countries of the tropical and semitropical world (Africa, Amazon, central and southern America; central, south and south east Asia; Pacific) that are home to more than half of the world's people (3.3 billion) and it is a persistent problem in most of these areas.

Globally, it is estimated to cause between 300 and 500 million cases and about 900,000 deaths annually, with more than 86% of the disease burden and 90 % of the deaths occurring in in sub-Sahara African regions (WHO, 2009). The disease plays a tremendous burden on individual families and national health systems as well it is an enormous global killer and places significant strain on economies around the world (ECA, 2005). In Africa, a child dies every 30 seconds due to malaria and those children who survive from the episodes of cerebral malaria may suffer from anemia and cerebral complications that affect their long-term growth and development (Opoka *et al*., 2009). Low birth weight in infants, often a result of malaria infection during pregnancy, undermines the chance of survival. In the eastern and southern African region, an estimated 30% of all recorded deaths during pregnancy are attributed to malaria infection (AMREF, 2005; WEF, 2006).

In addition, malaria affects individual businesses and the economy of countries either directly or indirectly. The direct individual economic impacts of the disease include; the value of lives lost, the value of time lost to sickness, the expenditures on medical care such as diagnosis, treatment and prevention and the direct social costs include government expenditures on malaria control and prevention (Gollin, 2007; Chuma *et al*., 2010). Whereas, the indirect costs are still greater which include changes in human settlement and labor patterns induced by the disease, consequences of the disease on fertility, demography, human capital investments and potentially on managerial quality and technology adoption. For example, skilled managers may prefer not to work in malarial regions, resulting in reduced productivity levels in that area (Amar and Sachs, 1999; Mia *et al*., 2011).

Data on the effects of malaria on economies and businesses are scarce.A number of companies have been reported that malaria has had severe impacts on operating costs in recent years. A survey conducted in Ghana, showed that 30 % of business leaders of the respondents reported that the disease had a strong impact on productivity (WEF, 2006). Similarly, malaria is estimated to cost $US 12 billion in lost Gross Domestic Product (GDP) every year in Africa and it assumed that, the disease has slowed the economic growth in the continent by 1.3 percent per year as a result of lost life and lower productivity what is known as “growth penalty” (Goodman *et al*., 2000; Gallup and Sachs, 2001). In contrary, some Southern European economies grew rapidly

following malaria eradication. Countries including Greece, Spain and Italy have experienced rapid economic growth after eradicating malaria from their land (Gallup and Sachs, 1999; Gallup and Sachs, 2001; WHO, 2006_b).

Several studies have also found that malaria can impede a child's schooling, which potentially results in long term harm to the economy of a country (WEF, 2006). The disease affects primarily through damaging cognitive abilities and absenteeism from schooling. And those children who survive from sever malaria results in neurological damage including; behavioral problems, language difficulties, seizure and epilepsy (Opoka *et al*., 2009). A study by Waruiru, *et al*., (1996) in Kenya reported that malaria was found to account for the causes of 16 % and 31 % of seizures and epilepsy in children respectively (Waruiru *et al*., 1996) and another report by Holding and Kitsao-Wekulo, (2004), documented 15 % of health related absenteeism from schools due to the pathology caused by malaria and found that primary school students missed an average of 20 school days per year which covers 10 % of the total school days of a one academic year (Holding and Kitsao-Wekulo, 2004).

Malaria is one of the leading public health problems in Ethiopia and it is the main causes of morbidity and mortality. *P. falciparum* and *P. vivax* are the two main species accounting for 60% and 40% of malaria cases respectively (FMOH, 2004). Approximately 75% of the country is malarious with about 68% of its population living in areas at risk of malaria (Jima *et al*., 2007). Approximately 4-5 million causes and more than 600, 000 confirmed cases are reported to cause 70,000 deaths each year (Nigatu *et al*., 2009). According to FMOH, (2011), in 2008/2009 malaria was the first cause of outpatient visits (12%), health facility admissions (9.9%) and inpatient deaths (FMOH, 2011).

The epidemiological pattern of malaria transmission is generally unstable and seasonal except perennial transmission along Awash River (FMOH, 2008; Nigatu *et al*., 2009). The level of transmission is also varying from place to place because of differences in altitude and rainfall patterns. But changes have been observed in the epidemiology of the disease through time. Although, malaria was known to occur in areas below 2000 m previously (Woyessa *et al*., 2002).

In the country, the disease mainly affects those living in rural areas due to the existence of abundant mosquito breeding sites, poor housing conditions and inability to afford control measures coupled with low awareness of the preventive methods (Nigatu *et al*., 2009). Meteorological factors are also important driving forces of malaria transmission by affecting both malaria parasites and vectors directly or indirectly. Generally, temperature, rainfall and humidity have been associated with the dynamics of malaria vector populations and therefore, with spread of the disease in the country (Waktola, 2008; Alemu *et al*., 2011).

Recently,there have been records in the reduction of malaria cases and malaria related deaths by up to 50% over the past decade (Jaenisch *et al*.,2010; Mmbando *et al*., 2010). For instance, country wide surveillance in Ethiopia and Eritrea revealed a 70% decline in malaria morbidity in 2010, with similar changes has been documented in parts of Kenya, Gambia, Rwanda, and Zambia (O'Meara *et al*., 2010).

According to World Malaria Report (2011), there were 106 countries with malaria endemic in 2010. Even though, Ninety nine of these countries had ongoing malaria transmission and an estimated 3.3 billion people at risk with 655,000 persons deaths in the year, mortality rates due to the disease have fallen by more than 25% since 2000, with the largest percentage reductions seen in the Europe (99%) followed by America (55%), Western Pacific (42%) and African Regions (33%). Surprisingly, the European Region reported no cases of *P. falciparum* malaria for the first time in 2009 (WHO, 2011).

In spite of this progress, malaria remains one of the major public health problems on the African continent with about 80% of malaria deaths occurring in African children under five years of age that is around 30% or more of outpatient visits and hospital admissions are reported to be caused by malaria. This again also places a heavy burden on individual families and national health systems (WHO, 2011).

1.6 Malaria prevention and control

WHO define malaria control as “reducing malaria morbidity and mortality to a lower level through deliberate efforts using the preventive and curative tools available today.” Globally,

different methods that target both the vector and parasite are used to overcome problems associated with malaria. According to WHO, (2006 and 2008), the control strategies of malaria rely on both preventive and case management. The tools used for prevention are long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), intermittent preventive treatment for pregnant women (IPTP), malaria vaccine and antimalarial drugs. Other vector control measures including larviciding, biological methods and environmental management can also be used when appropriate based on scientific evidence (WHO, 2006a; WHO, 2008).

IRS using insecticide such as DDT was responsible for the success of malaria control in the early 1950s and 1960s (NIAID, 2007). However, because of financial and operational constraints and applications carried on time intervals caused the emergence of insecticide resistance vectors and environmental pollution (Read *et al*., 2009). Personal protections using insecticide treated nets and repellants, prevents human and mosquito contact. But, due to behavioral change of the vector such as been diurnal feeder rather than nocturnal, this method is also not effective as expected too (Echhoff, 2011). In the other cases, biological controls using larvivorous fish such as the *Gambusia* fish is a voracious eater of mosquito larvae and the bacteria *Bacillus thuringiensis var israelensis* have been effective in the reduction of the number of vectors significantly, but it is difficult on large scale applications where the breading sites of the vectors are not easily manageable (WHO, 2006_a).

The other best, safe and cost effective method of malaria control is malaria vaccine. Research activities have been in progress for several years on this issue. And this day, hope has been renewed by the development of several new vaccine candidates and delivery systems (Graves and Gelband, 2009). Vaccines currently under evaluation include recombinant proteins, synthetic peptides (including multiple antigen peptides), DNA vaccines, inactivated whole parasites and vaccines comprising mixtures of a large variety of potential antigens (Loucq, 2009).

The SPf66 vaccine was the first malaria vaccine to be tested in clinical trial extensively in endemic areas. It is a synthetic peptide vaccine containing antigens from the blood stages of malaria linked together with an antigen from the sporozoite stage. This vaccine has had 10 trials in Africa, Asia and South America and results were initially promising but further trials showed

only a small effect in some trials and no effect in others(Graves and Gelband, 2009). The other vaccine is RTS, S is the most clinically advanced malaria vaccine candidate in the world. In clinical trials, it was the first to demonstrate that it can protect young children and infants in malaria endemic areas against infection and clinical disease caused by *P. falciparum*. The RTS,S vaccine candidate is a recombinant protein that induces the production of antibodies and T cells that are believed to diminish the malaria parasite's ability to infect, develop and survive in the human liver (MVI, 2011). Clinical trial of this vaccine has been conducted at 11 trial sites in 7 countries across sub-Saharan Africa showed that 3 doses of RTS, S reduced the risk of children experiencing clinical malaria by 56% and severe malaria by 47%. Although this much protection is promising vaccine, it is not as effective enough as what was expected (Barcelona, 2011).

Chemotherapy (antimalarial drugs) remains one of the most important tools for themanagement of malaria; that have played the greatest role in controlling the disease today than ever the other methods even though the control ishampered by the emergence and spread of parasites resistanceto the currently available antimalarial drugs (WHO, 2010_b).

Antimalarial drugs and drug resistance

Early diagnosis and effective treatment of malaria cases using antimalarial drugs is the basis of its control (WHO, 1993). In general, antimalarial drugs are used to treat malaria illness, prevent both infection and disease, eliminate dormant malaria parasites from the liver in the case of *P. vivax* and *P. ovale* malaria infection and to block malaria transmission (Pedro *et al*., 2011). In the context of malaria elimination or eradication, drugs have been applied for both treatment and prevention in situations where intensive surveillance has been used to identify cases and in mass drug administration (MDA) programs without regard for the presence of infection (Seidlein and Greenwood, 2003).

