ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

STUDIES ON ANTIMALARIAL ACTIVITY OF THE LEAF CONSTITUENT OF *OTOSTEGIA INTEGRIFOLIA* BENTH. (LAMIACEA) AGAINST *PLASMODIUM BERGHEI* IN MICE

BY

ABYOT ENDALE

August, 2012

Addis Ababa, Ethiopia

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A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfilment of the requirements for the degree of Master of Science in Pharmacognosy

Under the supervision of Kaleab Asres (Ph.D.) and Daniel Bisrat (Ph.D.), Department of Pharmaceutical Chemistry and Pharmacognosy

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LIST OF ACRONYMS

AIDS: Acquired Immuno Deficiency Syndrome

ACT: Artemisinin combined treatment

ANOVA: One Way Analysis of Variance

DEPT: Distortional Enhancement Polarization Transfer

EHNRI: Ethiopia Health and Nutritional Research Institute

FOMH: Federal Ministry of Health-Ethiopia

HIV: Human Immunodeficiency Virus

ILAR: Institute for Laboratory Animal Research

MOP: Malaria Operational Plan Ethiopia

OECD: Organisation for Economic Co-operation and Development

PCV: Packed Cell Volume

SEM: Standard error of the mean

SPSS: Statistical package for social science

WHO: World Health Organization

^{1}H NMR: Proton Nuclear Magnetic resonance

^{13}C NMR: Carbon thirteen Nuclear Magnetic Resonance

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ABSTRACT

Medicinal plants play an important role in the treatment of malaria especially in developing countries where resources are limited. A continued search for other effective, safe and cheap plant-based antimalarial agents thus becomes imperative in the face of these difficulties. The leaves of *Otostegia integrifolia* Benth. are used traditionally as malarial remedy in Northern Ethiopia. This study was aimed to investigate the antimalarial potentials of the crude leaf extract, solvent fractions as well as isolated compound(s) *in vivo* in *Plasmodium berghei* infected mice to give scientific proof to the ethnobotanical claims.

Leaves of *O. integrifolia* were collected and dried under the shade. The dried plant material was pulverized and extracted with 80% methanol. The dried crude extract was then further fractionated with different solvents. Both the crude extract and fractions were evaluated for their antimalarial activity *in vivo*, in 4-day suppressive assays against *P. berghei* in mice. Column chromatography was used to isolate compound/s from the most active fraction. The isolated compound was evaluated for its antimalarial activity *in vivo,* in 4-day suppressive assay against *P. berghei* and its structure elucidated using spectroscopic methods (MS and NMR). Preliminary phytochemical screening was performed using standard procedure. Acute and sub-acute toxicity studies of the crude extract were carried out in Swiss albino mice prior to antimalarial activity tests. No sign of toxicity was observed up on administration of the 80% methanol extract of the plant up to the highest dose (5000 mg/kg) given. Preliminary phytochemical screening showed the presence of saponins, flavonoids and other phenolic compounds in the hydroalcoholic leaf extract. The hydroalcoholic leaf extract (200, 400 and 600 mg/kg/day) and solvent fractions (200, 400 and 600 mg/kg/day) exhibited significant ($P<0.01$) antimalarial activity compared to the negative control group in four day suppression test with a considerable mean survival time. The highest parasite suppression (80.52%) was observed at 600 mg/kg /day for the crude extract, while the ethyl acetate fraction was found to be the most active among the solvent fractions and showed highest suppression (60.15%) at 600 mg/kg/day. The isolated compound also showed significant antimalarial ($P<0.001$) activity at the tested doses (25, 50 and 100 mg/kg/day) with chemosuppression of 50.13, 65.58, and 73.16%, respectively. Structural elucidation revealed that the isolated compound is a labdane type diterpenoid, 15,16-epoxy-3α,9α-dihydroxy-labda-13(16),14-diene. The crude extract, solvent fractions and the isolated compound possess considerable antiplasmodial activities which justify the use in ethnomedicine and can be exploited in the control of malaria.

1. INTRODUCTION

1.1. Epidemiology of malaria

Malaria is a febrile hemolytic disease caused by protozoa of the genus *Plasmodium* and transmitted by female Anopheles mosquitoes. Human malaria is caused by four species of the genus *Plasmodium* namely: *P. falciparum, P malariae, P. ovalae* and *P.vivax.* The four species differ in microscopic appearance, clinical features, geographical distribution and the potential for development of resistance to antimalarial drugs. *P. falciparum* is the most dominant and pathogenic of the four human malaria parasites and is responsible for almost all malaria mortality in tropical and subtropical countries (Kaur *et al*., 2009).

Malaria is endemic in most of South-East Asia, Latin America, and sub-Saharan Africa and *P. falciparum* and *P. malariae* are found worldwide. *P. vivax* is responsible for over 50% of malaria outside Africa, notably in South-East Asia and Central and South America, and has a particularly strong impact on the Indian sub-continent (Ashley *et al*., 2006; Scholar, 2007). Malaria remains one of the most important diseases of developing world, killing 1-3 million people and causing disease in 300-500 million people annually worldwide (Kalra *et al*., 2006). In 2009, there were an estimated 225 million cases of malaria worldwide. The vast majority of cases (78%) were in the African Region, followed by the South-East Asia (15%) and Eastern Mediterranean Regions (5%) (WHO, 2010). There were 781,000 estimated malaria death globally in 2009. It is estimated that 91% of deaths were in the African Region, followed by the South-East Asia (6%) and Eastern Mediterranean Regions (2%). About 85% of deaths globally were children under 5 years of age (WHO, 2010). A total of 109 countries were endemic for malaria in 2008, 45 within the WHO African Region (WHO, 2008). It is the second leading cause of death from infectious diseases next to HIV/AIDS in Africa (WHO, 2009).

Malaria is endemic in Ethiopia with differing intensity of transmission, except in the central highlands which are malaria-free. The most recent epidemic occurred in 2003–2004 and approximately half of the cases were caused by *P. falciparum.* According to WHO report in 2010 there were 1.75 million malaria cases in the country in the year 2009 (WHO, 2010). The economic impact of malaria is very significant, as the country's economy is based on agriculture and peak malaria transmission coincides with the planting and harvesting season. Historically, malaria has forced people to inhabit the less agriculturally productive highlands. About 75% of the country is malarious, with about 68% of the country's total

population living in areas at risk of malaria. Approximately 70,000 people die of malaria each year (MOP, 2010).

1.2. *Plasmodium* life-cycle

The *Plasmodium* parasite exhibits a complex life cycle involving an insect vector (mosquito) and a vertebrate host (human) as shown in Figure 1. All four species exhibit a similar life cycle with only minor variations (Scholar, 2007). The infection is initiated when sporozoites are injected with the saliva of a feeding mosquito. Sporozoites are carried by the circulatory system to the liver and invade hepatocytes. The intracellular parasite undergoes an asexual replication known as exoerythrocytic schizogony within the hepatocytes. Exoerythrocytic schizogony culminates in the production of merozoites which are released into the bloodstream. A proportion of the liver-stage parasites from *P. vivax* and *P. ovale* go through a dormant period instead of immediately undergoing asexual replication. These hypnozoites will reactivate several weeks to months (or years) after the primary infection, and are responsible for relapses (Wiser, 2010). Merozoites invade erythrocytes and undergo a trophic period in which the parasite enlarges. The blood stage is responsible for the pathology associated with malaria. The intermittent fever paroxyms are due to the synchronous lysis of the infected erythrocytes. *P. malariae* exhibits a 72 hour periodicity, whereas the other three species exhibit 48 hour cycles. However, *P. falciparum* often exhibits a continuous fever rather than the periodic paroxysms. *P. falciparum* also is responsible for more morbidity and mortality than the other species. This increase virulence is due in part to the higher levels of parasitaemia associated with *P. falciparum* infections. In addition, more complications are associated with *P. falciparum* because of the sequestration of the trophozoites and schizont-infected erythrocytes in the deep tissues (Wiser, 2010).

Clinical manifestations occur at the erythrocytic stage and can include fever, chills, prostration and anaemia, as well as delirium, metabolic acidosis, cerebral malaria and multi-organ system failure, which may be followed by coma and death (Batista *et al*., 2009).

1.3. Treatment, prevention and control of malaria

The drug of choice for the treatment of malaria depends on various factors, including the severity of the infection, the patients' age, the degree of background immunity, parasite sensitivity, and the cost and availability of the drugs. Therapeutic goals include prophylaxis, treatment of the acute attack (clinical cure), and radical cure (Scholar, 2007; Wiser, 2010).

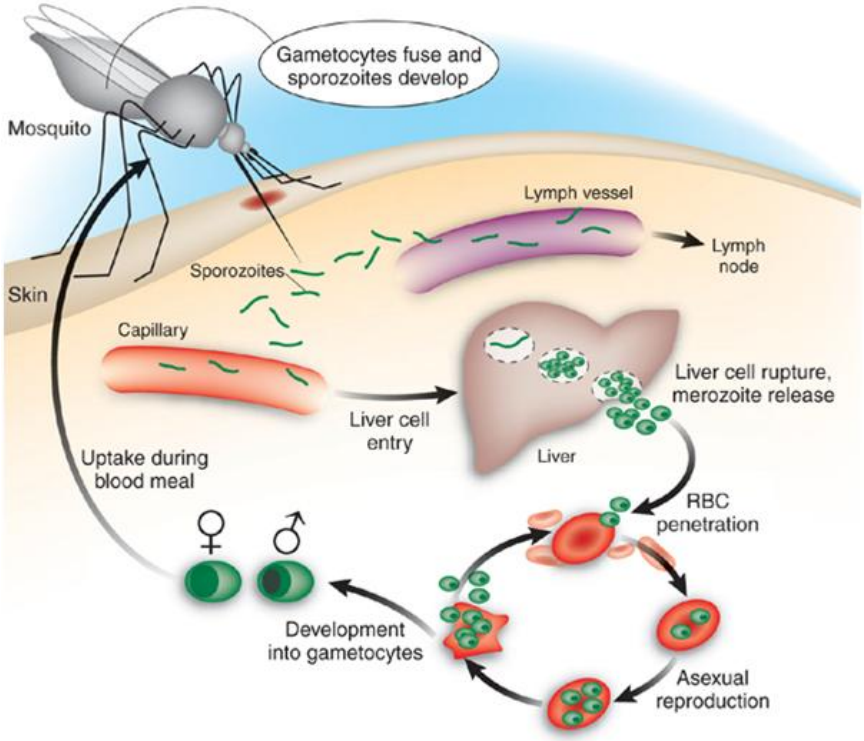

Figure 1. Life cycle of *Plasmodium* (Batista *et al.,* 2010).

Drugs that eliminate developing or dormant liver forms are called tissue schizonticides; those that act on erythrocytic parasites are blood schizonticides; and those that kill sexual stages and prevent transmission to mosquitoes are gametocides. Few available agents are causal prophylactic drugs (capable of preventing erythrocytic infection). However, all effective chemoprophylactic agents kill erythrocytic parasites before they increase sufficiently in number to cause clinical disease (Rosenthal, 2006).

For the prophylaxis in areas where malaria is endemic, mefloquine is the drug of choice, with doxycycline the usual alternative for those who cannot take mefloquine. In the few areas where chloroquine-sensitive strains of *P. falciparum* are found, chloroquine is used for prophylaxis. Chloroquine is also the prophylactic agent of choice to prevent infections due to *P. vivax, P. ovale,* and *P. malariae*. With the exception of drug-resistant strains of *P. falciparum*, the treatment of an acute attack is the same for all species. Treatment with a rapidly acting blood schizonticide must begin promptly if falciparum malaria is suspected. Chloroquine is the drug of choice for *P. malariae*, *P. ovale*, *P.vivax*, or chloroquine-sensitive *P. falciparum*. Quinine is the drug of choice for the treatment of chloroquine-resistant *P. falciparum* infections. For multidrug-resistant falciparum malaria, quinine is

given together with other blood schizonticides, such as antifolates or tetracyclines. For severe malaria, quinine or quinidine is given by i.v. injection. For recurrent attacks of *P. vivax, P. ovale,* or *P. malariae*, another course of chloroquine is given combined with, or followed by, a course of primaquine in the case of *P. vivax* or *P. ovale* (Scholar, 2007).

Artemisinin analogues, in particular artesunate and artemether, have recently shown great promise as rapidly acting and potent antimalarials (Rosenthal, 2003). Artemisinin-based combination therapy is being deployed worldwide in order to combat the spread of antimalarial drug resistance and remains highly effective in most parts of the world. Since 2001, 42 malaria-endemic countries, 23 of them in Africa, have adopted artemisinin based combination therapies recommended by WHO. Artemisinin-combined therapies (ACT) were also formally adopted as first-line treatment of uncomplicated malaria in Ethiopia from 2004 onwards (FMOH, 2004). However, treatment failure on this regimen was currently reported. For example, in Ethiopia, 7.5% treatment failure was reported (WHO, 2010).

Many malarial prevention and control strategies exist (such as the provision of prompt, effective malarial treatment, vector control and chemoprophylaxis), but none are appropriate and affordable in all contexts (Bloland, 2001). Some of the factors that contributed to this worst picture of malaria are high cost control programmes, emergence of new insecticide resistant strains of the vector, creation of new mosquito breeding sites, the problem of drug resistance (*P. falciparum*) to almost all currently available antimalarial drugs, lack of organized health infrastructures and the migration behaviour of people that increase the incidence and spread of malaria (WHO, 1996). The present global situation indicates a recent resurgence in severity of disease and that malaria could still be described as one of the important communicable diseases. Prevention and control strategies of malaria include vector control with residual insecticide spraying, larva control and personal protection measures such as use of insecticide treated bed nets, insect repellents and wearing appropriate clothing (Bloland, 20001; Ashley *et al*., 2006). Use of personal protective measures and behaviours to reduce the likelihood of being bitten by a female anopheline mosquito is a key to the prevention of malaria (Gkrania-Klotsas and Lever, 2007).

