
# Antimalarial activity of the root extract of *Lobelia giberroa* Hemsl. and its major constituent against *Plasmodium berghei* infection in mice.

By

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## Addis Ababa University

## School of Graduate Studies

This is to certify that the thesis prepared by Getnet Tadege, entitled: “Antimalarial activity of the root extract of *Lobelia giberroa* hemsl. and its major constituent against *Plasmodium berghei* infection in mice.” and submitted in partial fulfillment of the requirements for the Degree of Master of Science (in Pharmacognosy) complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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## Abstracts

Antimalarial activity of the root extract of *Lobelia giberroa* Hemsl. and its major constituent against *Plasmodium berghei* infection in mice.

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Addis Ababa University, 2021

*Lobelia giberroa* Hemsl. is an indigenous plant in Ethiopia. Ethiopian traditional healers use its different parts to treat malaria, bacterial and fungal infections, and cancer. For treatment of malaria, in particular, the traditional healers utilize the plant's root parts. This study was carried out to investigate the antimalarial activity of *L. giberroa*.

The roots of *L. giberroa* were extracted using 80% methanol and the hydroalcoholic extract was successively fractionated with hexane, ethyl acetate, methanol and water. Chromatographic and spectroscopic methods were used to isolate and identify the active compound from the methanol fraction. Acute oral toxicity study was conducted on the hydroalcoholic extract, solvent fractions, sub-fractions and the isolated compound. The hydroalcoholic extract and the isolated compound were evaluated for antimalarial activity using the standard four-day suppressive method, Rane's and prophylactic tests in *Plasmodium berghei* infected albino mice. The solvent fractions and sub-fractions from column chromatography were evaluated for antimalarial activity using four-day suppressive method.

The hydroalcoholic extract, solvent fractions, subfractions and the isolated compound were found to be safe at a dose of 2000 mg/kg. The hydroalcoholic extract of *L. giberroa* root and its methanol fraction exhibited the highest antimalarial activity and significantly increased the mean survival time of the treated mice. In fact, the hydroalcoholic extract exhibited 73.05%, 49.35% and 43.16% parasitemia suppression for four-day suppressive test, Rane's test and prophylactic

test, respectively. The methanol fraction demonstrated 64.37% parasitemia suppression for four-day suppressive test.

The isolated compound, characterized as lobetyolin, suppressed the level of parasitaemia by 39.96, 53.46 and 68.21% at a dose of 25, 50 and 100 mg/kg, in standard four-day suppressive test, respectively. Mice treated with 100 mg/kg/day lobetyolin survived longer (18.6 days) when compared with negative control group (6.8 days).

In conclusion, the current study supports the traditional use of the plant for the treatment of malaria and identified the main active compound lobetyolin, the potential to be an antimalarial lead for further development. The active ingredient, lobetyolin, was also isolated for the first time from the root of *Lobelia giberroa*. To the best of our knowledge, this is the first report on the biological activity of *L. giberroa*.

**Key words**; *Lobelia giberroa*, antimalarial activity, *in vivo*, solvent fraction, *Plasmodium berghei*, lobetyolin

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## List of Acronyms

| | |
|---|---|
| 2D NMR | Two-dimensional nuclear magnetic resonance |
| ACTs | Artemisinin-based combination therapies |
| ANOVA | Analysis of variance |
| API | Atmospheric pressure ionization |
| CC | Column chromatography |
| $CHCl_3$ | Chloroform |
| CRPV | Chloroquine resistant *P. vivax* |
| DAD | Diod array detector |
| DMSO-d6 | Deuterated dimethyl sulfoxide |
| $ED_{50}$ | Effective dose that suppress 50 % of parasitaemia |
| EPT | Distortionless enhancement by polarization transfer |
| ESI | Electrospray Ionization |
| EtOAc | Ethyl acetate |
| IR | Infrared |
| LC-MS | Liquid chromatography–mass spectrometry |
| LGCE | *Lobelia giberroa* crude extract |
| MeOH | Methanol |

| | |
|---|---|
| NMR | Nuclear magnetic resonance |
| OECD | Organization of economic cooperation and development |
| TLC | Thin-layer chromatography |
| TMS | Tetramethylsilane |
| UV | Ultraviolet |
| WHO | World health organization |
| α7-nicAchRs | Alpha-7 nicotinic receptors |