Based on the derivative materials they were synthesized, the currently available antimalarial drugs are grouped into three classes. Quinoline based antimalarials, which includes quinine and its derivatives such as chloroquine (CQ), amodiaquine, premaquine(PQ)and mefloquine. Quinine is the first pharmaceutical antimalarial drug isolated from the bark of *Cinchona calisaya* in

1820(Hobbs and Duffy, 2011). It has been used for more than three centuries and until the 1930s it was the only effective agent for the treatment of malaria. It is now only used for treating severe falciparum malaria through intravenous injection; because of its undesirable side effects (Parija and Praharaj, 2011).

In 1940, chloroquine was synthesized. It was cheaper, very effective and relatively safer (Bloland, *et al*., 2003). It is a 4-aminoquinolones derived from quinine used both for treatment and prophylaxis (Saxena *et al.,* 2003). CQ is still used as the first line malaria treatments for *P. vivax*infection in some parts of the world including Ethiopia (Ketema *et al*., 2009).Quinoline based antimalarial drugs act by interfering with the parasite's ability to eliminate the toxic byproducts of hemoglobin digestion (Hobbs and Duffy, 2011).

The second group of antimalarial drugs is the antifolates (Sulfadoxine-pyrimethamine) that have specific enzymes(dihydropteroate synthetase and dihydrofolate reductase) targets in the parasite (Bloland, 2001; Wells *et al*., 2009). Sulfadoxine-pyrimethamine (Fansidar®) became available in 1971 as second line therapy for malaria that inhibits the parasite's essential ability to synthesize tetrahydrofolate used for methylation reactions. Antifolates were demonstrated to be effective against malaria as early as the 1930s and sulfonamide components with better pharmacokinetic profiles, although resistance developed soon(WHO, 2010_b).

Unfortunately, the malaria parasites have developed resistance to all the above mentioned drugs in different parts of the world (NIAID, 2007). In response to this, WHO recommended artemisinin combination therapy (ACT) in recent years (WHO, 2010_b). Artemisinin is the third group of antimalarial and its semisynthetic derivatives (dehydroartemisinin (DHA), artemether, arteether, artesunate and artelinic acid) are derived from sweet wormwood(*Artemisia annua*) which is potent drug that rapidly kills young intraerythrocytic malaria parasites thereby preventing their development to more pathological mature stages (White, 2008) and are the most rapidly acting and effective drugs against multidrug resistant strains of the malaria parasite and have shown very rapid parasite clearance times and faster fever resolution than occurs with quinine (Bloland. 2001).

Drug resistant *Plasmodium*species is a major problem in malaria control (White, 1998). Resistance in*in vivo* has been reported against almost all antimalarial drugs.Resistance of *Plasmodium* parasites to currently available antimalarial medicines has been reported from different parts of the globe. It has been documented for *P. falciparum, P. vivax* and *P. malariae*. But,*P. falciparum* resistance has been observed in all today's available antimalarial drugs (amodiaquine, chloroquine, mefloquine, quinine, and sulfadoxine-pyrimethamine) (WHO, 2010_b) and more recently; in artemisinin derivatives along the Thi-Cambodia border historically a site where antimalarial drug resistance first reported (Noedl, *et al*., 2008; Dondorp *et al*., 2009).

Antimalarial drug resistance is "the ability of a malaria parasite to survive in the presence of higher concentration of a drug that normally destroys or prevents multiplication of cells of the same species" (WHO, 1986). The development of resistance to antimalarial drugs poses one of the greatest threats to malaria control and is the main cause of recent increases in malaria morbidity and mortality (Stepniewska, 2007; WHO, 2010_b).

P. falciparum resistant isolates were first reported in South East Asia and then South America in the early 1950s, chloroquine (CQ) was the mainstay of malaria therapy worldwide since 1940s (Young and Moore, 1961). CQ resistant falciparum strains had spread in all endemic areas of South America by 1970 and almost all in Asia and Oceania by 1989 (White, 1998). In Africa it was first reported in the eastern part in 1978 which then spread to the central and southern parts then into West Africa in 1983 and by the 1989 it was widespread in sub-Sahara African regions (Pickard and Wernsdorfer, 2002: Al-Mekhlafi *et al.,* 2011; Frosch *et al*., 2011). Since early 1960ssulphadoxine-pyrimethamine combination was used as a drug of choice to treat CQ resistant falciparum strains even though resistance to sulphadoxine-pyrimethamine (SP) was described from the Thai-Cambodian border in 1960 (Bjorkman and Howard, 1990). Malawi was the first African country to replace CQ as the first line treatment of uncomplicated malaria with the antifolate combination SP in 1993 followed by Kenya, Uganda and Tanzania (Bloland and Ettling, 1999).

1986 was the year when CQ resistant*P. falciparum* isolate was first reported in Ethiopia (Teklehaimanot, 1986; Tulu *et al*., 1996). The CQ resistance has led to a national antimalarial policy change in 1999 from CQ to adopting SP as a first line drug for the treatment of

uncomplicated falciparum malaria (Kassa *et al*., 2005). However, CQ still remains the first line treatment for *P. vivax* and *P. malarae* with quinine being second line (Ketema *et al*., 2009). According to Jima, *et al*., (2005) and FMOH, (2004); artemether-lumefantrine replaced SP in 2004 due to the increasing resistance of malaria parasites to SP (Jima *et al*., 2005; FMOH, 2004).

The first pharmaceutical antimalarial drug quinine remains the first line antimalarial option for treatment of patients with complicated malaria in Europe and Africa where there is a resistance to CQ (Alonso *et al*., 2011). However, *P. falciparum* resistance to quinine was first demonstrated in Brazil in 1880 and then in German railroad workers returning from Brazil (Pickard and Wernsdorfer, 2002). The clinical resistance to quinine therapy has been noticed sporadically in Southeast Asia and western Oceania but is less frequent in South America and Africa (Jelinek *et al*., 2001). In addition, CQ and mefloquine resistant strains of *P. falciparum* displaying reduced susceptibility to quinine are found in some focal areas in the Amazon basin that are considered to be malaria endemic areas (Pickard and Wernsdorfer, 2002).

In the other cases, CQ resistant*P. vivax* has been spreading throughout the world since the first report in Papua New Guinea and Iran in 1989 (Garg *et al*., 1995; Fairlamb *et al*., 2005). *P. vivax* accounts for 40% of the malaria cases in Ethiopia and the first line treatment of this infection is CQ (FMOH, 2004; Ketema *et al*., 2009). But this day, treatment failure of CQ against *P. vivax* infection has been reported from the country with the report by Teka *et al*., (2008) in 2006 after an *in vivo* CQ drug efficacy study taken place in Debre Zeit, Ethiopia (Teka *et al*., 2008). Another report revealed by Ketema *et al*., (2011), Among 87 malaria patients enrolled in Halaba district, South Ethiopia, the rise in treatment failure was documented (13%). These much of statistics signals the spreading of CQ resistant *P. vivax* (CRPv) strains to malaria endemic areas of the country (Ketema, *et al*., 2011).

Artemisinin based combination therapies are now recommended by the World Health Organization (WHO) as first line treatment of uncomplicated falciparum malaria in all areas where malaria is endemic (WHO, 2010_b). Replacing ineffective and failing treatments of CQ and SP with artemisinin based combination therapies (ACT) has reduced the morbidity and mortality associated with malaria significantly (Bloland, 2001). However, recently there have been signs that the efficacies of artemisinin based combination therapy and artesunate monotherapy have

declined in western Cambodia (Noedl, *et al*., 2008;Dondorp *et al*., 2009;Parija and Praharaj, 2011).

Therefore, there is an immediate need of developing new antimalarial drugs hopefully accompanied by the understanding of their mode of actions. And one of the best sources for developing new and efficient antimalarial drugs that can easily affordable by those poor living in the malarious endemic tropical countries is traditional medicinal plants and other natural products (Ginsburg and Deharo, 2011).

1.7 Antimalarial drugs from traditional medicinal plants

"Traditional medicine has remained as the most affordable and easily accessible source of treatment in the primary health care system of resource limited communities and the local therapy is the only means of medical treatment for such communities" (Yineger and Yewhalaw, 2007). Currently, an estimate about 80% of people in developing countries; especially those in Asia, Africa, Latin America and the Middle East use traditional medicine for the management of disease and to address their primary health care needs based largely on species of plants and animals (WHO. 2010_c). In some industrialized nations, use of traditional medication is equally significant; Canada, France, Germany, Italy, Belgium and the Netherlands has reported that more than 70% of their populations have used traditional medicines under the titles of "complementary", "alternative", or "nonconventional" medication (Hoareau and DaSilva, 1999; Robinson and Zhang, 2011)

Plants had been used for medicinal purposes long before recorded history. Ancient Chinese and Egyptian papyrus writings describe medicinal uses of plants (Hassan *et al*., 2009). Still the practices continue because of biomedical benefits as well as place in cultural beliefs in many parts of the world and have made a great contribution towards maintaining human health (Verma and Singh, 2008). These plants that may contain active materials for treating the body or that can be the origin of pharmacologically active drugs are called herbal medicine (Ahmad *et al*., 2009). Indigenous cultures, such as the African and Native American used herbs in their healing rituals (Hassan *et al*., 2009); while others developed traditional medical systems, the Ayurveda and Traditional Chinese Medicine (TCM) in which herbal therapies were used (Morgan, 2002) plus

people in different parts of the world tended to use the same or similar plants for the same purposes (Kraft, 2009).