1.4. Medicinal plants for malaria treatment

Plants have been used as a source of medicine throughout history and continue to serve as the basis for many pharmaceuticals used today (Ginsburg and Deharo, 2011). The first antimalarial drug, quinine (**1**), was extracted from the bark of the *Cinchona* (Rubiaceae)

species (Adebayo and Krettli, 2011). In 1967, Chinese scientists isolated a sesquiterpene lactone, artemisinin (**2**), from the leafy portions *Artemisia annua* which is responsible for its reputed medicinal action (Klayman, 1985). Although artemisinin and its analogues have provided much needed drugs for the treatment of chloroquine-resistant malaria, these are unavailable and/or unaffordable to many people who live in malarious areas. An alternative to manufactured drugs is the use of traditional medicines for the treatment of malaria. in the last decade there has been increasing interest in the potential of locally grown plants (especially *A. annua*) to provide much needed antimalarial therapy (Wright, 2005).

Scholars have been trying to isolate new lead compounds from traditionally claimed plants. One group of compounds that has been explored further is the quassinoids which are degraded triterpenes found in various species of Simaroubaceae, some of which are used traditionally for the treatment of malaria and other protozoal diseases (Wright, 2005). Quassinoids, such as glaucarubinone (**3**), from *Simarrouba amara* were found to be selective against *P. falciparum in vitro*. Cryptolepine (**4**) is an indole alkaloid isolated from *Cryptolepis sanguinolenta,* a West African climbing shrub used traditionally for malaria treatment. It has been investigated as a potential lead to new antimalarials (Wright *et al*., 2001). Even if cryptolepine has potent *in vitro* antiplasmodial activity, it failed to cure malaria in mice when given orally and was toxic when given by the intraperitonial (i.p.) route. For that reason, semi-synthetic analogues were synthesized. The most promising analogue prepared was 2,7-dibromocryptolepine (**5**), which is approximately nine-fold more potent than cryptolepine against chloroquine-resistant *P. falciparum* (strain K1) and when tested in mice infected with *P. berghei* 25 mg/kg/day given i.p., parasitemia was suppressed by 90% with no apparent toxicity to the mice (Wright *et al*., 2001).

Wide arrays of secondary metabolites have been investigated for their antimalarial potentials. To point out some, naphthylisoquinoline alkaloids dioncopeltine A (**6**) and dioncophylline B (**7**) isolated from the extracts of *Triphyophyllum peltatum* (Dioncophyllaceae), exhibited high antiplasmodial activity in *P. berghei* infected mice (Kaur *et al,* 2009). Labdane diterpene (**8**) isolated from the seeds of *Aframomum zambesiacum* showed antiplasmodial activity *in vitro* (Kaur *et al*., 2004), while clerodane type diterpenes gomphostenin (**9**) and gomphostenin-A (**10**) isolated from leaves of *Gomphostemma niveum* exhibited promising antimalarial activity *in vivo* (Sathe *et al*., 2010). The flavonone cis-3-

acetoxy-4',5,7-trihydroxyflavanone (**11**) isolated from the leaf extracts of *Siparuna andina* displayed high antimalarial activity *in vitro* (Bero *et al.*, 2009).

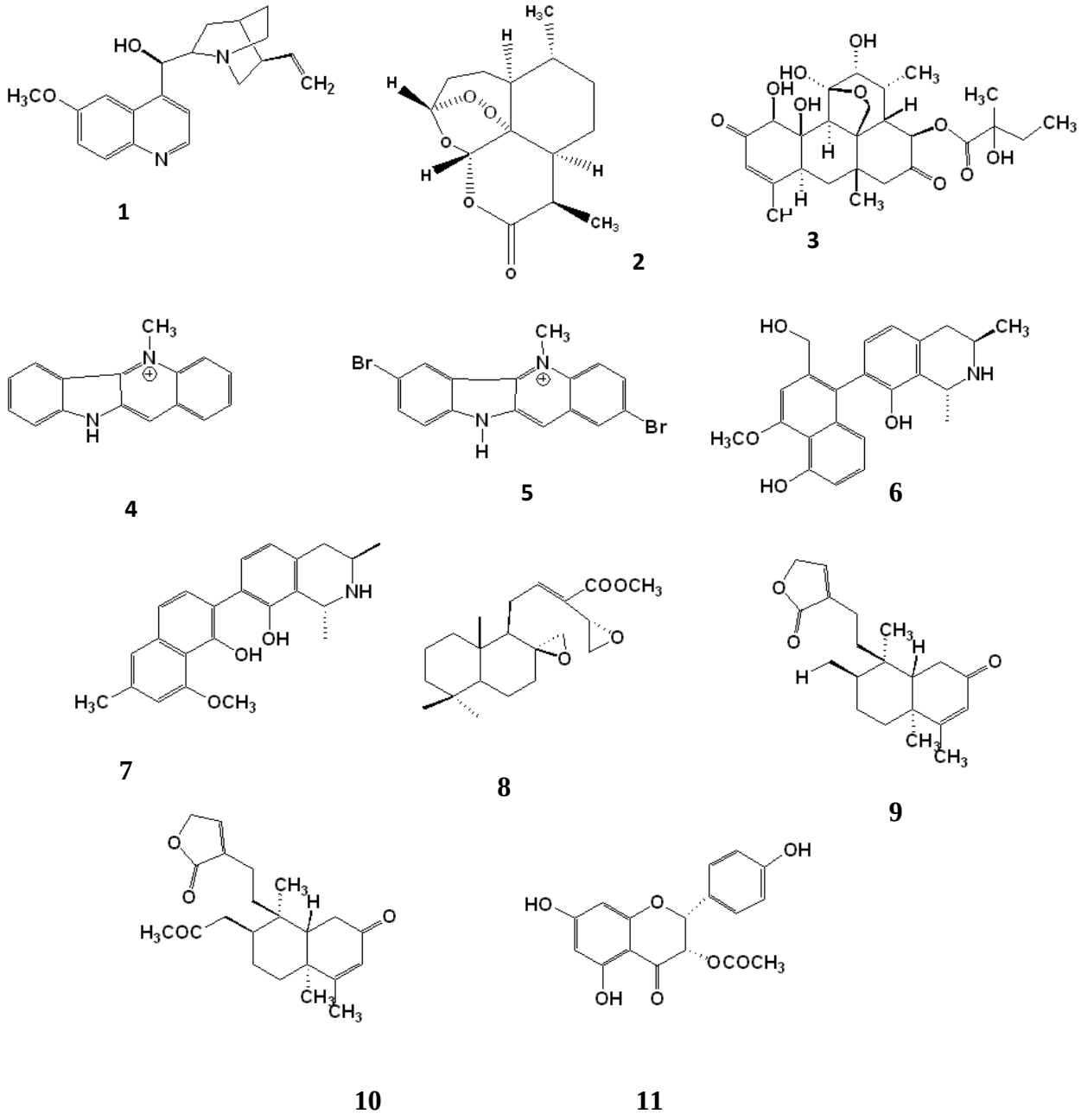

Figure 2. Some natural product and natural product-derived antimalarial agents

1.4.1. Ethiopian medicinal plants used for malaria treatment

Ethiopia has rich flora diversity and many of the plant species are used by indigenous people for medicinal purposes. Because traditional medicine is culturally entrenched, accessible, and affordable, up to 80% of the Ethiopian population relies on traditional remedies as a

primary source of health care (Kassaye *et al*., 2006). A large number of plants are used for the treatment of malaria in the traditional medical practice of Ethiopia.

A number of *in vivo* and *in vitro* studies have been conducted to evaluate antimalarial activities of Ethiopian medicinal plants, for instance, Assefa *et al*. (2007) reported that the methanol and aqueous extracts of *Cissampelos mucronata* (Menispermaceae), *Plumbago zeylanica L.* (Plumbaginaceae) and *Warburgia ugandensis* Sprague (Cannaleceaceae) possess antimalarial activities against *P. berghei in vivo* with chemosuppression of 47.5%, 30.5%, 34.6% , respectively. Bogale and Petros (1996) reported that among nine Ethiopian medicinal plants that are used to treat malaria the *in vitro* antimalarial activities of *Withnia somnifera* and *Vernonia amygdalina* against *P. falciparum* are substantial. Asres and Balcha (1998) reported that the acetone and methanol extracts of the stem bark of *Comberatum molle* inhibit schizont maturation of *P. falciparum*. Dikasso *et al*. (2006) evaluated the hydroalcoholic extracts of the roots and aerial parts of *Asparagus africanus* and showed that they possess significant antimalarial activity in the Swiss albino mice. Similarly, the methanolic extracts of *Clerodondrum myricodies* (Lamiaceae), *Dodanea angustifolia* (Sapindaceae) and *Aloe debrana* (Aloaceae) have showed potent antimalarial activity against *P. berghei* in mice with chemosuppression value of 82.5%, 84.52%, 73.95%, respectively (Deressa *et al*., 2010).

A handful of compounds have been isolated and characterized from plants used in Ethiopia traditional medicine for the treatment of malaria. From the acetone fraction of the stem bark of the *Combretum molle* the ellgitannin punicalagin (**12**) has been isolated. Punicalagin showed significant activity against *P. falciparum in vitro* with an IC_{50} value of 27.73 μg/mL (Asres *et al*., 2001). 10-(Chrysophanol-7'-yl)-10-(ξ)-hydroxychrysophanol-9-anthrone (**13**) and chryslandicin (**14**) isolated from the dichloromethane root extract of *Kniphofia foliosa* were shown to strongly inhibit the growth of *P. falciparum in vitro* with ED_{50} value of 0.26 and 0.537 μg/mL, respectively (Wube *et al*., 2005). Wube *et al*. (2010) also evaluated the dichloromethane extract of *W. ugandensis* Sprague (Cannaleceaceae) on chloroquine sensitive and chloroquine resistant *P. falciparum* strains *in vitro*. They reported that the antimalarial activity of the extract is due to the presence of drimane and colaratane sesquiterpenes. Among the compounds tested against the chloroquine-sensitive strain, 11α-hydroxymuzigadiolide (1**5**), mukaadial (**16**) and muzigadiolide (**17**) were found to be the most active with IC_{50} values of 6.4, 6.4 and 7.2 μM, respectively. On the other hand, mukaadial (**16**), muzigadiolide (**17**), 6α,9α-dihydroxy-4(13),7-coloratadiene-11,12-dial (**18)**,

and ugandensidial (**19)** exhibited potent activity against the chloroquine-resistant strain K1 with IC_{50} values of 7.3, 7.9, 11.0 and 10.6 μM, respectively. In addition, mukaadial (**16**), muzigadiolide (**17**) and 6α,9α-dihydroxy-4(13),7-coloratadiene-11,12-dial (**18)**, showed almost similar activity on both strains of *P. falciparum*.

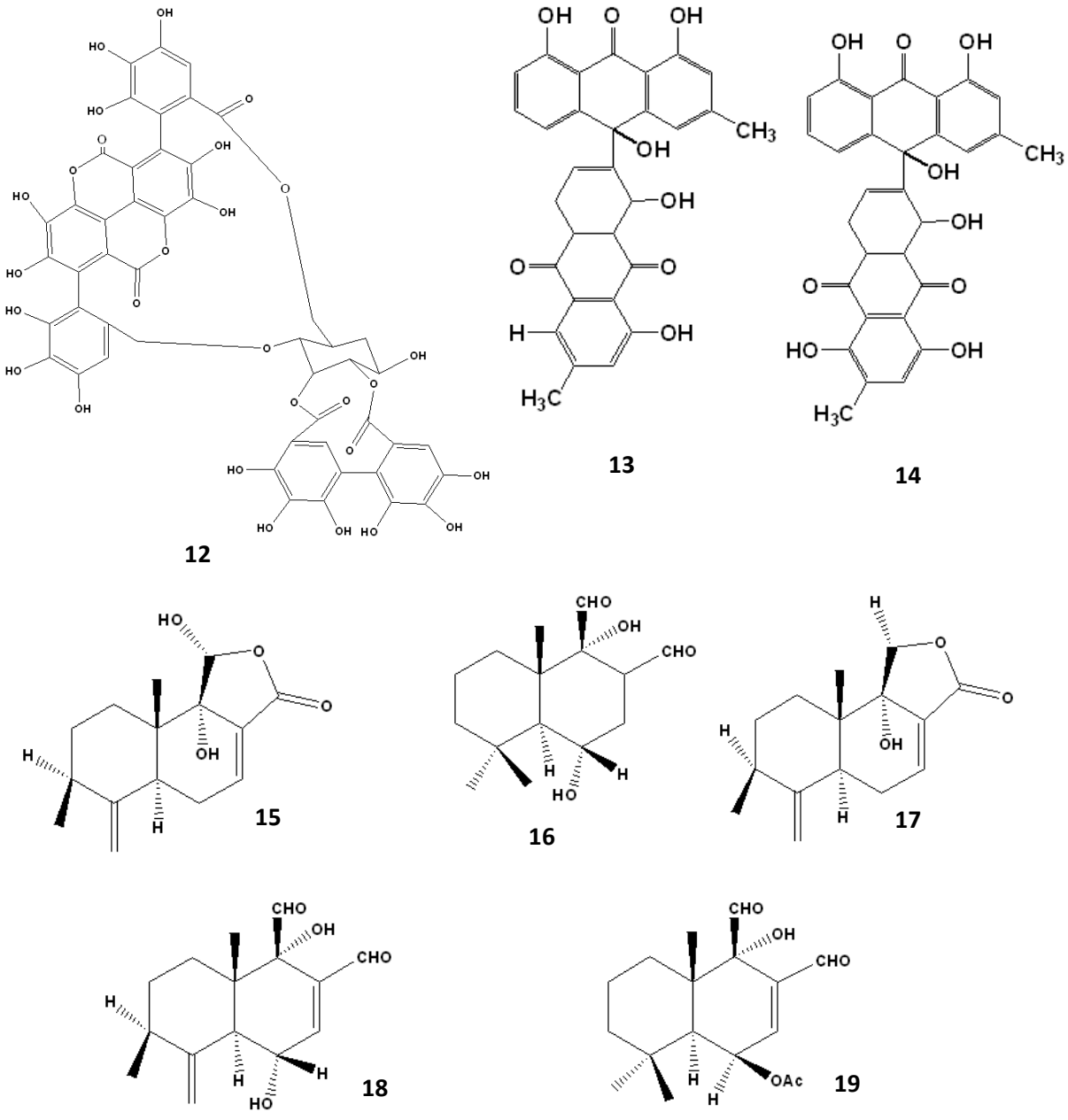

Figure 3. Structures of antimalarial active compounds isolated from medicinal plants used in Ethiopian traditional medicine for the treatment of malaria

1.5. The family Lamiaceae

The Lamiaceae is a commonly encountered family. Members of the family are mostly herbaceous, sub-shrubs or less often trees. When crushed they usually emit, mostly pleasant odours. Their stems are usually square and they contain abundant flowers which are quite attractive. Many members of the family are used as culinary or medicinal herbs, as sources of volatile oils and in some cases for the preparation of constituents of the volatile oils such as menthol and thymol (Evans, 2002; Sebald, 2006).