# 1. Introduction

## 1.1. Malaria

Malaria is a protozoal disease transmitted by the bite of infected female Anopheles mosquitoes of the genus *Plasmodium* and family Plasmodidae (Sutherland and Polley, 2011). Human malaria is caused by *P. falciparum, P. vivax, P. ovale, P. malariae,* and *P. knowlesi*. with *P. falciparum* causing the most fatal and severe type of malaria disease (Tangpukdee *et al.*, 2009). Malaria is common in tropical and subtropical regions of the world and mainly affects children and pregnant women (Hanboonkunupakarn and White, 2015). Malaria is preventable and treatable. But it continues to have a devastating impact on people's health and livelihoods throughout the world (Fischer *et al.*, 2020). Even though there is a significant progress in malaria control during the last decade, huge burden of malaria is still persisting especially in sub-Saharan African countries (Korenromp *et al.*, 2013).

There were 29 million malaria cases reported which resulted in 409,000 deaths in 2020 alone (WHO, 2020). Although malaria cases are reported from 87 countries globally, Africa contributes for 94% of total cases, particularly the sub-Saharan Africa (Al-Awadhi *et al*,. 2021).

According to the world malaria report of the year 2016, in Ethiopia the number of people living in high transmission (1 case per 1000 population) and low transmission (0-1 cases per 1000 population) areas was estimated to be 27,000,000 (27%) and 40,600,000 (41%), respectively (WHO, 2016). In most parts of Ethiopia, the distribution of malaria is seasonal with variable transmission and prevalence rates. It is affected by the large diversity in altitude, rainfall and population movement (Derbie and Alemu, 2017).

## 1.2. Antimalarial natural products

About half a million medicinal plants are available around the world and some could be of significant importance for present and future treatment of malaria, which is a leading cause of death in developing countries ( Hassan, 2012). The first antimalarial drug was quinine (**1**) which was isolated from the bark of *Cinchona* (Wallaart *et al.*, 1999). Since then, malaria has been treated with quinoline based drugs such as chloroquine (**2**), mefloquine (**3**) and primaquine (4) (Wallaart *et al.*, 1999).

Artemisinin and its semisynthetic derivatives are a group of drugs used in the treatment of malaria due to *P. falciparum* (Pinheiro *et al.*, 2018). Artemisinin (**5**), a sesquiterpene lactone containing an endoperoxide bridge, has been isolated from the arial parts of *Artemisia annua* (Klayman, 1985). Because of poor pharmacological profile of Artemisinin, scientists rapidly developed derivatives from dihydroartemisinin (**6**) (Saxena *et al.*, 2013) with better properties, in particular better solubility in oil or water (Magueur *et al.*, 2004). The semisynthetic ethers or esters, such as artemether (**7**), arteether (**8**) and artesunate (**9**) are being widely used for therapy in Asia and in Africa. Another drug Artelinate (**10)** is in clinical development (Magueur *et al.*, 2004) (Figure 1). These endoperoxides have several advantages such as desired antimalarial effectiveness against resistant *P. falciparum* over existing antimalarial drugs (Brossi *et al.*, 1988; Magueur *et al.*, 2004).

Although, artemisinin and its analogues have provided much needed drugs for the treatment of chloroquine-resistant malaria, they are unavailable and/or unaffordable to many people who live in malaria epidemic areas (Wright, 2005). Artemisinin and its semisynthetic analogs artemether,

artether, and artesunate are potent antimalarial agents especially used in the regions where resistance has developed to other antimalarial agents (Oliveira *et al.*, 2009).