Plant based drugs provide an outstanding contribution to the modern therapeutics (Ginsburg and Deharo, 2011). It is estimated that at least 25% of all modern medicines are derived either directly or indirectly from medicinal plants primarily through the application of modern technology to traditional knowledge. In the case of certain classes of pharmaceuticals such as antitumoral and antimicrobial medicines, this percentage may be as high as 60% (Sucher, 2008). The development of useful and widely used drugs like digoxin and digitoxin from digitalis leaves, quinine from the cinchona bark, reserpine from *Rauwolfia serpentine*, morphine from *Papaver somniferum*, coaine from *Erythroxzion coca* and the anticancer vincristinerand viblastinefrom *Cartharathus troseus* of Madagascar and again the anticancer compound bruceatin from the Ethiopian plant*Brucea antidysentrica* are just to name a few examples of the contributions of traditional medicinal plants to the modern therapeutics agents (Bekele, 2007).

Many plant species continue to be used in traditional medicine for the treatment of malaria and many people depend up on such remedies as they cannot afford or do not have access to standard antimalarial drugs (Colin, 2005). It have been played a critical role in the history of malaria with Peruvian bark (*Cinchona* spp.); being the first effective treatment for this complications (Yarnell and Abascal, 2004). The long established use of quinine and the more recent introduction of artemisinin and their derivatives as highly effective antimalarials; demonstrates that plant species are important sources for the discovery of new antimalarial agents (Colin, 2005). Recently, the WHO estimated that 80% of people worldwide rely on herbal medicines for some part of their primary health care (WHO, 2010_c); especially developing countries where malaria is endemic depend strongly on traditional medicine as a source for inexpensive treatment of this disease. However, scientific data to validate the antimalarial properties of these herbal remedies are scarce (Ramalhete *et al*., 2008).

Over 1,277 plants belonging to 160 families were reported in 2004 to be used traditionally for the treatment of malaria following an extensive survey of the literature(Willcox and Bodeker, 2004). In traditional practice several plants are often used in combination. Crude extracts of

some of them have been screened in*in vitro* tests and others as *in vivo*for their antimalarial activity. Single active antimalarial constituents have been successfully characterized from some extracts following the pharmaceutical industry paradigm of drug discovery (Rasoanaivo *et al*., 2011).

An aqueous-ethanol leaf of *Verbena hastate* extract of this plant atdoses of 200 to 800mg/kg b.wt resulted in 64 to 72% inhibition of parasitaemia in the suppressive test, 60 to 74% parasitemia inhibition in repository test and a mean survival time of 21 to29 days representing 69 to 86% inhibition of parasitemia in the curative test *in vivo* have been reported (Akuodor *et al*., 2010).

Among ten plants found in Iranthat are used by traditional healers for the treatments of malaria, the ethanolic crude extracts of three of them (*Boerhavia elegans, Solanum surattense* and *Prosopis juliflora)* showed promising antimalarial activity against *Plasmodium falciparum:* both chloroquine resistant and sensitive strain) *in vitro* and 44.1 to 66.2% *Plasmodium berghei* (ANKA strain) parasitaemia inhibition *in vivo* with no toxicity effects (Ramazani *et al*., 2010)

*Acacia nilotica*of the family *Fabaceae*is a thorny tree commonly used in Northern Nigeria for the treatment of cough, diabetes and malaria. Aqueous root extract of *A. nilotica* was analyzed for antimalarial activity in mice. Suppressive test, curative and prophylactic effect was studied in chloroquine-sensitive *P. berghei berghei* NK-65 infected mice. The mice were administered with 100, 200 and 400mg/kg b.wt. All doses of the extract produced significant dose dependent chemo suppressive activity against the parasite in the suppressive, curative and prophylactic tests up to 79.5 % inhibition of parasitaemia (Alli *et al*., 2011).

A report by Bickii *et al*., (2007); among fifteen crude extracts from the stem bark and seeds of four medicinal plants served as antimalarial treatment by Cameroonians, *Entandrophragma angolense*, *Picralima nitida*, *Schumanniophyton magnificum* and *Thomandersia hensii* tested *in vitro* for their antimalarial activity against the chloroquine-resistant *P. falciparum* W2 strain and the results showed that the extracts of these plants possessed some antimalarial activityespecially the methanol extract of *Picralima nitida* demonstrating the highest activity of (10.9 ± 1.1 IC_{50} (µg/ml))*in vitro*(Bickii *et al*., 2007).

In Ethiopia, traditional medicine has started long ago to combat disease through curing of diseases and also with the protection and promotion of human physical, spiritual, social, mental and material wellbeing as well (Mesfin and Obsa, 1994). Over 85 % of the rural population and poor urban centers and animal husbandry employ many of the available plants as well as products from wild animals and minerals as their primary source of healthcare in the fight against various physical and mental health problems (Kassaye *et al*., 2006; Bekele, 2007). In Ethiopia, it was estimated that there are more than 7000 species of flowering plants of which 600 species of them are medicinal plants (Getahun, 1976).

In different parts of the country people are using traditional herbal medicine for addressing their primary health care need and to treat various ailments and physical damages. For instance: - *Calendula officinal* (for haemorroide), *Eucalyptus globules* (for skeletal musculo problem), *Taraxacum official, Lactuca spp., Marubium vulgaris,* and *Cynara scolymus* (for chronic cough, asthma and hepatitis) (Getahun, 1976). Giday *et al*., (2007) also reported that *Calpurnia aurea* (for amoebiasis, giardiasis, malaria, diarrhoea, rabies, diabetes, hypertension) and *Clerodendrum myricoides, Dodonea angustifolia* and *Aloe debrana* (Deressa *et al*.,2010),A*sparagus africanus* Lam (Dikasso *et al*., 2006). *Kniphofia foliosa* (Animut*et al*., 2005),*Cissampelos mucronata, Clerodendrum myricoides,Gnidia stenophylla,Vernonia bipontini,Euclea scimperi,Solanum incanum, Plumbago zylanica, Warburgia ugandensis and Kalanchoe petitiana* (Assefa *et al*., 2007) are some of plant species in Ethiopia used for the treatment of malaria traditionally.

Since malaria is the number one public health problem in Ethiopia and the majority of people are living in malaria risk rural areas coupled with the inaccessible and unaffordable antimalarial drugs; they strongly depends on traditional medicines particularly herbal remedies to treat the disease (Bekele, 2007; Kassaye *et al*., 2006). There are huge numbers of plant species used by the people to treat malaria in different parts of the country such as *Carica papaya* in asendabo district, Jimma zone (Parvez and Yadav, 2010). In the northwestern Ethiopia, *Aloe* sp, *Azadirachta indica, Calpurnia aurea, Carica papaya, Croton macrostachyus, and Zehneria scabra are* also used (Gidey *et al.,* 2007).

Otostegia integrifolia is among the plant species used as traditional herbal remedies in the Ethiopia folk medicine. For instance:- in Ethiopian plateau, the smoke of burning branches

andleaves of this plant is used as an insecticide, disinfectant and 10 days after giving birth mothers are smoked before being released from confinement (Wilson and Mariam, 1979). As Andemariam, (2010) reported *O. integrefolia* is also used for the treatment of tonsillitis, uvulitis and hypertension in Eritrea and the Northern parts of Ethiopia (Andemariam, 2010); whereas, in Southern Ethiopia, the root juice serves as antiascariasis (Paraz and Yadav, 2010). Another ethno botanical study by Gidey *et al*., (2007); among the Shinasha, Agaw-awi and Amhara people in the Northwestern Ethiopia, traditional healers use the leaves of the plant for malaria treatments.

Literature reviews on the photochemistry of *Otostegia integrifolia* has revealed that a total of 40 phytochemical constituents including monoterpenes, sesquiterpenes, diterpenes and their derivatives were identified as the major constituents isolated from the essential oil and chloroform extract of the air dried leaves (Tesso, 2004). These constituents have biological activity and are used for the treatment of human diseases especially those of terpines and terpine derived pharmaceuticals. Among the pharmaceuticals, the anticancer drug Taxol and the antimalarial drug Artimesinin are two of the most renowned terpine based drugs (Zhang, and Demain, 2005). However, the safety and effectiveness of these herbs has not been scientifically evaluated except for a very few.

Currently, *Plasmodium spps*are becoming resistant to the majority of the available antimalarial drugs especially *P. falciparum* (for all) (WHO, 2010_b) and the infection is one of the most serious causes of morbidity and mortality globally; particularly in sub-Saharan Africa (SSA) (Kabanywanyi *et al*., 2008; Roca-Feltrer *et al*., 2008). Therefore, there is a need to develop new and effective antimalarial medicine that is easily affordable by poor living in malarious tropical countries. And this day, there is a focus to traditional medicinal plants and other natural products having high antimalarial activities (Ginsburg and Deharo, 2011). Consequently, it is important that traditional medicinal plants should be investigated in order to establish their safety and efficacy as well to determine their potential as source of new plant based drugs (Bandaranayake, 2006; Soetan and Aiyelaagbe, 2009).

Hypothesis

- Crude extract of the leaf of *O. integrefolia* possess some antimalarial effect.

2 OBJECTIVES

2.1 General objective

- To evaluate antimalarial activity of *Otostegia integrefolia* leaf extracts in Swiss albino mice.

2.2 Specific objectives

- To assess acute toxicity (LD_{50}) and sub-acute toxicity of crude extracts of the plant material in mice.
- To determine percent parasite suppression due to the antimalarial effect of extracts of this plant against CQ sensitive strain of *P. berghei* in mice.
- To evaluate the effect of the extracts on packed cell volume, body weight and survivability of *Plasmodium berghei* infected mice.