1.5.1. Biological activity of the Lamiaceae

Due to their essential oils content, several species of this family show antimicrobial activity (Asghari *et al*., 2006). For instance the essential oils obtained from dried leaves of *Lavandula bipinnata* showed activity against selected Gram positive and Gram negative bacterial and fungal strains (Hanamanthagouda *et al*., 2010). Hossain and Rahman (2011) evaluated the antibacterial activity of six Lamiaceae essential oils against pathogenic and food spoilage bacteria. All the essential oils showed inhibitory effects on most of the strains tested. In addition, different extracts from plants belonging to this family also exhibit activity against different strains of bacteria and fungi (Marino *et al*., 2001; Askun *et al*., 2009; Joshi *et al*., 2010; Radulovic *et al*., 2010).

Potent antioxidant activities of different species from the family Lamiaceae have been reported. For example aqueous extracts of four commonly consumed herbs, i.e. *Origanum vulgaris* L., *Rosmarinus officinalis* L., *Salvia officinalis* L. and *Thymus vulgaris* L. were shown to have potent antioxidant activity (Dorman *et al*., 2003). Furthermore, different solvent extracts and volatile oils from member of the family were found to exhibit significant antioxidant and free radical scavenging activities (Tepe *et al*., 2005; Armata *et al*., 2006; Matkowski and Piotrowska, 2006; Sharififar *et al*., 2009; Samec *et al*., 2010).

A number of Lamiaceous plants were found to exhibit antimalarial activity. For example, the methanol extract of the aerial part of *Perovskia abrotanoides* and solvent extracts of *Salvia* species have been reported to have significant antiplasmodic activity on chloroquine resistant *P. falciparum* strain (Kamatou *et al*., 2008; Esmaeili *et al*., 2009). Betulafolientriol oxide (**20**) and salvigenin (**21**) isolated from *Salvia radula* also exhibited significant antiplasmodic activity (Kamatou *et al*., 2008). The ethyl acetate extract of aerial parts of

Teucrium ramosissimum and its isolated compounds, homalomenol C (**22**), 4β-hydroxy-11,12,13-trinor-5-eudesmen-1,7-dione (**23**), oxo-T-cadinol (**24**) and 1β,4β,6β-trihydroxy eudesmane (**25**), displayed a significant *in vitro* antiplasmodial activity against *P. falciparum* (Henchiri *et al*., 2009).

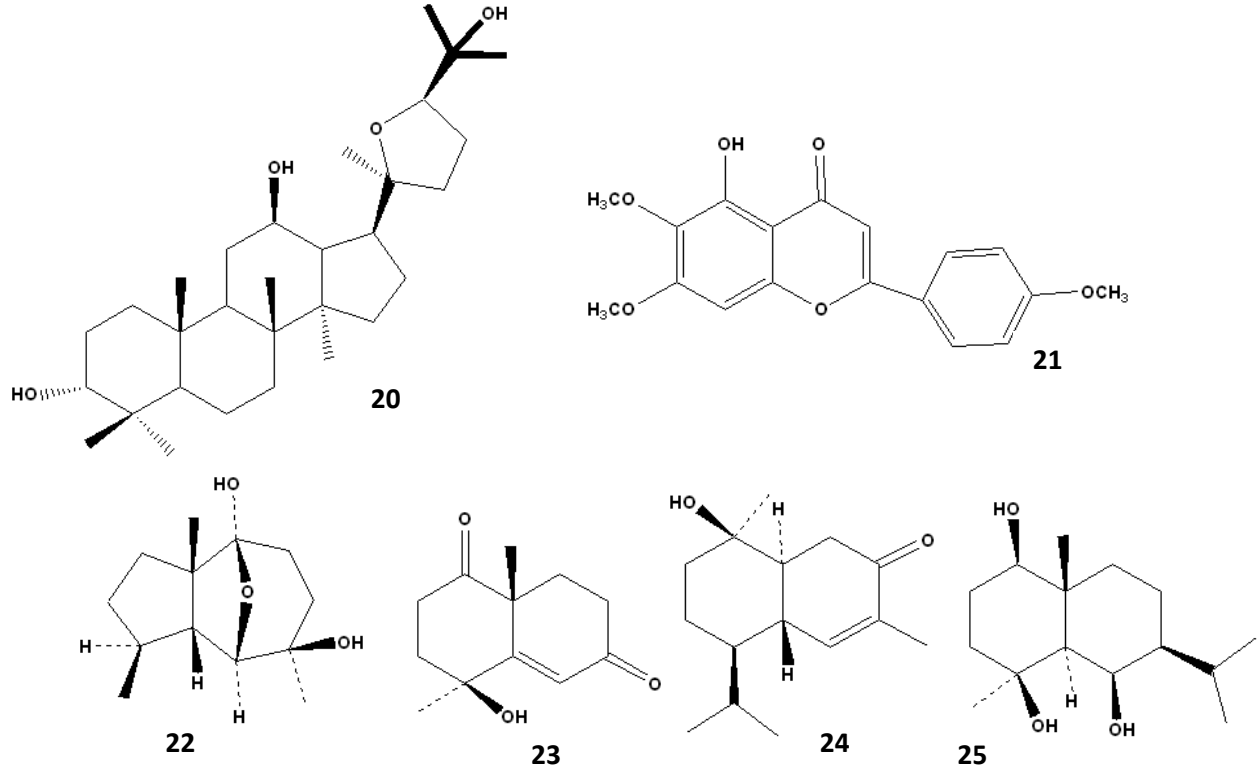

Figure 4. Representative antimalarial compounds isolated from plants belonging to the family Lamiaceae

1.6. The genus *Otostegia*

The genus *Otostegia* (Lamiaceae) comprises about 15 species. It is endemic to the northern part of tropical Africa and South Western and Central Asia. Five species of this genus have been reported to occur in flora of Ethiopia and Eritrea (Sebald, 2006).

1.6.1. Ethnobotonical use

In Iran *O. persica* is traditionally used for the treatment of malaria, fever and diabetes (Ayatollahi *et al*., 2009). In Pakistan the juice of the fresh leaves of *O. limbata* (Bth.) is taken orally for the treatment of inflammation of the gum and scabies, while powder of dried leaves is mixed with butter and layered on wounds and boils (Abbasi *et al*., 2010). In Saudi Arabia infusion of the flowering branches of *O. fruticosa* is used as a remedy for sun-stroke

(Rahman *et al.,* 2004), while in Eritrea the leaf and stem of *O. fruticosa* is used for arthritis, tonsillitis and gynaecological problems (Andemariam, 2010).

1.6.2. Pharmacological activity

1.6.2.1. Antioxidant and antidiabetic activity

The methanolic extract of *O. persica* was found to exhibit strong antioxidant activity. Kaempferol (**26**) and quercetin (**27**) isolated from the same extract showed significant antioxidant activity (Shrififar *et al*., 2003; Yassa *et al*., 2005). Antiglycation activity of *O. persica* has also been reported. Activity guided fractionation of the active fraction leads to the isolation of 3',7-dihydroxy-4',6,8-trimethoxyflavone (**28**) which was shown to be responsible for the antiglycation properties of the plant. *O. persica* also possesses antihyperglycaemic action against stereptozotocin induced hyperglycaemia in mice (Asgarpanah *et al*., 2010).

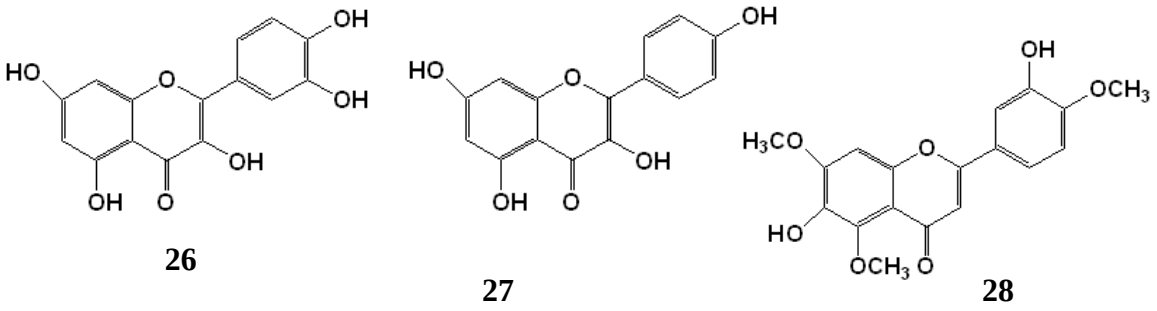

Figure 5. Flavonoids from the genus *Otostegia*

1.6.2.2. Antimicrobial activity

Antimicrobial activities of the methanolic, chloroform and hexane extracts of *O. persica* were tested on several microorganisms. These extracts showed antimicrobial activity against Gram-positive strains including *Enterococcus fecalis, Listeria monocytogens, Staphylococcus aureus,* and *Staphylococcus epidermidis* with the methanolic extract showing the highest activity (Asghari *et al*., 2006). The essential oil from *O. fruticosa* was reported to exhibit significant antimicrobial activity against Gram-positive and Gram-negative bacteria as well as a number of fungal strains (Aboutabl *et al*., 1995).

1.6.2.3. Antimalarial activity

Two species from this genus was found to exhibit antimalarial activity. The methanolic extract of the fruits and leaves of *O. persica* and *O. michauxii* were found to be active *in vitro* with IC_{50} value of 31.1 and 44.6 ug/mL, respectively, in chloroquine resistant *P. falciparum* (Esmaeili *et al*., 2009).

1.6.2.4. Other biological activities

The methanolic extract of *O. persica* was reported to alleviate morphine withdrawal syndrome in mice (Hajhashemi *et al*., 2004), while the leaf extract from *O. olgae* showed good insectoacaricidal activity (Chermenskaya *et al*., 2010).

1.6.3. Phytochemicals isolated

1.6.3.1. Terpenes

Terpenes are the main constituents of the genus *Otostegia*. Labdane diterpenes otostegin A (**29**), otostegin B (**30**) and 15-epi-otostegin B (**31**) were isolated from the aerial part of *O. fruticosa* (Al-Musayeib *et al*., 2000), whereas the aerial part of *O. persica* yielded clerodane type (**32-34**) and the tetracyclic diterpene, limbatenolide C (**35**) (Ayatollahi *et al.,* 2009). From the leaf essential oil of *O. fruticosa*, thymol (**36**) and γ-terpinene (**37**) were isolated (Aboutabl *et al*., 1995).

1.6.3.2. Flavonoids

Flavonoids were isolated from *O. persica* by bioactive guided fractionation during screening for antioxidant and antiglycan activity. As mentioned earlier compound **26** and **27** were isolated from the active methanolic extract, whereas **28** were identified during testing for antiglycan activity of *O.persica* (Shrififar *et al*., 2003; Yassa *et al*., 2005).

1.6.3.3. Iridoids

Iridoids are monoterpene lactones, which can occur as glycoside, with one or more sugar molecule attached or as aglycones (no sugar attachment). The iridoid glucoside 8-O-acetylharpagide (**38**) was isolated from the aerial parts of O. *fruticosa* (Al-Musayeib *et al*., 2000).