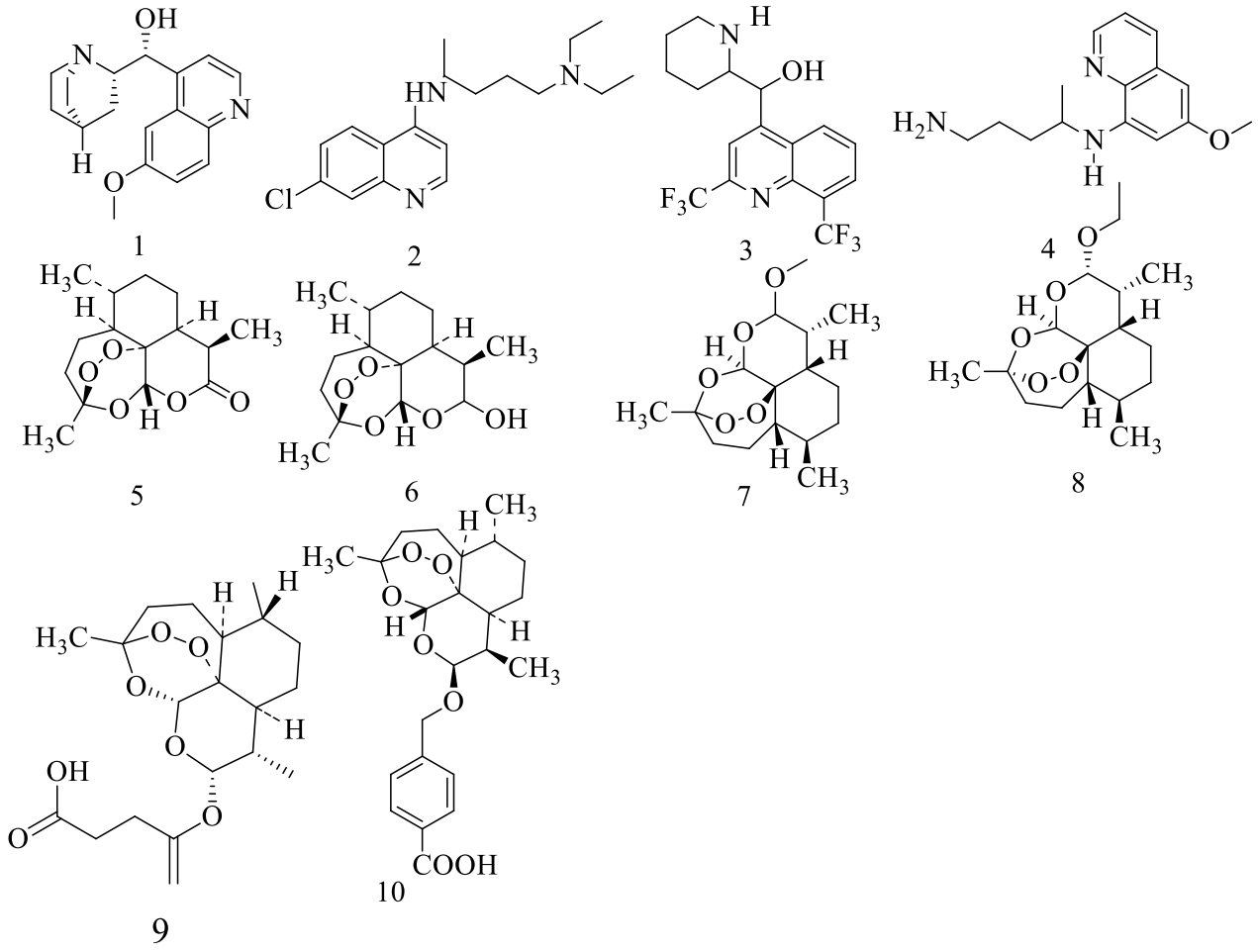



**Figure 1:** Natural products and natural product-derived antimalarial compounds (Onori, 1984; Klayman, 1985; Wiesner *et al.*, 2003).

## 1.3. Genus *Lobelia*

*Lobelia* (Campanulaceae, Lobelioideae subfamily) is a genus of flowering plants comprising of 415 species with a distribution primarily in tropical to warm temperate regions of the world and a few species extending into cooler temperature regions (Stolom *et al.*, 2016). The genus *Lobelia*

comprises a substantial number of large and small annual, perennial and shrubby species, hardy and tender, from a variety of habitats, in a range of colors (Brochmann *et al.*, 2018).

Many species appear totally dissimilar from each other. However, all have simple, alternate leaves and two-lipped tubular flowers, each with five lobes. The upper two lobes may be erect while the lower three lobes may be fanned out (Marasinghe *et al.*, 2019). Flowering is often abundant and the flower color is intense, hence their popularity as ornamental garden subjects (Garbuzov and Ratnieks, 2014). Some species of this genus have circular leaf spots, centrally white greyish, with distinct dark brown margins, becoming subcircular to irregular, tissue collapsing in the necrotic areas and often torn (Marasinghe *et al.*, 2019).

This genus has a wide distribution, in Africa, North America, South America, Asia, Australia, New Zealand, Caribbean, Hawaii and other regions (Kokubugata *et al.*, 2012). *L. chinensis* is one of the species in genus *Lobelia,* distributed widely in East Asian countries (Kuo *et al*., 2011). *L. inflata* is the most investigated species belonging to this genus, which is widely distributed in North America and cultivated in the Netherlands, Poland and Russia (Mohrig *et al.*, 2013). From this genus *L. rhynchopetalum* and *L. erlangeriana* are endemic Ethiopian plants, whereas *L. giberroa* is an indigenous medicinal plant in Ethiopia (Thulin, 2006).