3.3 Crude extracts preparation of the plant material

The fresh leaf of the plant was cleaned, cut into pieces and air dried under shade in Biomedical Laboratory, Faculty of Life Sciences, Addis Ababa University for a month. Then, the dried leaf was ground in to a coarse powder using an electrical cross bitter mill (IEC, 158VDE0660, Germany) and the powdered plant material was packed in plastic bag and kept until extraction done. The coarsely powdered plant material was weighed by sensitive digital weighing balance (AND: FX-320, Japan) and crude extracts were prepared by cold maceration technique; soaking the plant powder in 1**:**8 (w/v) of distilled water, methanol and chloroform in separate Erlenmeyer flasks and placed on a platform shaker of 120 rpm for 72 hours at room temperature. After 72 hours, the mixtures were first filtered using cotton and then passed through Whatman grade no 1 filter paper. For water extract; the filtrate was frozen in refrigerator overnight and dried in a lyophilizer (CHRIST, 3660Osterode/harz/, France) to get a freeze dried product. While, the methanol and chloroform extracts were concentrated in rotary evaporator (Buchi type TRE121, Switzerland) distillation flask at a temperature of 45°C and 60 rpm. Finally, the weights of the dry extract was measured to determine the percentage yield of each extracts and all the extracts were stored separately in screw caped glass bottle at -20ºC until used.

3.4 Experimental animals (Swiss albino mice)

Animals used in this study were 6 to 8 weeks old males and virgin females (for toxicity tests) and only male Swiss albino mice (for antimalarial tests) weighing 23 to 32g obtained from the Animal Facility Centre, Faculty of Life Sciences of Addis Ababa University and purchased from Ethiopian Health and Nutrition Research Institution (EHNRI). They were housed in plastic cages with saw dust as beddings and given food and water *ad libitum*.Each mouse was used only once.

3.5 *In vivo* acute and sub-acute toxicity tests

Toxicity test of *O. integrefolia* leaf crude extracts were carried out using Lorke's method (Lorke, 1983). Twenty mice were randomized into four groups of five mice per group and given 500, 1000 and 2000mg/kg b.wt orally by dissolving each dose with 0.4ml of vehicles (20% of

dimethyl sulfoxide (DMSO) and dH_2O) the fourth group (control) was given 0.4ml of the respective vehicle for each mouse. Administration of extracts was continued for the next three consecutive days in a 24 hour schedule. For acute toxicity studies; gross physical and behavioral changes such as hair erection, lacrimation, reduction in motor activity and feeding plus mortality within 24hours was observed. For sub-acute toxicity,bodyweight and packed cell volume (PCV) parameters were considered at D_0 and D_4 for each extract dose levels.

3.6 *In vivo* antimalarialactivity (suppressive) tests

3.6.1 *Plasmodium berghei*infection

CQ-sensitive strain of the rodent parasite*P. berghei* was kept alive by continuous intraperitoneal (IP) passage in mice on a weekly bases. Percent parasitaemia of the donor mouse was first determined (about 20 to 30% Para) and blood was collected by gentle cardiac puncher from the donor mouse using syringe after it has been scarified using chloroform. Then, 1ml of blood was diluted with 4ml of physiological saline (0.9%) and 1ml of the dilution contain 5×10^6 of infected erythrocytes. Therefore, each mouse was infected on day zero (D_0) intraperitoneally with 0.2ml dilution of infected blood (standard inoculum) containing approximately $1x10^6$ *P. berghei* parasitized red blood cells (Krettli *et al*., 2009).

3.6.2 Extract administration

Twenty five mice were divided into 5 groups of 5 mice per group randomly for each extract and three hours after parasite inoculation, the three groups were administered 200, 400 and 800 mg/kg b.wt extract by dissolving the extract in 0.2ml of the respective vehicle for each mouse orally by using gavage (an oral needle) for four consecutive days starting from day 0 in a 24hr schedule. Two control groups were used. The positive control was treated daily with 25mg/kg b.wt CQ while the negative control group was given 0.2ml of the respective vehicle.

3.6.3 Parasitological study

A standard 4-day suppressive test was employed against CQ sensitive *P. berghei* infection in mice. On the 5thday (D_4) of the experiment, fresh blood samples were collected from the tail snip of each mouse and thin smear on to a microscope slide to made a film (Krettli *et al*., 2009). The blood films first fixed with methanol for 30 seconds and stained with 10% Giemsa for 25 min and parasitaemia examined under light microscopy with an oil immersion objective of 100x magnification power (WHO, 1991). In each microscopic slide, 5 to 10 field of visions along the tail part of the film for each mouse were examined. Then, percent parasitaemia and suppression were calculated for each dose level of treatments by comparing with the parasitaemia in infected negative control according to the following formula as indicated by Samanta *et al*., (2011).

$$\%\, parasitaemia = \frac{Number\ of\ infected\ RBCs}{Number\ of\ infected\ RBCs + Number\ of\ uninfected\ RBCs}\ \mathbf{X\ 100}$$

$$\%\, suppression = \frac{Para\ in\ negative\ control - Para\ in\ treated\ group}{Para\ in\ negative\ control}\ \mathbf{X\ 100}$$

3.6.4 Determination of mean survival time

Mortality was checked daily and the number of days from the time of parasite inoculation up to death was recorded for each mouse in the treatment and control groups throughout the follow up period and the mean survival time (MST) for each group calculated as presented by Samanta *et al*., (2011).

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

3.6.5 Body weight and PCV determination

Body weight (b.wt)and Packed Cell Volume (PCV) values of each mouse was measured at day 0 (D_0) and day 4 (D_4) in those *P. berghei* infected, extract treated, and negative control groups to determine the effectiveness of the extract in preventing loss of body weight and reduction in PCV by the parasite. For weight loss determination, each mouse in a group was measured using sensitive digital weighing balance (AND: FX-320, Japan) and for PCV,¾ volume of heparinized microhaematocrit tube filled with fresh blood taken from the tail snip of each mouse in separate microhaematocrit tube, sealed with sealantand centrifuged a microhaematocrit centrifuge (HAWKSLEY1500, England) at 12,000rpm for 4 minute. Then, the average body weight and average PCV were compared with the control groups at a fixed time (D_4) and over time in each group. According to Bull *et al., (*2000), mean body weight and PCV value were calculated respectively as:-

$$\textbf{Mean body weight} = \frac{\textbf{total weight of mice in a group}}{\textbf{total number of mice in that group}}$$

$$\textbf{PCV} = \frac{\textbf{volume of erythrocyte in a given blood x100}}{\textbf{total blood volume}}$$

4 STATISTICAL ANALYSIS

Data were analyzed using window software; IBM SPSS, version 19. For sub-acute toxicity tests, the Student's independent t-test was conducted to compare the PCV and body weight variations within a group taking the same dose extracts at D_0 and D_4. For the antimalarial efficacy tests, statistical analysis was undertaken by one way analysis of variance (ANOVA) to compare the levels of parasitaemia, body weight, PCV values and survival times of the *P. berghei* infected mice between the control and extract treated groups at a fixed time. In addition, Student's paired t-test was also carried out to determine percent change of PCV and body weight of *P. berghei* infected mice between D_0 and D_4. All the results were presented as the Mean±SEM(Standard Error of the Mean) and statistical significance was considered at a 95% confidence interval ($P<0.05$).

5 RESULTS

5.1 Percent extract yield of the plant material

Percent yields of the leaves of *O. integrefolia* aqueous, methanol and chloroform crude extracts are indicated in table 1. The aqueous and the methanol extracts yielded 8.27% (6.2g) and 5.67% (3.4g) respectively. And the chloroform extract accounted for 9.2% (4.6g) yield in a 1**:**8 w/v ratios of the plant powder per solvent reagents. Not only the amount of yields; but also the physical nature of each extract wasdiffering. The water and chloroform extracts were moist and semisolid.Whereas, the methanol extract was dried powder.

Table 1: Percentage extract yield from the leaves of *O. integrefolia* crude aqueous, methanol and chloroform extracts

Solvents for extraction	Dry powder (g)	Volume of solvent (ml)	Ratio (w/v)	Yield in (g)	Yield in (%)
dH_2O	75	600	1:8	6.2	8.27
MeOH	60	480	1:8	3.4	5.67
$CHCL_3$	50	400	1:8	4.6	9.2

5.2 *In vivo* acute and sub-acute toxicity tests

Acute toxic effects of aqueous, methanol and chloroform crude extracts of the leaves of *O. integrefolia* were conducted through oral administration of a single dose of 500, 1000 and 2000mg/kg body weight (b.wt) of mice. No mortality of mice was recorded within 24 hours. In addition, gross physical and behavioral changes were demonstrated and physical signs of acute toxicity such as depression, decrease in feeding activities and hair erection were examined for the first 3 to 5 hours after being administered with 1000 and 2000mg/kg body weight extracts. Then after, they returned to their normal conditions and they have been physically active during

a follow up observation period. This signifies that the oral median lethal dose (LD_{50}) of the plant extracts were greater than 2000mg/kg body weight.

The three crude extracts did not cause deaths in mice the four days treatment with 500, 1000 and 2000mg/kg b.wt. In addition, body weight and PCV were also compared at a fixed time between groups and overtime within each group. The Student's independent t-test (Table 2) showed no statistically significant difference ($P>0.05$) in both parameters measured when compared within each group between pre (D_0) and post (D_4) treatment even though the means of both parameters in treated groups were relatively reduced on D_4 than from D_0. An increased mean of weight and

Table 2: Effect of aqueous crude extract from the leaves of *O. integrefolia* on the PCV and bodyweight of healthy Swiss albino mice (n=5).