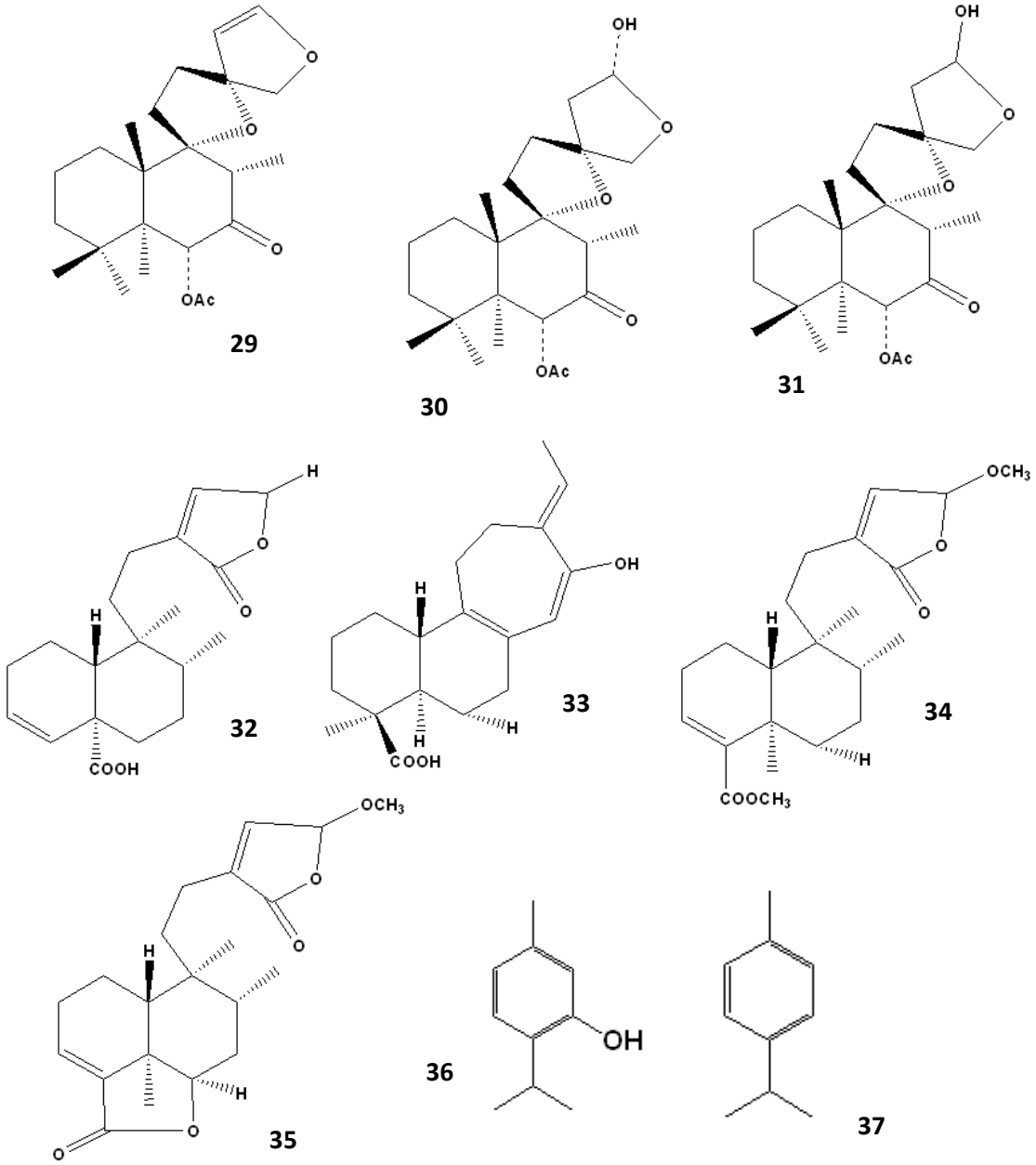

Figure 6. Some terpenoids isolated from the genus *Otostegia*

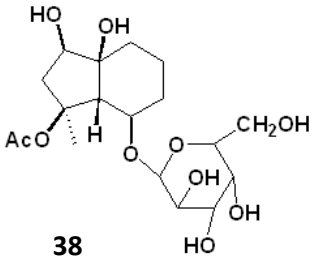

Figure 7. Iridoid glycoside isolated from the genus *Otostegia*

1.6.4. *Otostegia integrifolia* Benth.

O. integrifolia (known as "*Tinjut*" in Amharic) is a shrub which grows up to 3 m tall, often with paired spines at the nodes. Its leaves are sessile or shortly petiolate. The blade is bluish greyish-green, oblanceolate to lanceolate shaped, and reaches 2-9 cm long (Figure 9). The plant grows in the wild but is also cultivated in gardens. It growth on montane bush lands and wood lands over grazed slopes at altitudes ranging from 1, 300 to 2, 800 m. The plant is endemic to Ethiopia, Eritrea and Yemen (Sebald, 2006).

Figure 8. Morphological view of *Otostegia integrifolia*

1.6.4.1. Ethnobotonical use

The plant has insecticidal properties and is often used as fumigant for pots and houses (Waka *et al*., 2004; Mohagheghzadeh *et al*., 2006; Karunamoorthi *et al*., 2009). In Eretria it is used for the treatment of tonsillitis, uvulitis, and hypertension (Andemariam, 2010). The roots are used for treating lung diseases (Tesso and Konig, 2004). In Northern Ethiopia the leaves are used for the treatment of malaria (Giday *et al*., 2007), whereas in Central Ethiopia the leaves and steam part are used for treatment of stomach-ache (Teklehaymanot *et al*., 2007).

1.6.4.2. Phytochemical constituents

Tesso and Koning (2004) have reported the phytochemical contents of *O. integrifolia*. From the dried leaves of the plant 40 constituents have been isolated by hydrodistilation. The distillate was shown to be composed of monoterpenes, sesquiterpenes, diterpenes and their derivatives. *trans*-Sabinol (**39**), β-cyclocitral (**40**), dihydroedulan (**41**), theaspirane (**42**) and

(+)-axinyssene (**43**) (prenylbisabolane diterpenes) were the major components. The prefuranic and furanic labdane diterpenoids otostegindiol (**44**) and preotostegindiol (**45**) along with pentatriacontane and stigmasterol have also been isolated from the dried chloroform extract of the leaves of *O. integrifolia*.

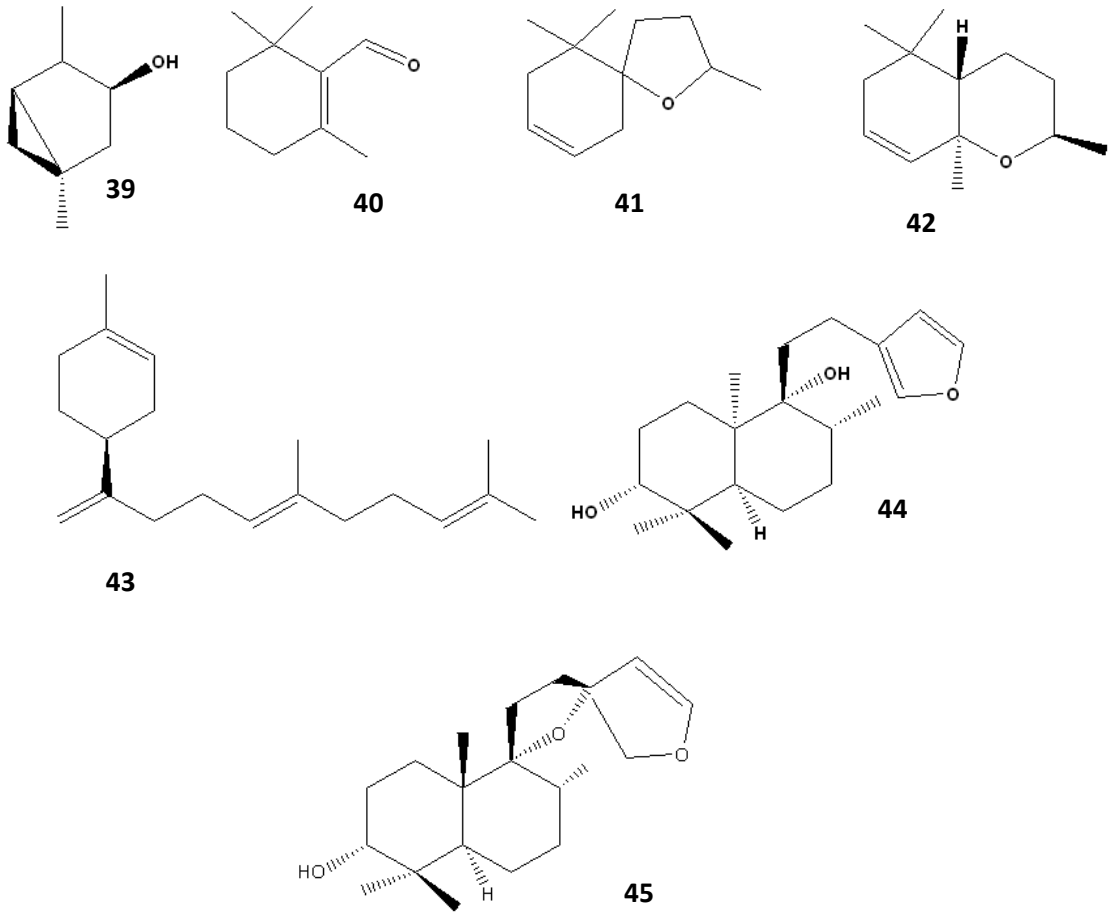

Figure 9. Compounds isolated from *Otostegia integrifolia*

1.7. Significance of the study

In sub-Saharan Africa where malaria is endemic and in other parts of the world, plants are extensively used for treating periodic fevers and malaria. The spread of multi drug-resistant *P. falciparum* has highlighted the urgent need to develop new antimalarial drugs, preferably inexpensive drugs that are affordable for developing countries, where malaria is prevalent.

Medicinal plants play an important role in the treatment of malaria especially in developing countries where resources are limited. They have in the past been the source of some of the

most successful antimalarial agents such as the quinolines and the endoperoxide artemisinin. One of the areas for the search for new antimalarials is the traditionally claimed antimalarial plants from the African flora. Medicinal plants have been the focus of many anti-infective drugs and alternative sources of antimalarial agents in various parts of the world since long time. Studies have been conducted on traditionally claimed medicinal plants in Ethiopia and elsewhere for scientific validation. This is because they have been part of human life since time immemorial; and a number of plant products have been in extensive use in ethnomedicine.

It has been claimed that about 80% of the Ethiopian population rely on medicinal plants for treating various illnesses including malaria. Plants and their extracts have immense potential for treatment of malaria. However, there is a need for scientific validation, standardization and safety evaluation of traditionally used medicinal plants before they are recommended for treatment of malaria.

Therefore, the list of traditionally used plants to control malaria must be backed by phytochemical studies and scientific authentication to develop an appropriate phytomedicine.

2. OBJECTIVES

2.1. General objective

- To investigate the *in vivo* antimalarial activity of the leaf extract of *Otostegia integrifolia* Benth.(Lamiaceae).

2.2. Specific objectives

- To study acute and sub-acute toxicity of the 80% methanolic extract of *O. integrifolia*;
- To evaluate the *in vivo* antimalarial activity of the hydroalcoholic extract;
- To prepare different fractions of the crude extract using hexane, chloroform, ethyl acetate, and water;
- To determine the antimalarial active fraction(s); and
- To isolate antimalarial active compound(s) from the active fraction(s).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Plant material

The leaves of *O. integrifolia* Benth. were collected in November 2010 from Chancho, 40 Km North of Addis Ababa, Central Ethiopia. The authenticity of the plant material was confirmed by Ato Melaku Wondafrash, the National Herbarium, Department of Biology, Addis Ababa University, where voucher specimen (collection number AE001) was deposited.

3.1.2. Chemicals, reagents and drugs

The following chemicals and reagents were used for the experiments. Giemsa, trisodium citrate, n-hexane, hydrochloric acid, Tween 80, potassium ferocynide, lead acetate (BDH, England), chloroform, ferric chloride (Fisher, UK), absolute methanol (Reagent Chemical Limited, UK), ethyl acetate (Research-Lab-Fine, India), acetic anhydride (Techno Pharm Chemical, India), chloroquine phosphate (Ethiopian Pharmaceutical Manufacturing, Addis Ababa-Ethiopia). All the chemicals were analytical grade and most of them were purchased from Pharmaceutical Fund and Supply Agency, Addis Ababa-Ethiopia, while the rest were obtained from Aklilu Lemma Institute of Pathobiology and Department of Pharmaceutical Chemistry and Pharmacognosy, School of Pharmacy, Addis Ababa University.

3.1.3. Instruments

^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance DMX400 FT-NMR spectrometer using TMS as internal standard, at the Department of Chemistry, Collage of Natural Sciences, Addis Ababa University, Ethiopia. The NMR data were collected using DMSO-d_6 as a solvent and the chemical shifts are reported in (δ ppm) downfield from TMS. EI-MS was performed by GC (Agilent 7890A system) coupled with MS operating at 70eV and mass scan range 40-600 u.

3.1.4. Experimental animals

Swiss albino mice of either sex weighing 24-30 g and age 6-8 weeks were obtained from Ethiopian Health and Nutritional Research Institute (EHNRI) animal house, Addis Ababa. All animals were housed in an air-conditioned room and were allowed to acclimatize for one week before the study. The animals were kept at room temperature and were exposed to a 12

h light/dark cycle. All the experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline (ILAR, 1996). Before and during the experiment, the mice were allowed free access to standard pellets and water *ad libitum*.

3.1.5. Parasite

Plasmodium berghei ANKA strain (chloroquine sensitive), was obtained from Aklilu Lemma Institute of Pathobiology. The parasite was subsequently maintained in the laboratory by serial blood passage from mouse to mouse on weekly bases.

3.2. Methods

3.2.1. Preparation of plant material

The leaves were cleaned, air-dried at room temperature and crushed into coarse powder. The powdered plant material (1000 g) was macerated in 80% methanol for 72 h with occasional stirring. The filtrate was separated from the mark using filter paper and the mark was re-macerated three times. The filtrates were combined and dried in an oven (Gallenkamp, England) at the temperature not exceeding 40 ^{o}C. The dried extract was transferred into vials and kept in a desiccator until use.

3.2.2. Fractionation

The dried hydroalcoholic leaf extract of *O. integrifolia* (20 g) was dissolved in distilled water (200 mL) and successively partitioned using different solvents of increasing polarity (n-hexane, chloroform, and ethyl acetate) in a separatory funnel as shown schematically in Figure 10. The different solvent fractions were concentrated under reduced pressure using rotary evaporator (Buchi Rota Vapor R-200, Switzerland) and dried in oven under 40 ^{o}C (Otsuka, 2006). The aqueous fraction was dried by lyophilizer. The dried fractions were then transferred into separate vials and stored in a desiccator for further use.

3.2.3. Chromatographic techniques

Among the three fractions tested, the ethyl acetate fraction showed the highest antimalarial activity in four day suppression test against *P. berghei* infected mice. Hence, this fraction was subjected to column chromatography on silica gel and eluted successively with n-hexane and ethyl acetate. A homogeneous compound designed A-1 was obtained in the 100% ethyl acetate fraction.