### 1.3.1. Ethnobotonical use

The species of the genus *Lobelia* are commonly used for the management of respiratory problems, cancer, inflammatory diseases, hepatic diseases, microbial infection, rheumatic disease, cathartic disease, muscle disorders, rabies, epilepsy and others as depicted in Table 1.

**Table 1**: Ethnobotanical uses of some members of the genus *Lobelia.*

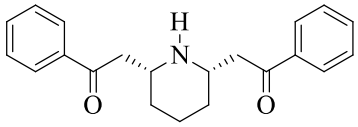
| Plant's name | Plant part used | Claim | References |
|---|---|---|---|
| *L. alsinoides* | Whole plant | Hepatic diseases | Binitha *et al.*, 2019 |
| *L. chinensis* | Whole plant | Anticancer, anti-inflammatory | Shibano *et al.*, 2001, |
| | Rhizome | Antiasthmatic | Felpin and Lebertn, 2005; Joshi *et al.*, 2011 |
| | Roots | Antirheumatic, antisyphilitic, cathartic and diuretic | Joshi *et al.*, 2011 |
| *L. flaccida* | Leaf | Pain and epilepsy | Stolom *et al*., *et al.*, 2016 |
| *L. inflata* | Whole plant | Respiratory and muscle disorders | Oyedemi *et al.*, 2020 |
| *L. nicotianaefolia* | Roots | Tonsillitis, stomachache | Prakasha and Krishnappa, 2006 |
| *L. pyramidalis* | Leaves and flowers | Intestinal spasms | Joshi and Edington, 1990 |
| | Leaves | Backpain, fever and antiasthmatic | Carolina *et al.*, 2010; Kumari *et al*., 2011 |
| *L. rhynchopetalum* | Leaves, stem and bark | Antimicrobial | Birhan *et al.*, 2017 |
| | Roots | Rabies, evil eye, malaria | Birhan *et al.*, 2017 |
| | Latex | Retained placenta, Donkey's wart | Birhan *et al.*, 2017 |

### 1.3.2.Phytochemistry

The major constituents of the genus *Lobelia* are reported to be piperidine alkaloids (Kuo *et al.*, 2011). In addition, *Lobelia* species have also been reported to contain polyacetylenes, anthocyanins, triterpene palmitate, polyhydroxylated piperidine, pyrrolidine alkaloids, and flavonoid glycosides (Shibano *et al.*, 2001).

The piperidine alkaloids of the genus include norlobelanine (**11**), lobeline (**12**), lobelanidine (**13**), lobelanine (**14**) (Yang *et al.*, 2014), sedinine (**15**) and pentylsedinine (**16**) isolated from different *Lobelia* species (Paz *et al.*, 2015). Among the piperidine alkaloids, pentylsedinine (**16**) comprise five carbons in the side chain and was isolated from *L. tupa* (Paz *et al.*, 2015). The pyrrolidine

alkaloids radicamine A (**17**) and radicamine B (**18**) were reported from *L. chinensis* (Paz *et al.*, 2015). Apigenin (**19**), luteolin (**20**), quercetin (**21**), linarin (**22**), and luteolin 3, 4-dimethylether-7-*O*-*β*-D-glucoside (**23**) are among the flavonoids isolated from *L. chinensis* (Yang *et al.*, 2014). Moreover, terpenoids isolated from the genus *Lobelia* were cycloeucalenol (**24**) from *L. chinensis*, β-sitosterol (**25**) from *L. davidii* and ursolic acid (**26**) from the aerial part of *L. sessilifolia* (Folquitto *et al.*, 2019). Chemical structures of some compounds reported from the genus *Lobelia* are given in Figure **2**.