Group	Dose (mg/kg)	Parameters	Treatments		P-value
			Pre (D_0)	Post (D_4)	
a	NC	PCV (%)	48.53±0.78	48.94±54	0.368
		Weight (g)	26.07±0.27	27.67±0.22	0.639
b	500	PCV (%)	51.40±0.80	50.50±1.46	0.124
		Weight (g)	27.27±0.26	26.87±0.46	0.435
c	1000	PCV (%)	51.42±1.26	49.96±1.33	0.906
		Weight (g)	29.73±0.90	30.80±0.32	0.098
d	2000	PCV (%)	51.15±0.70	51.39±1.06	0.538
		Weight (g)	31.23±0.55	29.60±0.57	0.906

Key: Values are presented as Mean±SEM (Standard Error Mean) D_0**:** Day zero

NC**:** Negative control PCV**:** Packed Cell Volume D_4**:** Day four

PCV were seen in 1000 and 2000mg/kg b.wt treated groups respectively and when the negative control was increased on D_4 than D_0. The overall analysis of variance among the treatment groups in comparison with the control group on D_4 was statistically insignificant ($P>0.05$) in both parameters undertaken. Similarly, analysis of variance of the weight and PCV values of groups treated with methanol extract of the leaves of *O. integrefolia* and the negative control groups on day four (Table 3) also showed no statistically significant difference ($P>0.05$). The Student's t-test results of the methanol extract of the plant showed no statistically significant difference between both parameters in all groups treated with different doses except the mean of PCV in group b: that significantly ($P<0.05$) decreased when compared with in that group over time. Generally means of the weights and PCV values of mice treated with methanol were relatively lower on D_4 than D_0.

Table 3: Effect of methanol crude extract from the leaves of *O. integrefolia* on the PCV and bodyweight of healthy Swiss albino mice (n=5).

Group	Dose (mg/kg)	Parameters	Treatments		P-value
			Pre (D_0)	Post (D_4)	
a	NC	PCV (%)	48.50±0.78	48.94±54	0.368
		Weight (g)	26.07±0.27	27.67±0.22	0.639
b	500	PCV (%)	50.93±0.80*	50.55±1.64*	0.043*
		Weight (g)	25.80±0.42	23.96±0.69	0.278
c	1000	PCV (%)	50.98±0.77	50.73±0.78	0.972
		Weight (g)	29.07±0.70	29.03±0.54	0.758
d	2000	PCV (%)	50.96±1.01	48.75±1.10	0.627
		Weight (g)	30.57±0.27	28.60±1.31	0.134

Key: *: $P<0.05$

As presented in (Table 4)chloroform extract was safe to mice up to a dose of 2000mg/kg b.wt. The Student's independent t-test comparison results of weight and PCV values of mice treated with 500, 1000 and 2000mg/kg b.wt were not statistically significant (P>0.05). However, the mean values of both parameters were relatively lower on D_0 than D_4 except a similar mean of weight seen at D_0 and D_4 in 500 mg/kg b.wt treated group. Analysis of variance on these parameters on day four of the extract treatment showed no significant differences.

Table 4: Effect of chloroform crude extract from the leaves of *O. integrefolia* on the PCV and bodyweight of healthy Swiss albino mice (n=5).

Group	Dose (mg/kg)	Parameters	Treatments		P-value
			Pre (D_0)	Post (D_4)	
a	NC	PCV (%)	48.50±0.78	48.94±0.54	0.368
		Weight (g)	26.07±0.27	27.67±0.22	0.639
b	500	PCV (%)	50.90±0.92	49.38±0.78	0.932
		Weight (g)	26.30±0.70	26.30±0.72	0.801
c	1000	PCV (%)	50.43±0.80	50.10±0.70	0.405
		Weight (g)	33.80±1.08	33.63±1.24	0.763
d	2000	PCV (%)	50.45±0.97	50.03±0.60	0.114
		Weight (g)	32.40±0.55	32.36±0.60	0.770

5.3 *In vivo* antimalarial activity (suppressive) tests

Antimalarial suppressive test results of aqueous, methanol and chloroform crude extracts of the leaves of *O. integrefolia* against *P. berghei* in Swiss albino mice are summarized in Tables 5, 6 and 7 respectively.All the extracts resulted in reduction of parasitaemia level significantly(P<0.05) in reference to their respective negative control. They caused varying

degrees of suppressive effects in a dose dependent manner. However, it did not clear the parasite completely on day four. Whereas, the positive control group (treated with 25mg/kg CQ)had no detectable parasitaemia on D_4 of post infection.

The crude aqueous extract induced a statistically significant ($P<0.05$)suppression of parasitaemia between 38.86 to 44.45% at oral doses of 200 and 800mg/kg b.wt respectively when compared with the respective negative control (treated with dH_2O) on day four of post infection (Table 5). In addition, these groups were observed with significantly lower percent parasitaemia levels of 27.91±0.96, 27.17±2.30 and 25.36±1.28% at the oral dose of 200, 400 and 800mg/kg extract respectively. While, the corresponding negative control parasitaemia level was 45.65±2.31%. The highest suppression of parasitaemia was observed at the dose of 800mg/kg body weight of mice and percent parasitaemia level was inversely proportional with concentration gradient of an extract.

Table 5:*In vivo* antimalarial suppressive test of aqueous extract of the leaves of *O. integrefolia* against *P. berghei* in Swiss albino mice (n=10).

Treatments	Dose (mg/kg)	%Parasitaemia	%Suppression
dH_2O	___	45.65±2.31	0.00
O. integrefolia (Aqueous extract)	200	27.91±0.96	38.86
	400	27.17±2.30	40.48
	800	25.36±1.28	44.45
CQ	25	0.00± 0.00	100.00

Key: Values are presented as Mean±SEM (Standard Error Mean)

CQ**:** Chloroquine diphosphate dH_2O**:** Distilled water

Statistically significant reduction of parasitaemia ($P<0.05$) was also observed in all groups of mice treated with methanol extract of the leaf of *O. integrefolia* when compared to the

corresponding negative control. Percent parasitaemia of the mice treated with 200mg/kg of the extract was 24.22±1.08%; 400mg/kg of the extract was 18.41±1.01%, the mice that received 800mg/kg was 13.57±0.70% (Table 6) and the level of the parasitaemia of the control mice (treated with 20% DMSO) was 31.39±1.94% on the fifth day (D_4) of post infection. The extract produced a dose dependent suppression ranged from 22.84 to 56.77% with the highest parasitaemia suppression observed in 800mg/kg b.wt extract treated group; showing concentration dependent suppression.

Table 6:*In vivo*antimalarial suppressive test of methanol extract of the leaves of *O. integrefolia* against *P. berghei* in Swiss albino mice (n=10).

Treatments	Dose (mg/kg)	%Parasitaemia	%Suppression
20% DMSO	___	31.39±1.94	0.00
O. integrefolia (MeOH extract)	200	24.22±1.08	22.84
	400	18.41±1.01	41.35
	800	13.57±0.70	56.77
CQ	25	0.00± 0.00	100.00

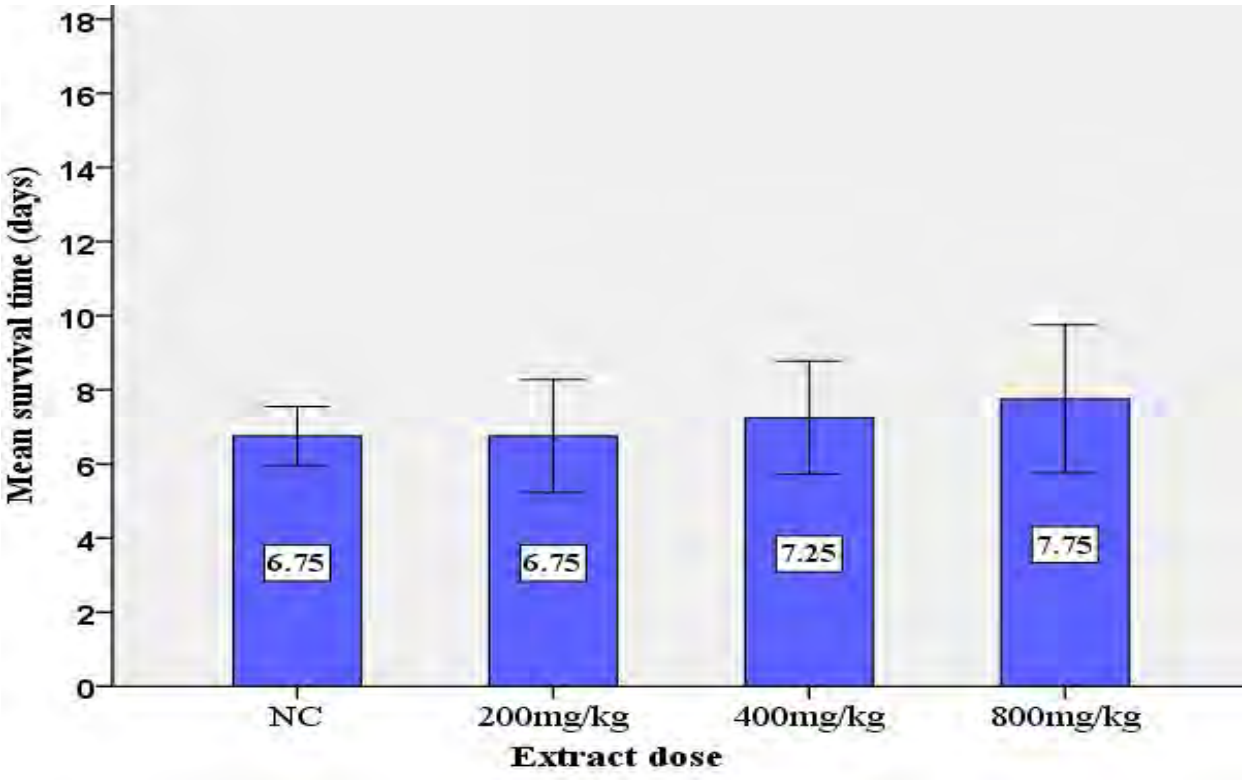
200, 400 and 800mg/kg b.wt (Table 7) of chloroform extracts of the plant materialalso caused a statistically significant ($P<0.05$) in a dose dependent parasitaemia suppressive effects in *P. berghei* infected mice when in comparison with the particular negative control (treated with 20% DMSO). Similarly, in all the doses of the extract administered; the experimental mice have by far lower parasitaemia than that of the corresponding negative control. In this extract, percent parasitaemia of the *P. berghei* infected mice were 21.09±0.87, 18.72±0.97 and 15.46±0.70% which resulted in suppression of 17.00, 26.33 and 39.16% at doses of 200, 400 and 800mg/kg b.w respectively when the respective 20% DMSO treated group was 25.41±0.86% parasitaemia plus a 100% parasite clearance observed in CQ phosphate administered group on the fifth day of post infection.