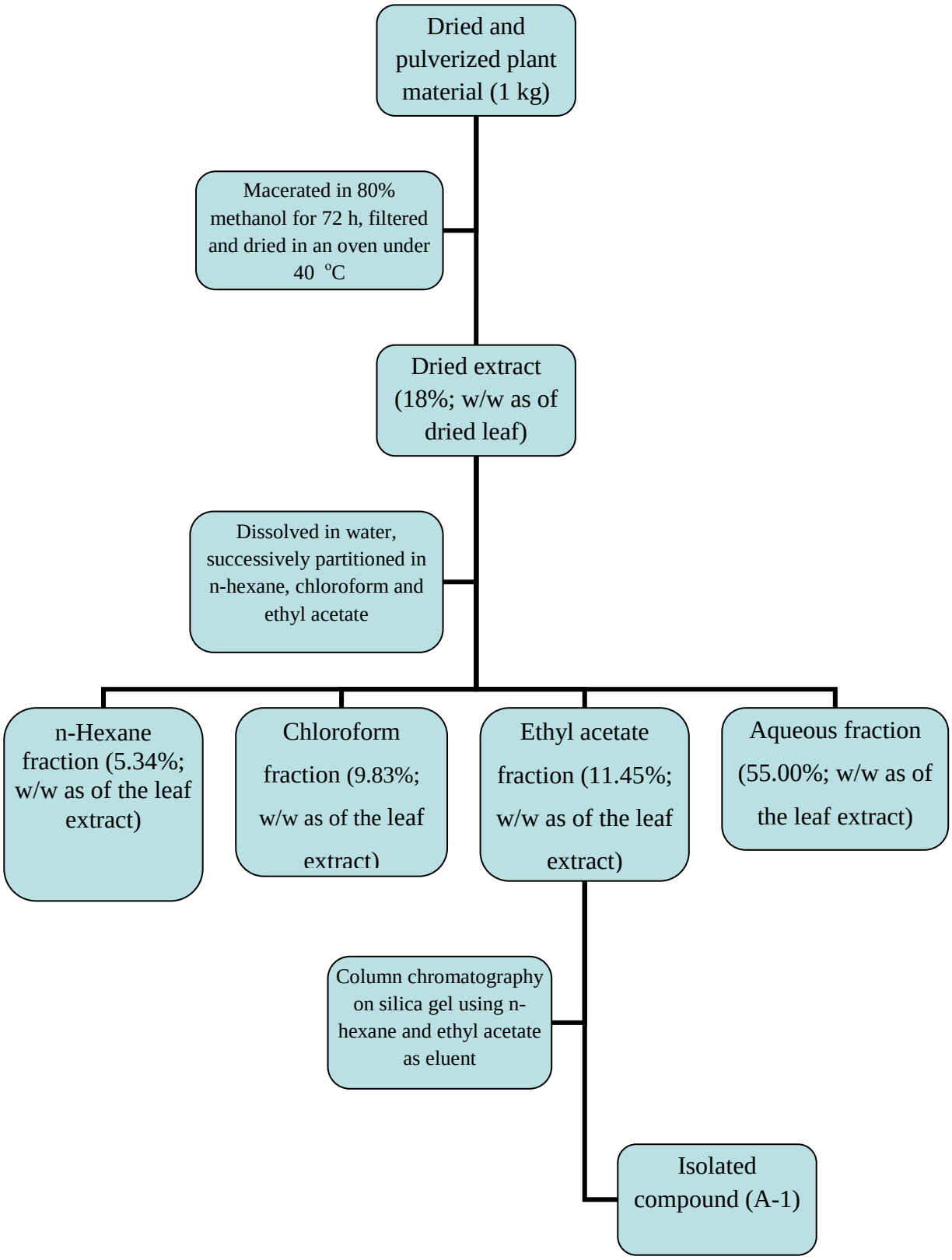

Figure 10. Schematic diagram of extraction, fractionation and isolation of a compound from the leaves of *Otostegia integrifolia*

3.2.4. Preliminary phytochemical screening

The 80% methanol or the aqueous extract of *O. integrifolia* was screened for the presence of alkaloids, flavonoids, polyphenols, tannins, saponins and steroidal compounds (Trease and Evans, 1989; Jones and Kinghorn, 2006).

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

Test for polyphenols (phenolic compounds): A mixture of one ml 1% $FeCl_3$ and one ml of 1% $K_3Fe(CN)_6$ was prepared immediately before this test. Then, to two ml of filtered solution of the aqueous macerate of plant material, three drops of a mixture of 1% $FeCl_3$ and 1% $K_3Fe(CN)_6$ were added. The final solution should form a green blue colour if it contains phenolic compounds.

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

Test for Tannins: The 80% methanol extract (3 g) was heated in a test tube with 10 ml of distilled water on a water bath for five minutes. After cooling, the solution was filtered through filter paper and 5 ml of 2% sodium chloride was added to the clear filtrate. The suspension was filtered (Whatman No. 1) and five ml of 1% gelatin was added to the clear filtrate. Then, the filtrate was observed whether it gives a precipitate which disappears upon addition of excess gelatin solution indicating the presence of tannins.

Test for steroidal compounds: The concentrated residue was dissolved in chloroform and treated with 3 drops of solutions of cold mixture of concentrated sulphuric acid and 1 ml of acetic anhydride. The formation of rose color (reddish brown) that changes to greenish blue indicates the presence of steroidal compounds.

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

3.2.5. Acute oral toxicity test

Female Swiss albino mice were used for acute oral toxicity study. Oral toxicity study was conducted as per the internationally accepted protocol drawn under OECD guidelines 423 (OECD, 2001). Nine mice were randomly divided into 3 groups of 3 mice per cage. The animals were physically active and were consuming food and water in a regular way. Before the administration of a single dose of the extract, the mice were fasted for 2 h. Then the mice in the first group were given distilled water while the second group were given the hydroalcoholic leaf extract of *O. integrifolia* 2 g/kg dissolved in distilled water orally, and the mice in the third group were provided with *O. integrifolia* leaf extract 5 g/kg dissolved in distilled water after following the first two groups for 14 days.

The mice were observed continuously for one h after administration of the extract; intermittently for 4 hours, over a period of 24 hours and for 14 days. Gross behavioural changes such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhoea, mortality and other signs of toxicity manifestation were observed (OECD, 2001).

3.5.2 Sub-acute toxicity

Sub-acute toxicity study testing was undertaken to evaluate if the extract has any effect on packed cell volume (PCV) and weight. Twelve healthy female Swiss albino mice were grouped into 4 groups of 3 mice per cage randomly. Before giving the extract, weight and PCV of each mouse were taken. The body weight of each mouse was determined in g using weighing balance while PCV, measure of the proportion of red blood cells to plasma, was determined by microhaematocrit reader (Hawksley, England). Then, group 1, 2 and 3 were given hydroalcoholic *O. integrifolia* leaf extract 200, 400 and 600 mg/kg/day at a volume of 0.2 ml dissolved in distilled water, respectively, while group 4 received distilled water for 4 consecutive days (OECD, 2001). On the fifth day weight and PCV of each mouse were measured. Weight and PCV before and after treatment were then compared for each group. The mice in each group were provided with food and water at all times during experiment.

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

3.2.6. Antimalarial activity test

3.2.6.1. Antimalarial activity test of the crude extract

The standard four-day suppressive method was used (Fidock *et al*., 2004). Blood was taken from a donor mouse with approximately 30% parasitemia and diluted in physiological saline to $5x10^7$ parasitized erythrocytes per ml. Swiss albino mice weighing 24-29 g were infected with 0.2 ml ($1x10^7$ parasitized erythrocytes) *P. berghei* intraperitoneally (i.p.) and randomly divided into five groups of five mice per cage with three test groups and two control groups (each for chloroquine as a standard drug and distilled water as a negative control).

The test extract were prepared in three different doses (200 mg/kg, 400 mg/kg, and 600 mg/kg of body weight) and chloroquine at 25 mg/kg in a volume of 0.2 ml. Each extract or the standard was administered as a single dose per day. The extract and the drug were given through oral route by using standard oral gavages. Treatment was started 3 h after infection on day 0 and was then continued daily for four days (i.e. from day 0 to day 3).

On the fifth day (D4) thin smears of blood films were obtained from the peripheral blood on the tail from each mouse. The smears were placed on microscopic slides (Westtmed Praxis, Germany), fixed with methanol and stained with 10% Gemsa at pH 7.2 for 15 min. The parasitaemia level was determined by counting the number of parasitized erythrocytes out of six random fields of the microscope (Olympus 6V20WHA2, Japan). Average percent parasitaemia and suppression were calculated by using the following formula (Fidock *et al*., 2004; Kalra *et al*., 2006).

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

$$\%\ \text{Suppression} = \frac{\text{Mean parasitemia of negative control} - \text{Mean parasitaemia of treated}}{\text{Mean parasitemia of negative control}} \times 100$$

3.2.6.2. Antimalarial activity test of fractions and isolated compound

The various solvent fractions of the dried hydroalcoholic extract of *O. integrifolia* leaves (chloroform, ethyl acetate and aqueous) were administered orally at three dose for each (200, 400, 600 mg/kg/day) to respective groups of five mice each 3 h post-infected intraperitoneally with 0.2 ml of infected blood containing about $1x10^7$ *P. berghei.* A negative control group was administered with 0.2 ml of distilled water while chloroquine (25 mg/kg/day) was given to the positive control group. The administration continued for four days (D0–D3). On the fifth day, thin films were prepared from tail blood of each mouse and the parasitaemia levels were determined by counting the number of parasitized erythrocytes in six random fields of the microscope. The average percentage chemosuppression was calculated as shown under section 3.2.6.1 (Fidock *et al*., 2004; Kalra *et al*., 2006).

Evaluation of antimalarial activity of the isolated compound was carried out using the above procedure. The compound was dissolved in 7% Tween 80/3% ethanol in distilled water as a vehicle and tested at the dose of 25, 50 and 100 mg/kg/day (Fidock *et al*., 2004).

The body weights of the mice were determined to observe whether leaf extract, solvent fractions or the isolated compound prevented weight loss that commonly reduced with increasing parasitaemia in infected mice. Weights were taken on day zero (D0) and day five (D4).

3.2.7. Data analysis

Data were analysed using Windows SPSS Version 16. The one-way analysis of variance (ANOVA) followed by Tukey's HSD *post-hoc* test, were used to compare results among and within groups for difference between initial and final results. Paired *t-test* was also used to compare some parameter between initial and final results. The results were considered significant when $P<0.05$.

4. RESULTS AND DISCUSSION

4.1. Percentage yields of crude extract and fractions

The 80% methanol leaf extract of *O. integrifolia* gave a greenish brown semi-solid, while the chloroform and ethyl acetate fractions were blue black paste, and the aqueous fraction reddish brown powder. The percentage yields of the crude extract and solvent fractions are shown in Table 1.

Table 1. Actual and percentage yields of the 80% methanol extract and solvent fractions obtained from the leaves of *Otostegia integrifolia*.

Extract	Actual yield (g)	Percentage yield (w/w)
80% methanol	180.60	18.06
Chloroform fraction	1.97	9.83
Ethyl acetate fraction	2.29	11.45
Aqueous fraction	11.00	55.00

Although traditionally water extract as infusions (steeping in cold water) is used, hydroalcoholic extraction was performed for this study. Water-soluble constituents present challenges for conventional isolation methods such as chromatography over silica gel, and water is not an ideal solvent for extraction of many biologically active plant constituents (Jones and Kinghorn, 2006). To the contrary, alcoholic (MeOH or EtOH) or hydroalcoholic extracts of plant materials contain a wide variety of polar and moderately polar compounds (Otsuka, 2006). So, in this study 80% methanol was used for extraction based on the above justifications. During fractionation, n-hexane was used to obtain a fraction containing nonpolar compounds, such as lipids and chlorophylls.

4.2. Phytochemical study

Preliminary phytochemical screening of the 80% methanolic leaf extract of *O. integrifolia* revealed that the extract contains phenolic compounds, saponins, and flavonoids while alkaloids, tannins and steroidal compounds were absent.

4.3. Acute toxicity

No sign of toxicity or mortality was observed in mice after oral administration of the hydroalcoholic leaf extract of *O. integrifolia*, even at doses as high as 5000 mg/kg, signifying that the oral LD_{50} was greater than 5000 mg/kg.

4.4. Sub-acute toxicity

In sub-acute toxicity study, the extracts were administered orally at different doses for three days (D0-D3), while distilled water was given to the negative control group. As shown in Table 2 no statistically significant differences ($P>0.05$) were observed when weight and PCV were compared in each group between pre-treatment (D-0) and post-treatment (D-4), as well as between extract administered and negative control groups. However, there were slight weight increments in all extract treated groups while reduction in weight was observed in vehicle treated group. Similarly, slight reduction in PCV was recorded in extract treated group while there was very small increase of this parameter on day 4 relative to day 0 in negative control group, which was statistically not significant. Generally the sub-acute toxicity test revealed the safety of the extract at the given dose.

Table 2. Sub-acute toxicity tests of the hydroalcoholic leaf extract of *Otostegia integrifolia* in mice.

Dose of extract (mg/kg/day)	Parameters	D0	D4
	Weight (g)	29.97±0.17	30.17±0.2
200	PCV (%)	60.00±1.52	57.00±0.58
	Weight (g)	29.1±0.47	30.33±0.25
400	PCV (%)	56.33±0.88	56.66±0.88
	Weight (g)	30.27±0.81	31.37±0.47
600	PCV (%)	55.00±2.65	53.67±0.88
	Weight (g)	30.63±0.54	30.50±0.12
Vehicle	PCV (%)	56.00±3.51	60.00±2.52

Data are expressed as means ± SEM for three mice per a group; D0: PCV and weight pre-treatment on day zero; D4: PCV and weight post-treatment on day five.