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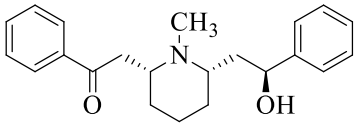


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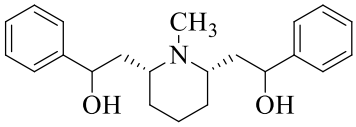


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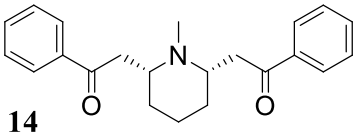


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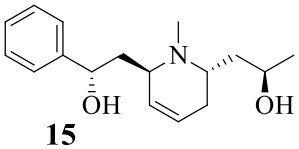


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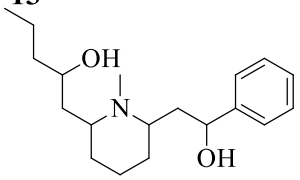


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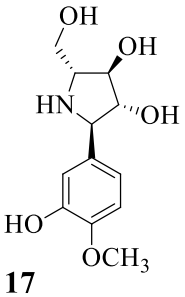


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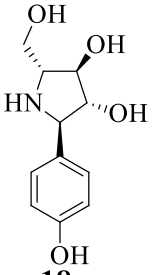


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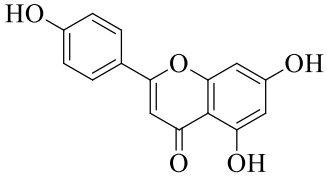


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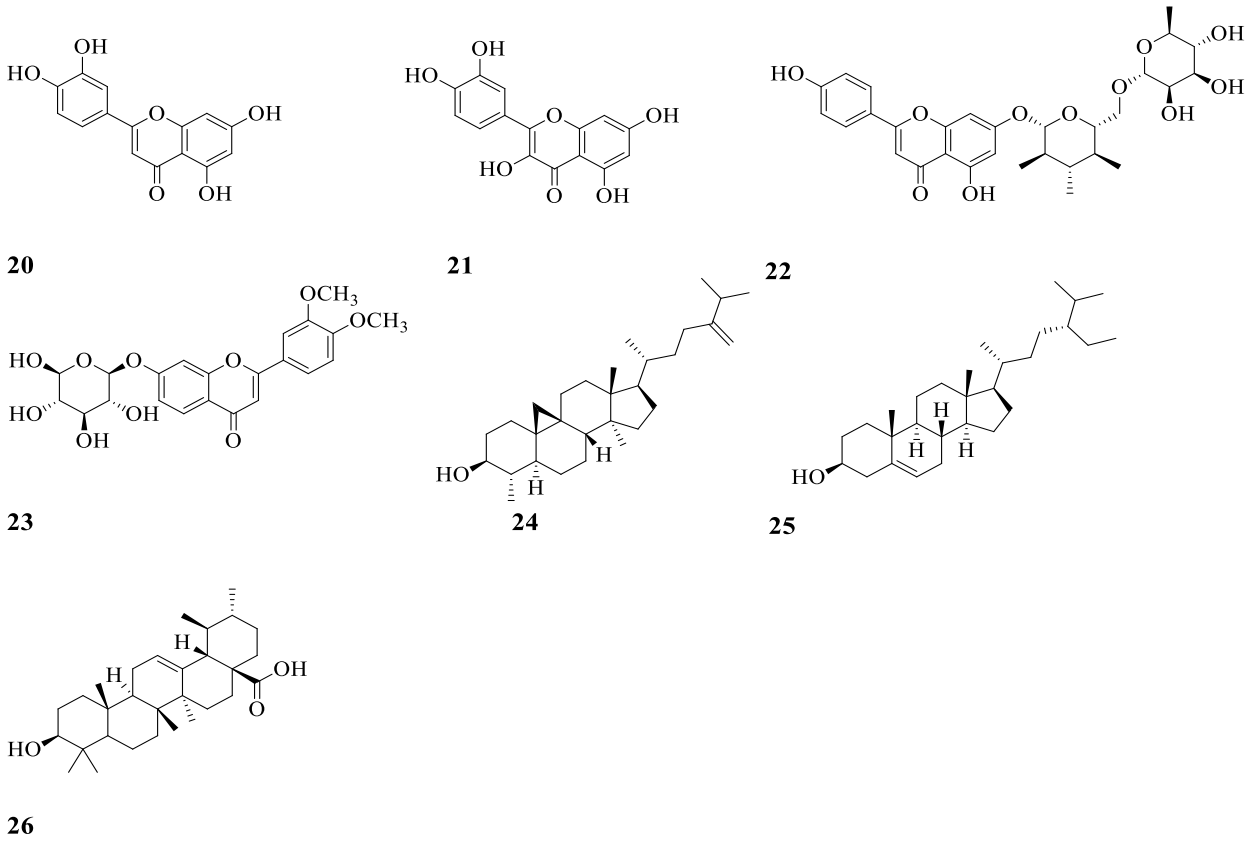


Figure 2: Chemical structures of some compounds isolated from the genus *Lobelia* (Li *et al.*, 2015; Yang *et al.*, 2014; Philipova *et al.*, 1998).