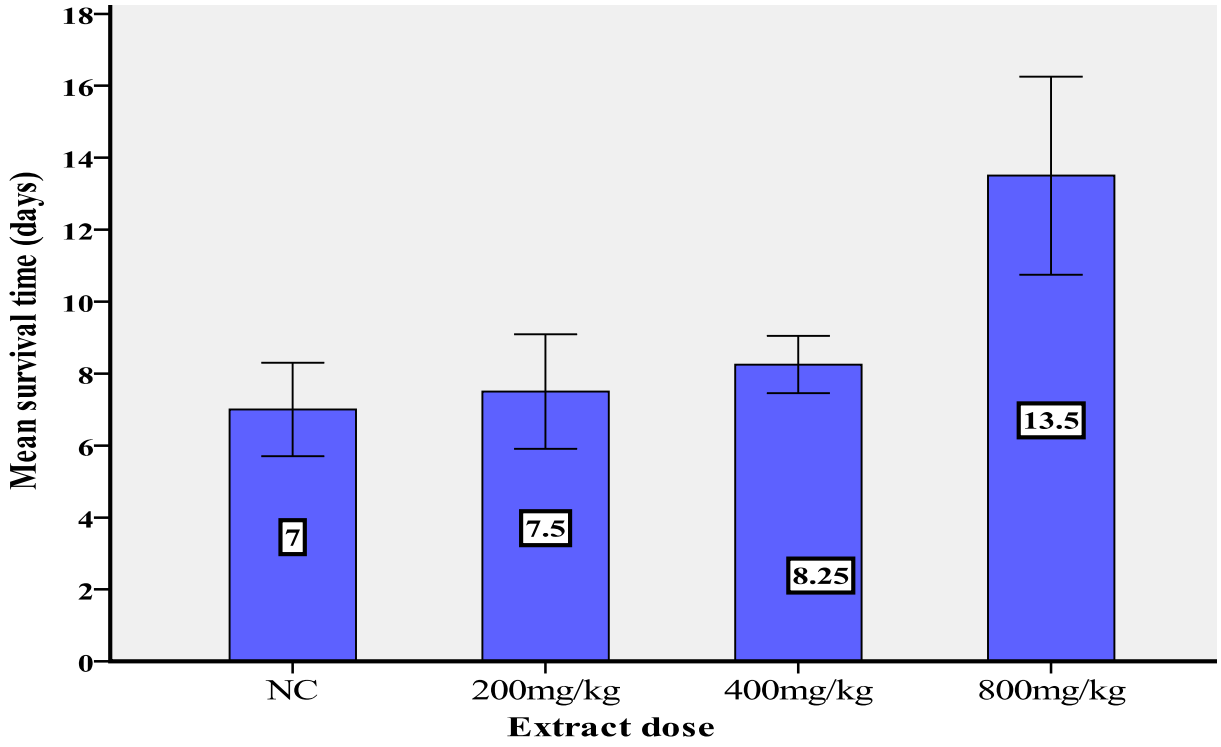
Table 7: *In vivo* antiplasmodial suppressive test of chloroform extract of the leaves of *O. integrefolia* against *P. berghei* in Swiss albino mice (n=10).

Treatments	Dose (mg/kg)	%Parasitaemia	%Suppression
20% DMSO	___	25.41±0.86	0.00
O. integrefolia ($CHCL_3$ extract)	200	21.09±0.87	17.00
	400	18.72±0.97	26.33
	800	15.46±0.70	39.16
CQ	25	0.00± 0.00	100.00

5.4 Determination of mean survival time

Majority of the treated groups lived longer than the corresponding negative control and in all cases; the highest survival time was recorded in mice that received 800mg/kg b.wt extracts. The mice treated with aqueous extract of the plant material had the mean survival time of 6.75±0.48, 7.25±0.48 and 7.75±0.63 at doses of 200, 400 and 800mg/kg b.wt treated groups, respectively, when the negative control mice lived 6.75±0.25 days (Figure 3). However, extract treated groups lived longer than negative control, it did not produce statistically significant ($P>0.05$) extension survival. The 200mg/kg b.w treated group lived equally with the corresponding negative control (6.75days).


Mean survival time (days)
18
16
14
12
10
8
6
4
2
0
6.75
6.75
7.25
7.75
NC
200mg/kg
400mg/kg
800mg/kg
Extract dose


Mean survival time (days)
18
16
14
12
10
8
6
4
2
0
7
7.5
8.25
13.5
NC
200mg/kg
400mg/kg
800mg/kg
Extract dose

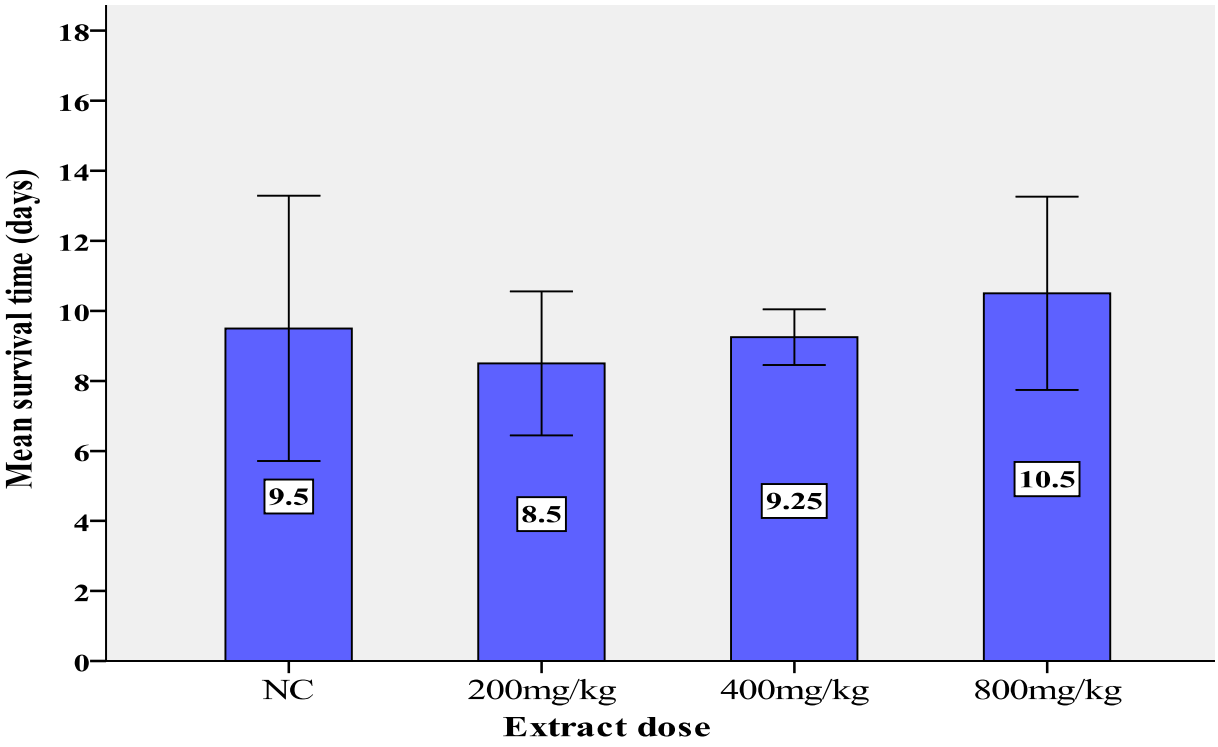

Mean survival time (days)
18
16
14
12
10
8
6
4
2
0
9.5
8.5
9.25
10.5
NC
200mg/kg
400mg/kg
800mg/kg
Extract dose

Table 8: Effect of aqueous, methanol and chloroform crude leaf extracts of *O. integrefolia* on the body weights of *P. berghei* infected Swiss albino mice (n=5).