Generally, toxicity is the main concern of indigenous therapeutic preparations. Sign of toxicity such as change in animal behaviour, lacrimation, weight loss, hair erection and mortality were not recorded both in acute and sub-acute toxicity testing. This fulfils the

criteria set by OECD (2001), lack of acute toxicity by the plant extract. Therefore, it can be said that the hydroalcoholic leaf extract of *O. integrifolia* is relatively safe for mice when given orally. Slight reduction in PCV observed in the extract treated groups in sub-acute toxicity test might be due to the presence of saponins in the crude extract which are known to cause haemolysis by increasing the permeability of the plasma membrane (Dewick, 2002; Jones and Kinghorn, 2006).

4.5. Antimalarial activity

4.5.1. Antimalarial activity of the crude extract

The results of this study indicated that the hydroalcoholic leaf extract of *O. integrifolia* possesses activity against *P. berghei* malaria parasite *in vivo*. The result also revealed that the extract has dose dependent activity. Thus, at doses of 200, 400 and 600 mg/kg/day, it caused 42.37%, 75.28% and 80.52% suppression, respectively. The chemosuppression was significant ($P<0.001$) when compared with the negative control. In the same assay, on day 4, chloroquine had a chemosuppression of 100% at the dose level of 25 mg/kg/day and showed significant ($P<0.05$) suppression when compared to extract treated groups (Table 3).

From the result shown in Tables 3 and 4, it is evident that hydroalcoholic leaf extract of *O. integrifolia* possesses blood schizontocidal activity in the early infection of mice by *P. berghei* parasite. The average percent of parasitaemia observed at a higher dose (600 mg/kg/day) was found to be 4.34±0.77 with 80.52% of chemosuppression. A dose of 400 mg/kg/day of the extract showed 6.00±0.53% parasitemia with 75.28% chemosuppression, while the lowest dose (200 mg/kg/day) lowered the average percent parasitemia to 14.04±2.36 with chemosuppression of 42.37%.

The mean survival time of mice of the given extract was shown to be 8.00±0.45, 11.00±0.95, 11.00±0.45 days, for doses of 200, 400 and 600 mg/kg/day, respectively, which was statistically significant ($P<0.001$), except for the lowest dose (200 mg/kg/day) when compared to vehicle treated mice (7.2±0.20 days). Furthermore, although statistically not significant, the mean survival time of mice treated with of 200 mg/kg/day was relatively longer than the negative control as shown in Table 4. All animals treated with the standard drug, chloroquine 25 mg/kg/day, survived more than 28 days.

Table 3. Percentage suppression of the hydroalcoholic leaf extract of *Otostegia integrifolia* against *Plasmodium berghei* in mice.

Drug/extract	Dose (concentration) mg/kg/day	% Parasitaemia ±SEM	% Suppression
Vehicle	0.2 ml	24.36±1.51	–
O. integrifolia	200	14.04±2.36*	42.37
O. integrifolia	400	6.00±0.53*	75.28
O. integrifolia	600	4.34±0.77*	80.52
Chloroquine	25	0.00*	100

*The mean value is significant (P<0.001) when compared with vehicle treated group; data are expressed as means ± SEM for five mice per a group.

Treatment of *P. berghei* infected mice with the hydroalcoholic leaf extract of *O. integrifolia* protected them from weight loss. The mean weight of each group improved on the fifth day of infection except the vehicle treated group. However, the improvement was not statistically significant (P>0.05) except in those mice treated with the highest dose (Table 5). Animals receiving higher doses of the extract gained more weight than those treated with the lower dose. All treatment groups showed higher weight increment than that of the standard drug chloroquine.

Table 4. Mean survival time of *Plasmodium berghei* infected mice after treatment with the hydroalcoholic leaf extract of *Otostegia integrifolia*.

Drug/extract	Dose (concentration) mg/kg/day	Survival time ± SEM (day)
Vehicle	0.2 ml	7.2±0.20
O. integrifolia	200	8.00±0.45
O. integrifolia	400	11.00±0.95*
O. integrifolia	600	11.00±0.45*
Chloroquine	25	28.00±0.00*

*The mean value is significant (P<0.001) when compared with vehicle treated group; data are expressed as means ± SEM for five mice per group.

The 4-day suppressive test is a standard test commonly used for antimalarial screening, and the determination of percent inhibition of parasitaemia is the most reliable parameter. This method has become popular during scientific evaluation of potential phytomedicines for early investigations of the *in vivo* efficacy test of antimalarial candidate (Ajaiyeoba *et al*.,

1999; Fidock *et al*., 2004; Dikasso *et al*., 2006). In this study, crude extract of *O. integrifolia* showed considerable antiplasmodial properties. The suppression activity of the crude extract increased with increase in the dose of the extract though this activity was not comparable to that of the standard drug which suppresses parasitaemia to non-detectable levels; perhaps this might be due to the crude nature of the extract (Okokon *et al*., 2007).

Table 5. Body weight of *Plasmodium berghei* infected mice after administration of the hydroalcoholic leaf extract of *Otostegia integrifolia*.

Drug/extract	Dose mg/kg/day	Weight D0 ±SEM	Weight D4 ±SEM	% change
Vehicle	0.2 ml	26.52±0.66	25.32±0.60	-4.52
O. integrifolia	200	25.34±0.59	26.54±0.43	4.74
O. integrifolia	400	26.54±0.51	28.90±0.62	8.89
O. integrifolia	600	26.40±0.40	28.76±0.43*	8.90
Chloroquine	25	28.44±0.79	28.64±o,74	0.70

*The mean weight pre-treatment is statistically significant (P<0.05) compared with post-treatment; data are expressed as means ± SEM for five mice per group; Weight D0: Weight pre-treatment on day zero; Weight D4: weight post-treatment on fifth day.

The antimalarial activity of *O. integrifolia* might be attributed to the presence of phytochemical constituents such as terpenes and flavonoids which have been implicated in antiplasmodial activities of many plants (Batista *et al*., 2009; Ene *et al*., 1009; Kaur *et al*., 2009). The results of the preliminary phytochemical screening also indicated a probable presence of flavonoids and other phenolic compounds. These chemical compounds may be acting singly or in synergy with one another to exert the observed antimalarial activity of the extract. Besides, two species from the genus *Otostegia* have been reported to exhibit moderate antiplasmodic activity *in vitro* in chloroquine resistant *P. falciparum* (Esmaeili *et al*., 2009), while many species from Lamiaceae are known to display antimalarial activity (Kamatou *et al*., 2008; Esmaeili *et al*., 2009: Henchiri *et al*., 2009). The observed antimalarial activity is also consistent with the traditional use of the plant as a herbal medication against malaria in Northern Ethiopia (Giday *et al*., 2007).

The much higher increment of body weight by the extract compared even to that of chloroquine might be attributed to the presence of several nutrients and immunomodulatory substances, in addition to antimalarial active compounds (Dikaso *et al*., 2006). This effect was also seen in sub-acute toxicity tests which resulted in slight increment of weight in

extract treated groups. The results clearly indicated that hydroalcoholic leaf extract of *O. integrifolia* prevented weight loss in a dose dependent manner compared to the controls.

4.5.2. Antimalarial activity of fractions

As shown in Table 6, all fractions possess blood schizontocidal activity in early infection of *P.berghei* with varying degrees of suppression. In the case of the aqueous fraction, the average percent of parasitaemia observed was found to be 22.02±1.15, 18.13±0.70 and 15.86±0.73 with 17.65%, 27.99%, 36.61% chemosuppression at doses of 200, 400, and 600 mg/kg/day, respectively. The chloroform fraction on the other hand, showed suppression of parasite growth from 22.02±1.15 to 14.67±0.58, 11.36±0.58 and 11.87±0.71, representing 33.82%, 48.41% and 46.10% of chemosuppression the doses of 200, 400 and 600 mg/kg/day, respectively. Highest suppression was observed by the ethyl acetate fraction, which showed suppression of parasite growth from 22.02±1.15 to 13.00±0.44, 10.72±0.38 and 8.79±0.81, representing 41.19%, 51.33% and 60.15% of chemosuppression at doses of 200, 400 and 600 mg/kg/day, respectively. All fractions showed statistically significant (P<0.01) difference on day 4 parasitaemia level, compared to the negative control. The highest level of inhibition (60.15%) was obtained by 600 mg/kg/day of the ethyl acetate fraction, while the lowest suppression (17.65%) was obtained after administration of 200 mg/kg/day of the aqueous fraction. The standard drug, chloroquine (25 mg/kg/day) completely cleared the parasite in the fifth day of infection.

The mean survival time was significant (P<0.05) for all fraction treated groups at the highest dose (600 mg/kg/day) employed, while the ethyl acetate fraction at doses of 400 and 600 mg/kg/day significantly increased the mean survival time when compared with the negative control group. As shown in Table 7, the survival time increased in dose dependent manner for all fractions. The longest survival time was observed in mice treated with the ethyl acetate fraction at 600 mg/kg/day (12.20±0.49 days), while the aqueous fraction (200 mg/kg/day) showed the shortest survival time (9.20±0.92 days). Although statistically not significant (P>0.05), the mean survival time of mice treated at lower doses of each fraction was relatively longer than that observed in the negative control group. This indicates that the average parasitaemia and survival time of mice treated with the extract were better than the negative control. The mean survival time of mice treated with each fraction was not comparable to that of the standard drug, chloroquine (25 mg/kg/day), which shows mean survival time of more than twenty-eight days.

Table 6. Percentage suppression of solvent fractions obtained from the hydroalcoholic leaf extract of *Otostegia integrifolia* against *Plasmodium berghei* in mice.

Solvent fraction /drug	Dose (concentration) mg/kg/day	% Parasitaemia ±SEM	% Suppression
Vehicle	0.2 ml	22.02±1.15	–
Chloroform fraction	200	14.67±0.58*	33.82
Chloroform fraction	400	11.36±0.58*	48.41
Chloroform fraction	600	11.87±0.71*	46.10
Ethyl acetate fraction	200	13.00±0.44*	41.19
Ethyl acetate fraction	400	10.72±0.38*	51.33
Ethyl acetate fraction	600	8.79±0.81*	60.15
Aqueous fraction	200	18.13±0.70**	17.65
Aqueous fraction	400	15.86±0.73*	27.99
Aqueous fraction	600	13.96±0.67*	36.61
Chloroquine	25	0.00±0.0*	100.00

**The mean value is significant (P<0.01) when compared with vehicle treated group; *the mean value is significant (P<0.001) when compared with vehicle treated group; data are expressed as means ± SEM for five mice for per group.

Treatment of *P. berghei* infected mice with solvent fractions obtained from dried hydroalcoholic leaf extract of *O. integrifolia* protected them from weight loss. All fractions at the given doses showed better weight increment than the negative control group. However, the protection was not in a dose dependent manner. The ethyl acetate fraction at a dose of 400 mg/kg/day showed the highest percent increment, while the aqueous fraction at a dose of 600 mg/kg/day showed the lowest. In addition, all fractions at the given doses showed better percent change than the standard drug except the 200 mg/kg/day and 600 mg/kg/day aqueous fraction. Moreover, no statistically significant (P>0.05) increments/decrements were observed in all fractions and controls when weight before and after treatment of each group was compared.

The ethyl acetate fraction was found to be the most active which might be attributed to the presence of more active secondary metabolites with antimalarial activity in it. Generally, medium polarity metabolites such as terpenes are expected to be present in ethyl acetate fraction (Otsuka, 2006; Hajnos, 2008). Sesquiterpenes and monoterpenes have been reported

to be present in the essential oil of the leaf while diterpenoids have been isolated from the leaf extract of *O. integrifolia* (Tesso and Konig, 2004). Diterpenoids, sesquiterpenoids and monoterpenoids have been implicated in antiplasmodial activity of many plants (Miyaoka *et al*., 1998; Loyola *et al*., 2004; Jullian *et al*., 2005; Wright, 2005; Bero *et al.,* 2009; Okokon and Nwafor, 2009*;* Sathe *et al*., 2010; Wube *et al*., 2010). The sesquiterpenoid artemisinin, the main active ingredient in the traditional Chinese antimalarial *Artemisia annua*, is one such example.

Table 7. Mean survival time of *Plasmodium berghei* infected mice after treatment with solvent fractions obtained from the dried hydroalcoholic leaf extract of *Otostegia integrifolia*.

Solvent fraction/drug	**Dose (concentration) mg/kg/day**	**Survival time ±SEM (day)**
Vehicle	0.2 ml	7.20±0.37
Chloroform fraction	200	9.40±0.24
Chloroform fraction	400	9.80±01.16
Chloroform fraction	600	10.20±0.49***
Ethyl acetate fraction	200	9.80±0.37
Ethyl acetate fraction	400	10.80±0.37**
Ethyl acetate fraction	600	12.20±0.49*
Aqueous fraction	200	9.20±0.92
Aqueous fraction	400	9.40±0.60
Aqueous fraction	600	10.60±0.60**
Chloroquine	25	28.00±00*

***The mean value is significant (P<0.05) when compared with vehicle treated group; **the mean value is significant (P<0.01) when compared with vehicle treated group; *the mean value is significant (P<0.001) when compared with vehicle treated group; data are expressed as means ± SEM for five mice per group.

Among the fraction tested, the aqueous fraction showed least antimalarial activity indicating that the active compound are not highly polar (Waako *et al*., 2005; Jones and Kinghorn, 2006; Muthaura *et al*., 2007).

Table 8. Body weight of *Plasmodium berghei* infected mice after the administration of solvent fractions of the dried hydroalcoholic leaf extract of *Otostegia integrifolia*.