## 1.3.3. Biological activities

The genus *Lobelia* possesses promising biological activities such as diuretic, choleretic, breathing excitement, anti-venom, anti-bacterial, and anticancer (Stansbury *et al*., 2013). *In vitro* experiments on alkaloids and flavonoids from different *Lobelia* species including *L. chinensis* indicated their contribution for cytotoxicity activity, but the mechanism of action has been searched rarely (Zheng *et al*., 2021). A plant preparation containing *L. chinensis* restrain tumor growth *in vivo* (Yang *et al.*, 2014). Furthermore, apigenin and luteolin isolated from *L. chinensis*

showed cytotoxic activity by stopping the cell growth cycle (Yang *et al.*, 2014; Kobayashi *et al.*, 2002).

Essential oils from *L. pyramidalis* and *L. flaccida* were reported to have activity against *Staphylococcus aureus* among the bacterial strains and *Trichophyton mentagrophytes* among the fungal strains (Mishra *et al*., 2011). Their antimicrobial activity may be related to the presence of perilla ketone and isophytol as the major compounds (Folquitto *et al.*, 2019).

The characteristic constituent of *Lobelia* is the alkaloid lobeline, which is known for its beneficial effects on the function of respiratory tract problems by stimulating breathing, supporting the cough reflex and improving vascular tone (Ziment and Tashkin, 2000). Lobeline which is isolated from *L. chinensis* is widely used in the treatment of CNS diseases (Rahman and Monem, 2014). Lobeline is shown to improve memory in rodents, probably due to its involvement in cholinergic mechanisms of neurotransmission, which is claimed to be the most common cause of Alzheimer's disease (Houghton and Howes, 2005).

Lobinaline (14), a complex binitrogenous alkaloid, which is extracted from *L. cardinalis* shows nicotinic acetylcholine receptor (nicAchR) binding profile. Purified lobinaline which is a potent free radical scavenger displayed agonist activity to nicAchRs in SH-SY5Y cells (Brown *et al.*, 2016; Subaranas *et al*., 1993).

The anti-inflammatory properties of metabolites from *L. chinensis* were also reported for the inhibition of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX- 2), tumor necrosis factor-α (TNF- α) and interleukin-6 (IL-6) from the NF-κB pathway (Li *et al.*, 2015). Flavonoids in *L. chinensis* possess anti-inflammatory activities *via* scavenging reactive oxygen species and

reducing pro-inflammatory cytokines, such as chlorogenic acid, apigenin, luteolin and buddleoside (Hung *et al.,* 2011; Li *et al.,* 2015).

## 1.4. *Lobelia giberroa* Hemsl.

### 1.4.1. Botanical description and distribution

*L. giberroa* Hemsl. is an indigenous plant species to Ethiopia which is known with local names: Jibera (Amharic), Maranga (Afaan Oromoo) and Shambato (Kef) (Thulin, 2006). Its habitats are moist dry montane forest, forest edges, stream sides or swamp edges; 1700-2800 m altitude. *L. giberroa* is 2-9 m tall when in flower (Kebede *et al.*, 2007), stem is erect, woody at the base with simple alternate leaves. Rosette-leaves of non-flowering plant are narrowly oblanceolate to oblong-ovate, double serrate, dentate or denticulate, more or less pubescent. *L. giberroa* has two-lipped tubular flowers, each with five lobes, flowering is often abundant and the flower color is intense, hence their popularity as ornamental garden subjects (Meehan, 1902) . The upper two lobes may be erect while the lower three lobes may be fanned out (Crous *et al.*, 2012).

*L. giberroa* ( Figure 3) is the most widespread species of the giant *Lobelia* (Knox and Palmer, 1998). *L. giberroa* occurs in Ethiopia, Eritrea, Sudan, Eastern Democratic Republic of Congo, Rwanda, Burundi, Zambia and Malawi (Abate, 1989) and it is the only *Lobelia* found on most large and small mountains throughout the region (Knox and Palmer, 1998).

Figure 3: *Lobelia giberroa* in its natural habitat.