Treatments	Dose (mg/kg)	Body weight		% Change
		Pre (D_0)	Post (D_4)	
Aqueous extract	0.2ml of dH2O	23.30±0.75	22.62±0.47	-3.18
	200	27.46±0.71	24.82±1.06	-2.82
	400	27.50±0.48	27.20±0.32	-0.30
	800	29.98±1.09	28.40±1.41	-3.13
MeOH extract	20% DMSO	29.28±0.79	27.98±0.90	0.58*
	200	27.40±0.95	26.00±0.79	0.48*
	400	29.18±0.55	27.68±0.60	0.12*
	800	30.10±1.20	28.98±1.13	0.28*
$CHCL_3$ extract	20% DMSO	26.08±0.59	23.70±0.49	-0.44
	200	29.65±0.81	28.50±0.89	-3.82
	400	32.32±1.95	28.45±1.71	-5.44
	800	31.80±1.11	28.45±0.37	-1.32

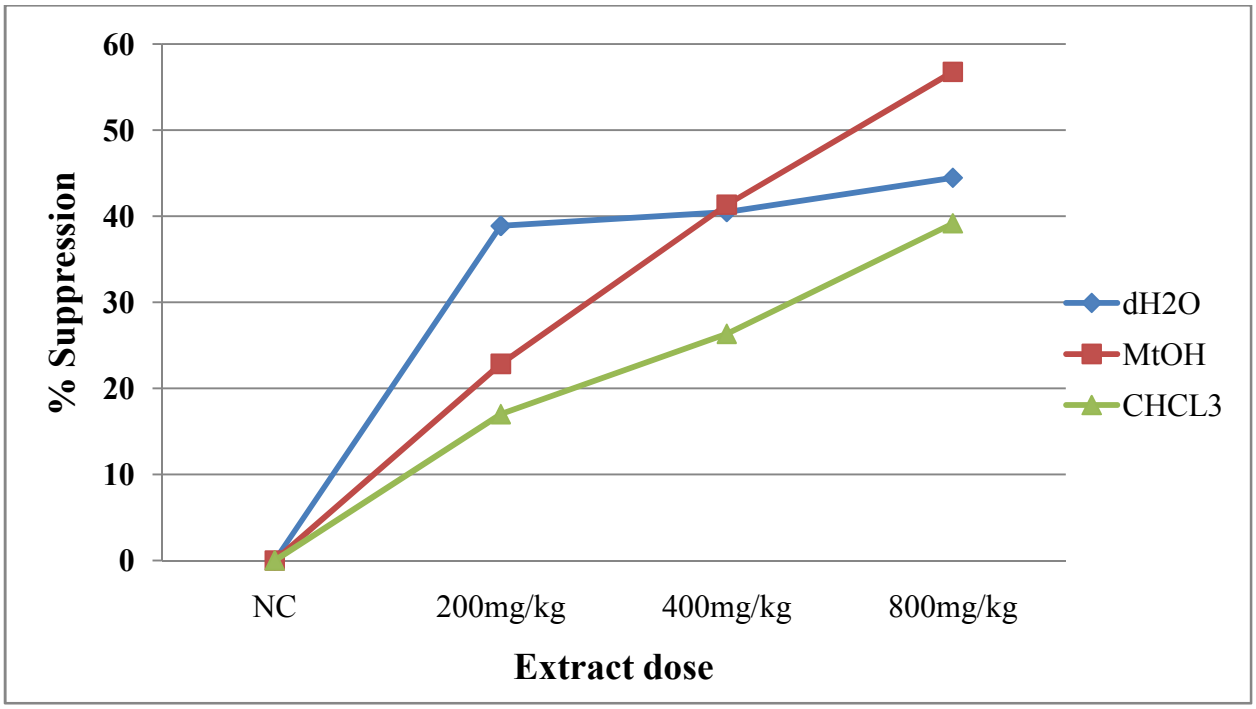
In the chloroform extract treated mice, loss of body weight was also observed with the highest loss measured in those which received 400mg/kg b.wt extract (Table 8).However, the difference was not statistically significant.

The crude extract of *O. integrefolia* did not prevent PCV reduction. 800mg/kg chloroform extract administered experimental *P. berghei* infected mice showed statistically insignificant increment of PCV values on D_4 than D_0. The Student's paired t-tests at all doses of the aqueous, methanol and chloroform extract treated mice have also showed statistically insignificant deviationsalthough reduction in level of PCV was seen on D_4 than from D_0 except 0.2ml of dH2O administered aqueous extract respective negative control group. In addition,analyses of variance conducted between the extract treated groups in comparison with the respective control have showedinsignificant deviation(Table 9).

Table 9: Effect of aqueous, methanol and chloroform extracts of *O. integrefolia*leaves on thepacked cell volume of *P. berghei* infected Swiss albino mice (n=5).

Treatments	Dose (mg/kg)	PCV		% Change
		Pre (D0)	Post (D4)	
Aqueous extract	0.2ml of dH2O	50.80±0.58	47.26±0.72	2.14*
	200	50.18±0.79	49.54±0.74	-1.89
	400	49.22±0.57	50.66±0.39	-2.78*
	800	51.00±0.45	51.28±0.40	1.39
MeOH extract	20% DMSO	49.68±1.51	49.96±0.98	5.07
	200	51.04±0.73	48.00±1.08	-1.68
	400	50.46±0.62	48.68±1.78	-3.02
	800	50.66±0.40	49.24±0.97	-1.98
$CHCL_3$ extract	20% DMSO	51.34±0.66	49.40±0.79	-1.08
	200	51.06±0.33	49.82±0.73	-1.01
	400	50.80±1.03	50.80±1.03	3.00
	800	50.40±0.53	52.50±1.16	2.00

In general, all extracts of the plant material produced dose dependent and statistically significant ($P<0.05$) parasitaemia suppression at all doses administered with the highest suppressive effect recorded at the dose of 800mg/kg b.wt of the methanol extract treated group and itrevealed 56.77% parasite suppression.While, aqueous and chloroform extracts with identical doses showed 44.45 and 39.16% parasite suppression respectively (Figure 6).


60
50
40
30
20
10
0
% Suppression
NC
200mg/kg
400mg/kg
800mg/kg
Extract dose
dH2O
MtOH
CHCL3

6 DISCUSSION

Even though medicinal plants are assumed to be safe, many of them are potentially toxic (Ajaiyeoba *et al.,* 2006). Therefore, evaluating the safety level of the herbal medicine is necessary for the determination of the safe dose that can be used for treatment (Verma and Singh, 2008). For this reason, the oral acute toxicity test in mice, such as mortality, gross physical and behavioral changes were observed in accordance with Center for Drug Evaluation and Research (CDER) guideline for the testing of chemicals in rodents. The absence of serious acute toxic symptoms such as mortality, impaired movement, listlessness, reduced motor activity, etc., within 24 hours and survival of mice after oral administration up to a dose of 2000 mg/kg body weight of the extract for a week indicates that the estimated oral median lethal dose (LD_{50}) is not toxic and the extract at 4000 mg/kg body weight is non-lethal (CDER, 1996). This suggests that acute oral administration of the extract is safe and also explains the reason why the plant is widely used in traditional treatment of malaria in Ethiopian folk medicine (Gidey *et al*., 2007).

Absence of sub-acute toxicity was also confirmed by administration of these extracts for four consecutive days in a 24 hour schedule up to a dose of 2000 mg/kg body weight of mouse. During the follow up period, no mortality was recorded at all doses that received 500, 1000 and 2000 mg/kg/day body weight of experimental mice. In addition, effect of the plant extracts on the body weight and PCV value of extract treated mice were carried out before and after the treatment. The result showed absence of statistically significant changes ($P>0.05$) when both parameters were compared over time (at D_0 and D_4) within each group and at a fixed time (at D_4) in comparison with the negative control (given the respective vehicles). The present study was in agreement with Flecknell (1987) in which both parameters were within the normal range of values set for mice with body weight and PCV between 25 to 40g and 32 to 54% respectively. Therefore, the present findings confirmed that all extract of the study plant was not toxic to the test mice at all doses less than or equal to 2000mg/kg body weight.

In the present study, all the three crude extracts confirmed statistically significant ($P<0.05$) parasitaemia suppression when compared with their respective negative controls. This antimalarial activity of *O. integrefolia* leaf extracts might be implicated with the presence of

phytochemical constituents such as terpinoids (monoterpenes, sesquiterpenes and diterpenes) which are again attributed in antiplasmodial activities of many other plants including the potent source of artimesinin, *Artemisia annua* (wormwood) (Tesso, 2004; Zhang and Demain, 2005). In addition, it also is probably due to the presence of other phytochemical ingredients such as flavonoids and steroids which are identified as major phytochemical constituent in the genus *Otostegia* (Ayatollahi *et al*., 2007).

The antimalarial action of the present study plant could be the result of a single or synergetic effect of these phytochemical constituents although the mechanisms of action of these extracts have not been explained. Whereas, some other plants extracts exert the antimalarial activity by causing through interfering with the parasites ability to eliminate the toxic byproduct of hemoglobin digestion (Red blood cell oxidation), directly killing young intraerythrocytic malaria parasites, inhibiting protein synthesis of the parasite or by other unknown mode of action (White, 2008; Bassy *et al*., 2009; Hobbs and Duffy, 2011).

All the aqueous, methanol and chloroform crude extracts of the study plant material showed different degree of parasitaemia suppression in dose dependent manner in which the highest inhibition was recorded in the methanol extract treated mice at a dose of 800 mg/kg of body weight. The present study was in agreement with the findings of Nateghpour *et al*., (2008) in which the ethanolic extract from *Otostegia persica*significantly (46.4%) suppressed *P. berghie* parasitaemia *in vivo* at a dose of 450 mg/kg body weight of mice. In addition, the combined effect of CQ and ethanol extract of *O. perisca* against CQ sensitive and CQ resistant strains of *P. berghei* showed a statistically significant ($P<0.05$) parasite inhibition against both strains of the parasite *in vivo* (Nateghpour *et al*., (2010); which could probably inhibits CQ resistant strains of human *Plasmodium* too.