Fractionating solvent/drug	Dose (concentration) mg/kg/day	Weight D0 ±SEM	Weight D4 ±SEM	% Change
Vehicle	0.2 ml	24.6±0.46	24.30±0.54	-1.53
Chloroform fraction	200	29.24±1.17	30.06±1.55	2.80
Chloroform fraction	400	24.40±0 .67	24.48±0 .47	0.33
Chloroform fraction	600	26.22±1.05	27.32±1.17	4.20
Ethyl acetate fraction	200	29.26±0.49	30.56±0.67	4.44
Ethyl acetate fraction	400	29.10±1.17	30.52±0.80	4.88
Ethyl acetate fraction	600	26.50±1.39	27.02±1.47	1.96
Aqueous fraction	200	26.14±0.64	26.18±0.48	0.15
Aqueous fraction	400	28.30±0.52	28.96±0.49	2.33
Aqueous fraction	600	28.38±0.57	28.02±0.34	-1.20
Chloroquine	25	28.44±0.79	28.64±0.74	0.70

Data are expressed as means ± SEM for five mice per group; Weight D0: weight pre-treatment on day zero; D4: weight post-treatment on day five.

Interestingly, none of the solvent fractions was found to be as active as the crude extract with respect to percent suppression. This could be explained by the possible synergistic effects that might have existed among the various components in the crude extract (Kamatou *et al*., 2008; Okokon and Nwafor, 2009; Batista *et al*., 2009).

The results of the antimalarial suppressive effects of the crude hydroalcoholic leaf extract and solvent fractions at 200, 400 and 600 mg/kg/day are summarized in Figure 11. Comparison of percent suppression showed that the 80% methanol extract has the highest effect at the tasted doses followed by the ethyl acetate and chloroform fractions with the aqueous fraction displaying the least suppressive.

4.6. Structural elucidation of A-1

From solvent-solvent partition of crude extract, the ethyl acetate fraction was found the most active in four-day suppressive assay. This fraction showed one major constituent on TLC with R_f value of 0.59 (hexane/ethyl acetate 7:3). Column chromatography of this fraction over silica gel eluting with hexane and ethyl acetate gradients led to the isolation of A-1.

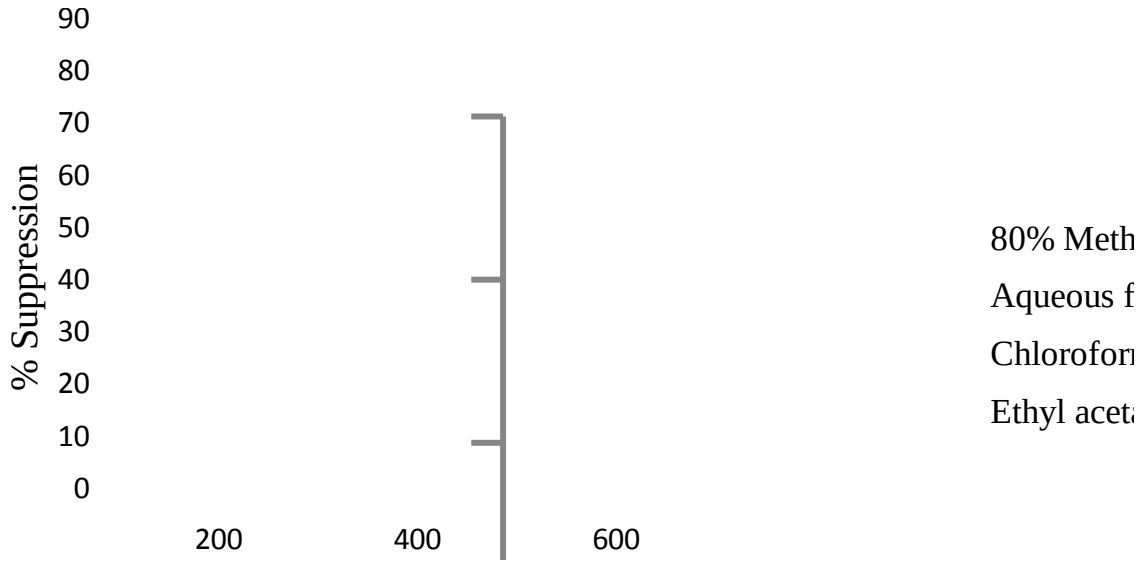

Figure 11. Percentage parasitaemia suppression of the hydroalcoholic and solvent fractions leaf extract of *Otostegia integrifolia*.

Compound A-1 was obtained as a dark-brownish amorphous solid with R_f value of 0.59 (hexane/ethyl acetate 7:3). The electron ionization mass spectrum (EI-MS) of A-1 exhibited molecular ion peak at m/z 320 (Figure 12). Fragment peaks at m/z 81 and 95 in the mass spectrum indicated the presence of a β-monosubstituted furan ring which is further confirmed by ^{1}H NMR. The MS data in combination with ^{1}H, ^{13}C NMR and DEPT spectra led to a molecular formula of $C_{20}H_{32}O_3$ for A-1.

The ^{1}H NMR spectrum of A-1 showed a typical β-mono-substituted furan ring signals at δ 6.32 (1H, *s*, H-14), δ 7.37 (1H, *s*, H-16) and δ 7.50 (1H, *s*, H-15) as shown in Figure 13. The presence of an oxymethine proton was also evident from ^{1}H NMR spectrum, where a singlet at δ3.16 (1H, s, H-3) was observed. In addition to these, allylic proton at δ 2.38 (2H, *t, J* = 8.83 Hz) was also observed.

The ^{13}C NMR along with DEPT spectra of A-1 showed signals for twenty different carbon atoms (Figures 14 and 15), corresponding to four methyl groups (δ 16.57, 16.83, 21.54, 29.39), six methylene groups (δ 21.82, 22.93, 25.12, 25.92, 31.21, and 35.77), six methines including three olefinic carbons and one oxymethine carbon (δ 36.42, 39.23, 74.33, 111.3, 143.2, 138.7) and four quaternary carbons (δ 37.69, 42.95, 76.34, 126.32) as shown in Table 9.