### 1.4.2. Ethnobotanical uses of *Lobelia giberroa* Hemsl.

In Ethiopia aqueous drink prepared from powdered roots of *L. giberroa* is used in the treatment of malaria and eye problems (Kassahun *et al*., 2019). The roots are also used for the treatment of impotence and epilepsy (Chekole, 2017).The stem and bark of *L. giberroa* are claimed to possess antimicrobial and antimalarial activities (Geyid *et al.*, 2005). The latex of the plant is used for the treatment of cancer (Abate, 1989). Furthermore topical application of the leaf preparation of L. giberroa is used by Tanzanian traditional healers for the treatment of fungal infections (Larrosa *et al.*, 2011).

## 1.5. Significance of the study

Malaria is still the world's most important parasitic disease that is responsible for the death of more people than any other communicable disease except tuberculosis (Visser *et al*., 2014).

Treating the large number of people infected with malaria has resulted in widespread resistance to current antimalarial drugs ( Guerin *et al.*, 2002; White, 2004). Thus, there is an urgent need for the discovery and development of novel, safe and effective antimalarial drugs to meet with the challenges of drug resistance (Navarro *et al*., 2010; Wang *et al.*, 2020).

To solve wide spread drug resistance, isolation of antimalarial natural products from traditional medicinal plants is considered as a leading strategy (Pieters *et al*., 2005). Thus, determining the antimalarial activity of plants and isolation of their active constituents is important (Cragg and Newman, 2013).

The roots of *L. giberroa* are traditionally used to treat malaria by Amhara and Oromo ethnic groups in central Ethiopia (Knox and Palmer, 1998). To the best of my knowledge, no scientific investigation has been performed to substantiate this claim. Thus, we conduct this study to determine the antimalarial activity of the root of *L. giberroa*.

# 2. Objective

## 2.1. General objective

To investigate *in vivo* antimalarial activity of the root extract of *L. giberroa* Hemsl. and perform bioassay guided isolation of active antimalarial compound(s).

## 2.2. Specific objectives

- To conduct acute oral toxicity study on the hydroalcoholic extract, solvent fractions and isolated compound from the root of *L. giberroa*.
- To investigate *in vivo* antimalarial activity of the hydroalcoholic extract and solvent fractions of *L. giberroa* root.
- To isolate, characterize and determine the antimalarial activity of the active constituent of root hydroalcoholic extract of *L. giberroa*.

# 3. Materials and Methods

## 3.1. Materials

### 3.1.1. Plant material

The roots of *L. giberroa* Hemsl. were collected from Menagesha Suba Forest, located 45 km South West of Addis Ababa. The fresh root was covered with plastic sheets during transportation. The plant was authenticated by Mr. Melaku Wondafrash, a senior botanist at the National Herbarium, College of Natural and Computational Sciences Addis Ababa university (AAU), where a voucher specimen was deposited with a voucher number of GT001

### 3.1.2. Chemicals, Reagents and Drugs

Hexane, chloroform, ethyl acetate (all from Sigma-Aldrich Co., MO, USA), methanol (Carlo Erba, France) and distilled water (Pharmaceutics laboratory, AAU) were the solvents used for extraction, fractionation and isolation. Chromatographic separation was performed by analytical TLC on Silica gel 60 F254 (0.2 mm thick), column chromatography on Silica gel 60 (70 - 240 mesh) (Merck KGaA, Darmstadt, Germany). The following chemicals and drugs were used during *in vivo* antimalarial activity test: Trisodium citrate (BDH Chemicals Ltd, England), Geimsa (ESJAY Chemicals, Maharashtra, India), pure chloroquine phosphate (kindly supplied by Ethiopian Pharmaceutical Manufacturing Factory, EPHARM, Ethiopia), Tween 80 (UNI-CHEM chemical reagents, India).

### 3.1.3. Instruments

$^{1}H$ and $^{13}C$ NMR spectrum were recorded on Bruker Avance DMx400 FT-NMR spectrometer using TMS as internal standard, at the Department of Chemistry, College of Natural Sciences,

Addis Ababa University, Ethiopia. Mass spectrometry was performed using an Agilent 1100 series system (Agilent system, USA), and ionization of sample was carried out using ESI-API (capillary voltage, 4000 V; fragmentor, 160 V; drying gas temperature, 350 ºC; gas flow ($N_2$), 10 l/min; nebulizer pressure, 50 psig). The purity of the compound was determined by using Agilent LC-MS system with diod array detector (DAD). IR spectrum was recorded on a 26 Perkin-Elmer 65 IR spectrometer College of Natural and Computational Sciences, Department of chemistry, Addis Ababa university. The optical rotation of the isolated compound was measured by using automatic polarimeter (RUDOLPH AUTOPOL® IV).