Moreover, the antibacterial activity of the species within the genus *Otostegia* such as *O. perisca*and *O. lambata in vitro* confirmed (Asghari *et al*., 2006; Anwar *et al*., 2009)support the present study in which the genus is rich in active antibiotic phytochemical ingredients that could probably suppress parasitaemia of the malaria parasites.

According to the review by Krettli *et al.,* (2009), an antimalarial compound is considered as an active compound when it reduces percent parasitaemia by ≥ 30% that supports the result of parasite suppression of the present study. Extracts of this study plant material suppress parasitaemia of *P. berghei* in mice significantly even though it did not clear the parasite totally. Similar studies on other species of plants such as *Acacia nilotica* (Alli *et al*., 2011), Mo*rinda lucida* (Unekwuojo *et al*., 2011), *Clerodendrum myricoides, Dodonea angustifolia* and *Aloe debrana* (Deressa *et al*.,2010) and A*sparagus africanus* (Dikasso *et al*., 2006) reported significant parasitaemia suppression. The present study therefore, is yet an evidence showing the relevance of traditional herbal medicine of Ethiopia are promising for the developments of new and easily affordable plant based antimalarial drugs.

The present study was carried out to determine the antimalarial potency of the different extracts of the leafof *O. integrefolia*.The results obtained from a standard 4-day suppressive test against chloroquine-sensitive strain of *P. berghei* in Swiss albino mice showedthat the crude aqueous, methanol and chloroform extracts of the plant materialpossess significant suppressive effect which is an indication that the plant extracts are possibly effective against human malaria parasites too.

Furthermore, the mean survival time of mice treated with all the extracts of the plant material was longer compared to the respective negative controls, confirming that the crude extracts of the study plant suppressed *P. berghei* and probably reduce the overall pathogenic effect of the parasite on the study mice. Therefore, the plant material used to suppresses the *Plasmodium* parasites until the patient get modern medical treatments. However, in those mice treated with 200 and 400mg/kg body weight of chloroform extract treated groups lived shorter. This could possibly be due to the depressing effects of the chloroform extract on appetite and food intake. Even though, extract treated mice lived longer time than the ones fed with vehicle with the highest survival time at a dose of 800mg/kg body weight in all cases; it was not significantly longer than the negative controls.

Body weight loss is one feature of rodent malaria infections (Perlmann and Troye-Blomberg, 2007). The crude extracts in this study did not prevent body weight loss of *P. berghei* infected

mice. The result of the present study showed in all group received the extract and those of untreated mice, loss of bodyweight was recorded when compared within each group at D_4 than from D_0. However, there was insignificant body weight loss when extract treated groups were compared with those of the untreated ones. The loss of body weight in those extract treated mice might possibly be due to the depressing effect of the crude extract of the plant material on appetite with the highest lost recorded in 400 mg/kg body weight methanol extract treated group. In a study by Mengiste*et al.* (2012), mice treated with crude extracts of *Dodonaea angustifolia* and *Bersama abyssinica* showed a lower body weight pattern as compared with the non-treated ones which is in agreement with the present study. However, the result of the this study on body weight loss of *P. berghei* infected mice was not in agreement with the findings of Dikasso *et al.,*(2006) and Debebe, (2009) where extracts of *Asparagus africanus* prevented body weight loss of *P. berghei* infected mice.

The PCV values of *P. berghei* infected mice that were treated with all the extracts of the study plant reduced on the fifth day of post-infection.However,the reduction was not statistically significant. Whereas, statistically significant ($P<0.05$) differences were recorded in 400 and 800mg/kg body weight of methanol and 200mg/kg body weight chloroform extract treated mice over time within the respective group. The present study is in agreement with the work of Mengiste*et al.* (2012), in which the crude extracts fromother species of plant such as *Dodonaea angustifolia* and *Bersama abyssinica* did not prevent reduction PCV values. On the other hand the finding of Debebe, (2009), extracts from *Asparagus africanus*prevented the reduction significantly ($P<0.05$).

7 CONCLUSION AND RECOMMENDATIONS

7.1 Conclusion

The spread of resistant malaria parasites to the available antimalarial drugs call for new chemotherapeutic agents to control the disease. The present study in the search for new antimalarial plants and it was found that *O. integrefolia* extracts have antimalarial activity *in vivo*against *P. berghei* in Swiss albino mice.Both the safety and efficacy test of the crude aqueous, methanol and chloroform extracts of the plant material suggested that the plant is not toxic and did cause neither acute nor sub-acute toxic symptoms up to the highest dose (2000mg/kg b.wt) administered to the experimental mice; likewise, the antimalarial test results of this study showed that the crude extracts of the plant material possessed potent antimalarial activities. The methanol and the water extracts showed the highest antimalarial effects, thus justifying the traditional usage of this plant as malaria remedy and the use of water and ethanol (local alcohols) as common solvents in traditional medicine. The plant extract produced statistically significant ($P<0.05$) parasitaemia suppression in dose dependent manner during a standard 4-days suppressive test and prolonged the survival time of *P. berghei* infected mice.

7.2 Recommendations

Based on the present findings, the following recommendations are made.

- *In vitro* investigation of the plant need to be undertaken against resistant strain of *P. falciparum* to determine as a potential source of antimalarial agents against resistant strain of the human malaria parasites.
- Further pharmacological screening with bioassay guided chemical fractionations of the methanol and water crude extracts of the study plantwould permit the isolation and identification of antimalarial active compounds.
- Chronic toxicity tests of the crude extracts and isolated fractions need to be undertaken in *in vivo* and cytotoxicity tests in *in vitro* experiments.

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Appendix

Photographs showing the different laboratory activities


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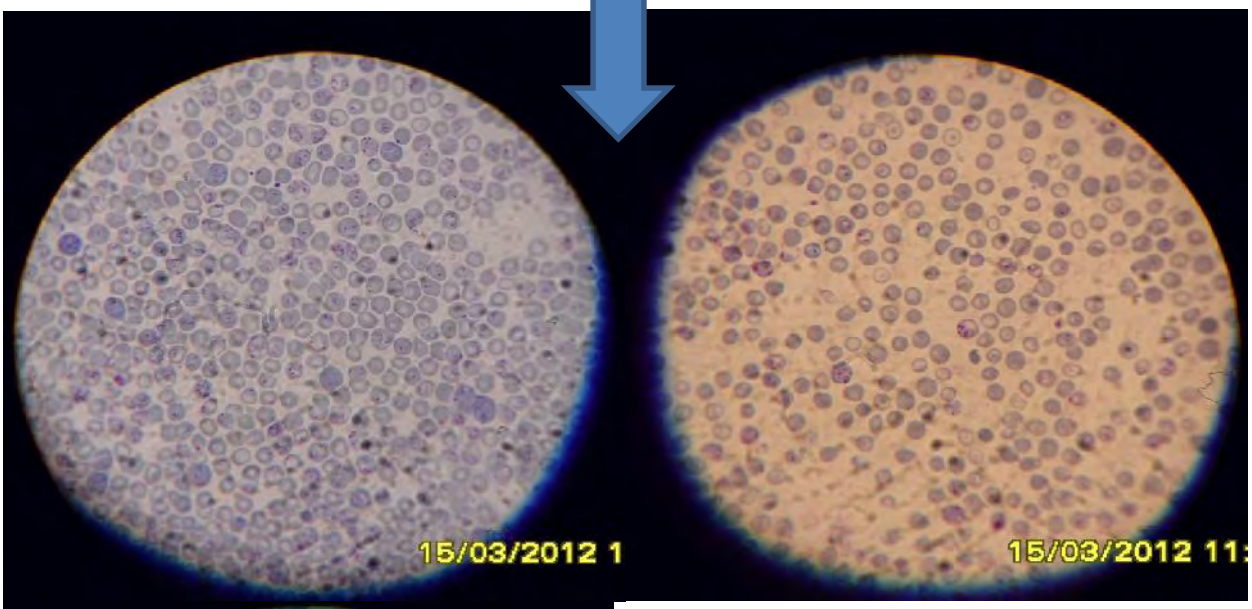
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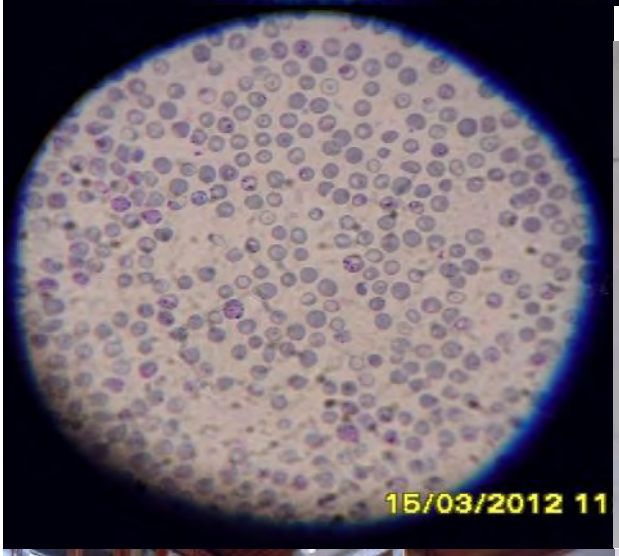
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DECLARATION

I, the undersigned, declare that this thesis is my own original work. It has not been presented in other university, colleges or institutions seeking for similar degree or other purposes. All source of materials used for the thesis has been duly acknowledged.

Name: Solomon Yeshanew Alamirew

Signature: _____

Date: _____

Advisor: Yalemtsehay Mekonnen (Prof.)

Signature: _____

Date: _____