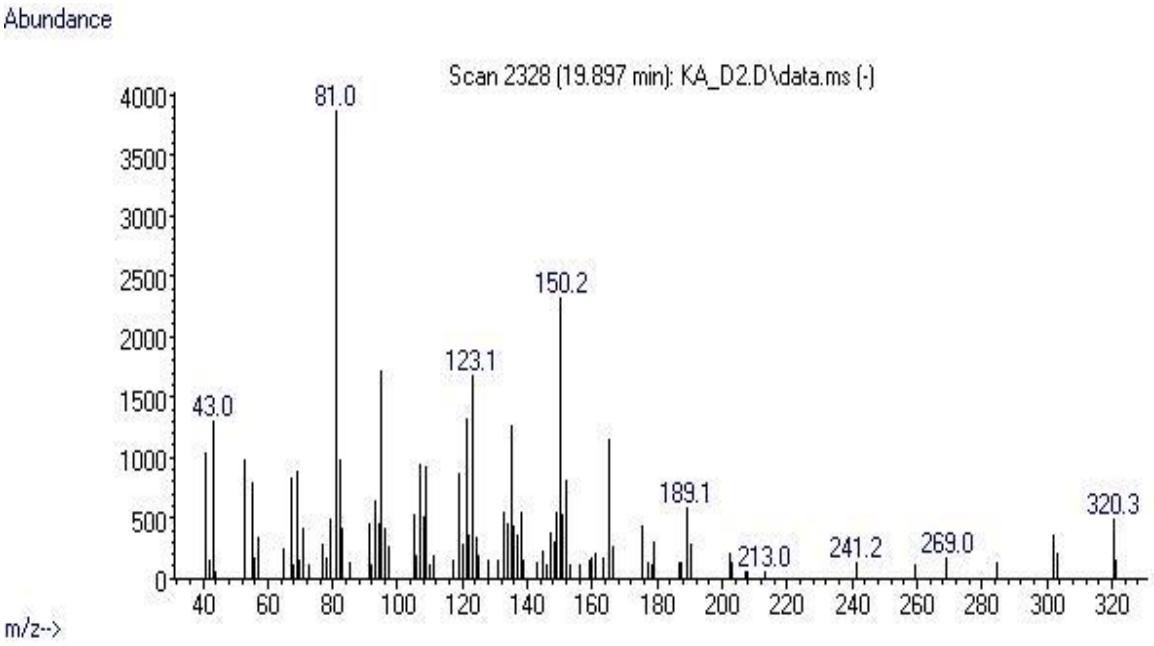

Figure 12. Mass spectrum of compound A-1

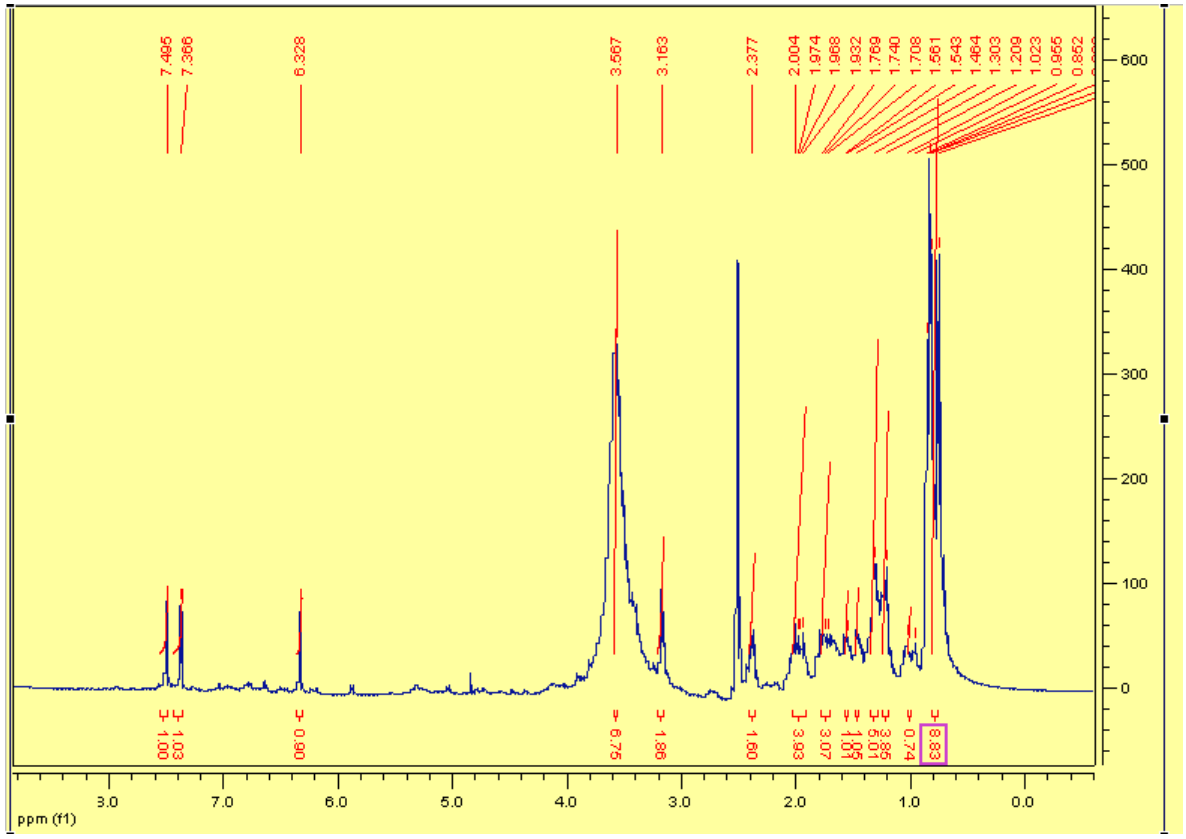

Figure 13: ^{1}H NMR spectrum of compound A-1 in dutrated dimethyl sulfoxide

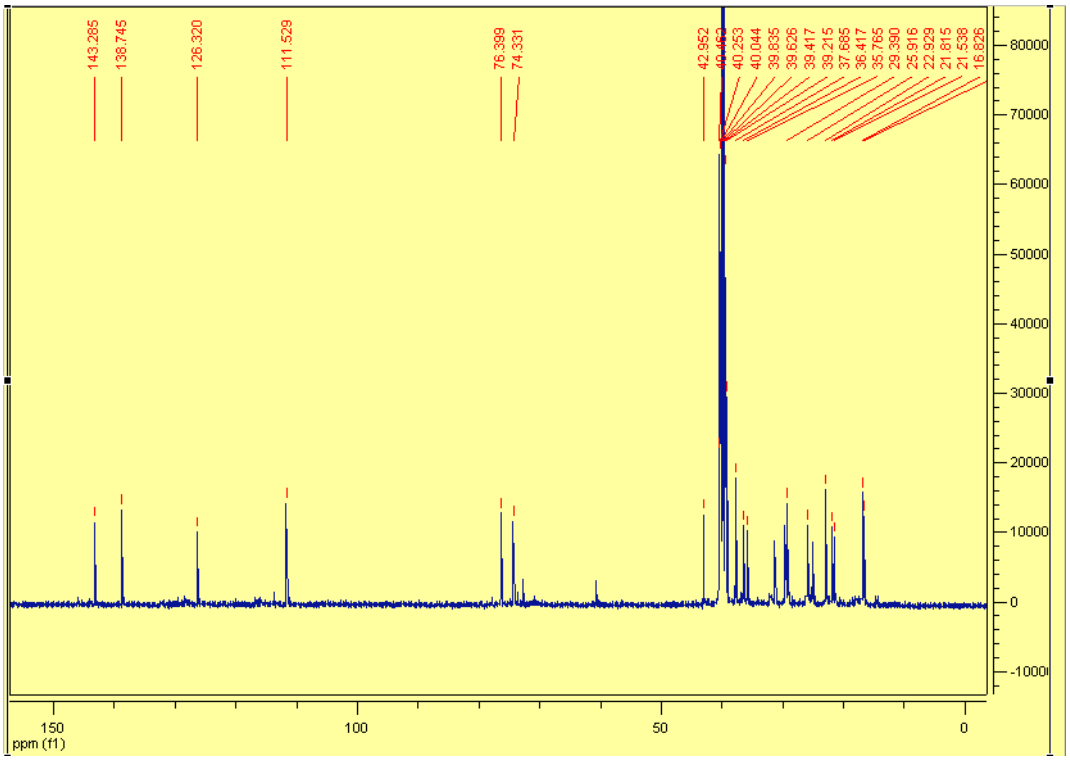

Figure 14: ^{13}C NMR spectrum of compound A-1 in dutrated dimethyl sulfoxide

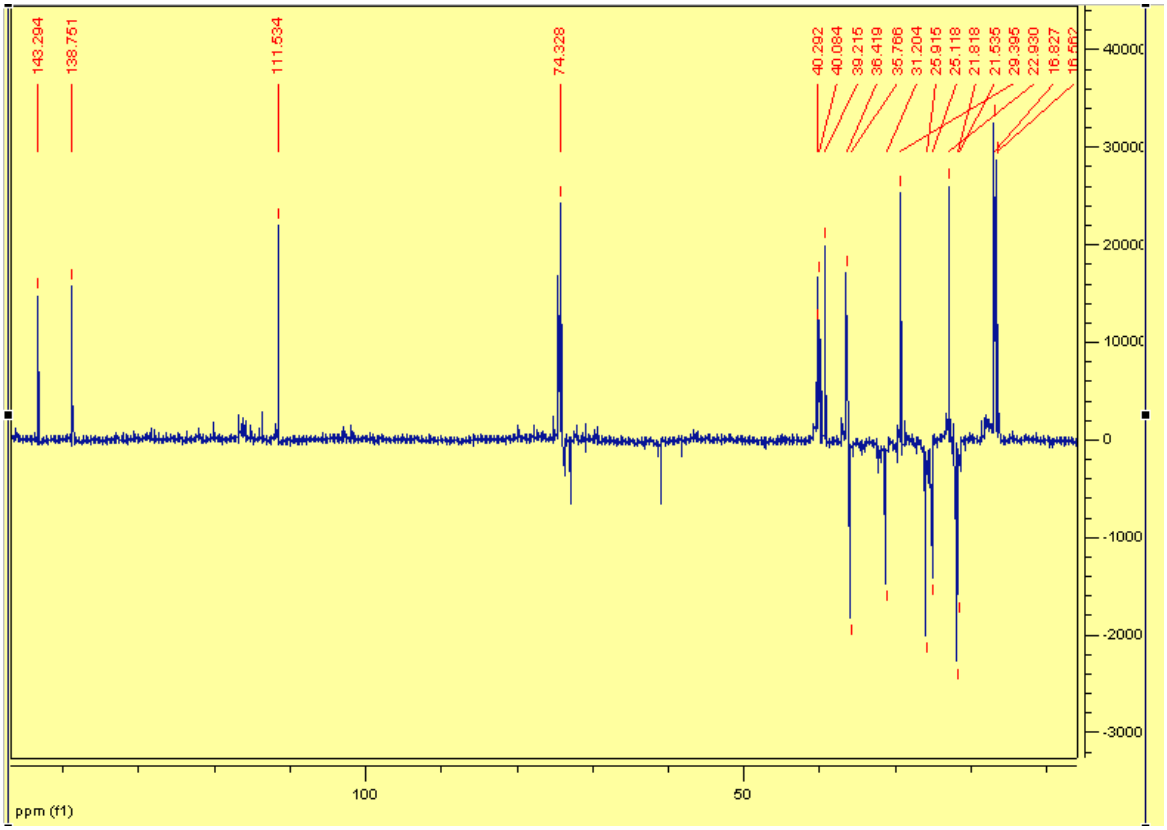

Figure 15: DEPT-135 spectrum of compound A-1 in dutrated dimethyl sulfoxide

Therefore, compound A-1 was finally identified as the labdane type diterpenoid, otostegindiol (15,16-epoxy-3α,9α-dihydroxy-labda-13(16),14-diene) (**44**), by comparing its spectroscopic data with those reported in the literature (Tesso and Konig, 2004).

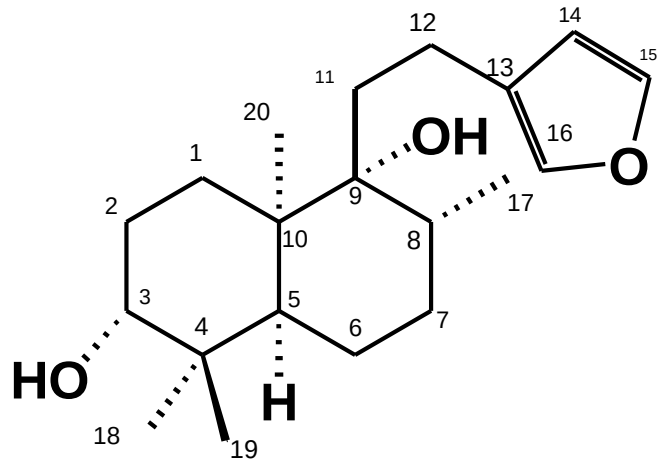

Figure 16: The structure of isolated compound **(**otostegindiol)

Table 9. ^{1}H NMR and ^{13}C NMR spectral signals of the isolated compound and otostegindiol (Tesso and Konig, 2004).

Assignment	^{1}H NMR (ppm)		^{13}C NMR (ppm)	
	Otostegindiol (Literature)	**Isolate (A-1)**	**Otostegindiol (Literature)**	**Isolate (A-1)**
1	1.25 1.93	1.21 1.93	25.1	25.12
2	1.64 1.96	1.56 1.96	25.6	25.92
3	3.38	3.16	76.3	74.33
4	–	–	43.2	42.95
5	1.90	1.96	39.8	39.23
6	1.34 1.50	1.30 1.54	21.6	21.82
7	1.35 1.46	1.31 1.48	31.4	31.21
8	1.79	1.77	37.1	36.42
9	–	–	77.5	76.34
10	–	–	37.8	37.69
11	1.67 1.91	1.71 1.97	35.5	35.77
12	2.46	2.38	21.9	22.93
13	–	–	126.1	126.32
14	6.27	6.32	111.3	111.53
15	7.34	7.50	143.2	143.29
16	7.22	7.37	138.9	138.75
17	0.92	0.93	16.5	16.57
18	0.97	0.99	22.5	22.9
19	0.87	0.88	29.0	29.39
20	0.96	0.95	16.8	16.83

4.7. Antimalarial activity of otostegindiol

The isolated compound (otostegindiol) was tested against *P. berghei* in mice and showed a dose dependent chemossepressive effect at various doses employed in this study. The chemosuppression was 50.13%, 65.58% and 73.16% at doses 25, 50 and 100 mg/kg/day, respectively (Table 10). The chemosuppression produced by otostegindiol was significant ($P<0.001$) when compared to the negative control group. The standard drug (chloroquine 25 mg/kg/ day) caused 100% suppression and this value was significantly ($P<0.05$) higher than the chemosuppression values obtained in otostegindiol treated groups.

In the untreated infected mice, death was first observed with a mean survival time of 8.20±0.58 day post-infection followed by the lowest dose (25 mg/kg/day) on the first day of 9.80±0.80 post infection. Mice receiving highest dose of the isolate (100 mg/kg/day) lived longer with a mean survival time of 12.20±0.80 days post-infection, significantly ($P<0.01$) beyond the mean survival period of the untreated mice. The mean survival time was also significant ($P<0.05$) at a dose of 50 mg/kg/day with 11.20±0.58 days. As shown in Table 11, the mean survival time of mice receiving the isolated compound in a dose dependent manner. Although the mean survival time of mice receiving the lowest dose of the isolated compound (25 mg/kg/day) and the negative control group was not statistically significant, it was slightly higher in compound treated groups.

Table 10. Percentage suppression of otostegindiol isolated from the ethyl acetate fraction of the leaves of *Otostegia integrifolia* against *Plasmodium berghei*.

Drug/Isolate	**Dose (concentration) mg/kg/day**	**% Parasitaemia ±SEM**	**% Suppression**
Vehicle	0.2ml	26.93±1.34	–
Otostegindiol	25	14.43±0.69*	50.13
Otostegindiol	50	9.96±0.52*	65.58
Otostegindiol	100	7.76±0.19*	73.16
Chloroquine	25	0.00*	100

*The mean value is significant ($P<0.001$) when compared with the negative control; data are expressed as means ± SEM for five mice per group.

Table 11. Mean survival time of *Plasmodium berghei* infected mice after treatment with otostegindiol.

Drug/Isolate	Dose (concentration) mg/kg/day	Survival time ± SEM (day)
Vehicle	0.2 ml	8.2±0.58
Otostegindiol	25	9.80±0.80
Otostegindiol	50	11.20±0.58***
Otostegindiol	100	12.20±0.80**
Chloroquine	25	28.00±00*

***The mean value is significant (P<0.05) when compared with the negative control; **the mean value is significant (P<0.01) when compared with the negative control; *the mean value is significant (P<0.001) when compared with the negative control; data are expressed as means ± SEM for five mice per group.

In addition to antimalarial activity, the otostegindiol protected the infected mice from weight loss. All otostegindiol treated groups increased weight when compared to the negative control group though the increase was not statistically significant (P>0.05). Compound treated groups also showed better percent increment compared to the standard drug chloroquine (Table 12). Animals receiving smaller doses of the isolated compound gained less weight than those treated with higher doses. The highest percent increments (3.99%) were seen in the group taking 100 mg/kg/day, while the negative control group showed a decrease in weight (-3.28%).

Table 12. Body weight of *Plasmodium berghei* infected mice after the administration of otostegindiol.

Drug/extract	Dose (concentration) mg/kg/day	Weight D0 ±SEM	Weight D4 ±SEM	% Change
Vehicle	0.2ml	25.20±0.21	24.40±0.26	-3.28
Otostegindiol	25	25.04±0.68	25.56±0.47	2.03
Otostegindiol	50	24.50±0.96	25.08±0.72	2.31
Otostegindiol	100	23.06±0.42	24.02±0.43	3.99
CQ	25	25.48±0.29	25.62±0.46	0.52

Data are expressed as means ± SEM for five mice per group; D0: weight pre-treatment on day zero; W4: weight post-treatment on day five.

As mentioned earlier, the isolated compound was identified as a labdane type diterpenoid. Many diterpenoids are known to exhibit antimalarial activities (Miyaoka *et al*., 1998; Loyola *et al*., 2004; Jullian *et al*., 2005; Sathe *et al*., 2010). Labdane diterpenoids isolated from the seeds of *Aframomum zambesiacum* (**8**) and the leaves of *Nuxia sphaerocephala* have been reported to possess antiplasmodial activity against chloroquine resistant *P. falciparum in vitro*. In addition, the labdane type diterpene, ozic acid, and other constituents isolated from the dichloromethane bark extract of *Pycnanthus angolensis* (Myristicaceae) have been shown to exhibit *in vitro* antimalarial activity against *P. falciparum* (Kaur *et al*., 2004; Batista *et al*., 2009).

According to Deharo *et al*. (2001) and Munoz *et al. (*2000) an *in vivo* antiplasmodial activity can be classified as moderate, good, and very good if an extract displays percent parasite suppression equal to or greater than 50% at a dose of 500, 250 and 100 mg/kg body weight per day. Based on this classification, the crude 80% methanol leaf extract and the ethyl acetate fraction of *O. integrifolia* exhibited a moderate antiplasmodial activity. Furthermore, a mean group parasitaemia level of less than or equal to 90% of the negative control groups usually indicates that the test compound to be active in standard screening studies (Dikaso *et al*., 2006). Therefore, this study indicates that hydroalcoholic extract and all fractions were active in four day suppression test.

The mechanism of action of the extract needs to be elucidated although various mechanisms of action have been postulated for antimalarial activity of natural products. The suggested mechanisms of action for some antimalarial compounds isolated from plants are:

i. inhibition of hemozoin polymerization in the parasite,
ii. intercalation with the parasite DNA,
iii. inhibition of *Plasmodium falciparum* lactate dehydrogenase(pfLDH), an essential enzyme for energy generation within the parasite through glycolysis, and
iv. alkylation (Wright *et al*., 2001; Akuodor *et al*., 2010; Adebayo and Krettli, 2011)

The extracts and the isolated compound could have elicited their action through any of the above mentioned mechanism or by some other means yet to be determined.

4. CONCLUSION

In this study, the acute and sub-acute toxicity, and antiplasmodial evaluations of the 80% methanol extract and solvent fractions of *O. integrifolia* were carried out. A labdane type diterpenoid identified as 15,16-epoxy-3α,9α-dihydroxy-labda-13(16),14-diene was isolated from the most active ethyl acetate fraction. From the results of this study it can be concluded that the plant extract is relatively safe to mice. Phytochemical screening of the total leaf extract of *O. integrifolia* indicated the possible presence of flavonoids, phenolic compounds and saponins. Both the hydroalcoholic leaf extract and solvent fractions showed significant antimalarial activity on four day suppression test at the tested doses. Although the ethyl acetate fraction was much more potent than the other fractions, its activity was less than that of the crude extract. Evaluation of the antimalarial effect of the major compound isolated from the ethyl acetate fraction indicated that the compound was highly active. Furthermore the hydroalcoholic extract, fractions and isolated compound prolonged the survival time of infected mice and prevented weight loss when compared to the untreated croups. The results reported in this study illustrate that correlations exist between the traditional use of leaf extracts of *O. integrifolia* and genuine antimalarial activity.

RECOMMENDATIONS

Based on the present study the following recommendations are proposed:

- Investigation of crude extract and the isolate on other *in vivo* and *in vitro* models.
- Isolation of other minor compounds from the ethyl acetate fraction and investigate their antimalarial activities.
- Beside the leaf, other parts of the plant like the roots might require investigations for their antimalarial activity; and
- Further toxicological studies including chronic toxicity tests must be carried out.

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DECLARATION

I, the undersigned, declare that this thesis is my original work, has not been presented for a degree in this and any other universities and that all sources of materials used for this thesis have been duly acknowledged.

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