### 3.1.4. Experimental Animals and Parasites

Healthy 6-8 weeks old Swiss albino mice of either sex weighing 24-28 g were used during the experiment. Two hundred healthy Swiss albino mice of either sex were obtained from Ethiopian Health and Nutritional Research Institute (EHNRI) animal house, Addis Ababa. The mice were allowed to acclimatize to the environment for one week before the experiment. One hundred forty-four Swiss albino mice were also obtained from Addis Ababa University, College of Health Sciences, Department of Pharmacology and Clinical pharmacy animal house. All animals were housed in an air-conditioned room, kept at room temperature, exposed to a 12 h light/dark cycle, allowed access to standard pellets and water *ad libitum*.

Chloroquine sensitive *Plasmodium berghei* ANKA strain was obtained from Mekelle University, College of Health Sciences, Department of Pharmacology. The parasite was maintained by serial passage of blood from infected mice to non-infected mice on weekly basis. All procedures followed were in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH Guidelines for Care and Use of Laboratory Animals, 1996). Ethical approval (approval

number ERB/SOP/356/13/2021) was obtained from the Institutional Review Board of the School of Pharmacy (SOP), Addis Ababa University (AAU) (Annex 7).

## 3.2. Methods

### 3.2.1. Preparation of Plant Material

The roots of the plant were carefully washed with distilled water to remove dirt, dried under shade at room temperature (25–27$^{o}$C) with optimal ventilation for 15 days and crushed into coarse powder. The powdered plant material (1000 g) was macerated for 72 h with intermittent shaking and stirring. The filtrate was separated from the mark using filter paper Whatman number 1 and the mark was re-macerated two times with a fresh solvent. The organic solvent was removed by using rotary evaporator. The extract was further dried using a lyophilizer to remove water. The dried extract was transferred into vials and kept in a desiccator until use.

### 3.2.2. Fractionation

The hydroalcoholic extract was successively extracted with solvents of differing polarity (hexane, ethyl acetate, methanol and water) using Soxhlet apparatus. The organic solvents were removed by using rotary evaporator and the aqueous fraction was dried using a lyophilizer. Dried hexane, ethyl acetate, methanol and aqueous fraction were collected and percent yield calculated. The dried fractions were kept in separate vials and stored in a desiccator until used for the experiment.

### 3.2.3. Chromatographic techniques

The methanol fraction was subjected to column chromatography. The column was packed with silica gel (particle size of 0.2-0.5 mm) and conditioned overnight. To pack the column, the stationary phase was suspended in chloroform and poured into the column. After conditioning,

the sample was loaded on top of the column. Elution was started with 100% $CHCl_3$, followed by $CHCl_3$: $CH_3OH$ (9:1), $CHCl_3$: $CH_3OH$ (8:2) with repeated elution. The collected eluates were further analyzed using analytical Thin Layer Chromatography (TLC). The solvent system used was $CHCl_3$: MeOH (4:1). Developed chromatograms were visualized under UV (254 nm).

### 3.2.4. Structural elucidation

Chemical structure of the isolated compound was determined using NMR, LC-MS and IR. NMR spectrum was recorded at room temperature on Bruker Avance 400 FT-NMR spectrometer operating at 400.13 MHz for $^{1}H$ NMR and 100.6 MHz for $^{13}C$ NMR using DMSO-D6 as a solvent. The region from 0 to 20 ppm for $^{1}H$ and 0 to 205 ppm for $^{13}C$ was used for scanning. Signals were referred to an internal standard tetra methyl silane (TMS). Chemical shifts are reported in δ (ppm) units and coupling constants (*J*) in Hz. Multiplicities of $^{1}H$ NMR signals are indicated as *s* (singlet), *d* (doublet), *dd* (doublet of doublets), *t* (triplet) or *m* (multiplate).

Mass spectrometry was recorded and Purity of the compound was determined to be greater than 95 % using Agilent LS/MS system with diod array detector (DAD) as described in section 3.13. IR spectrum was recorded on a 26 Perkin-Elmer 65 IR spectrometer. Heteronuclear multiple bond correlation (HMBC) spectrum was taken for the isolated compound.

Methods used in this study are summarized in figure 4.

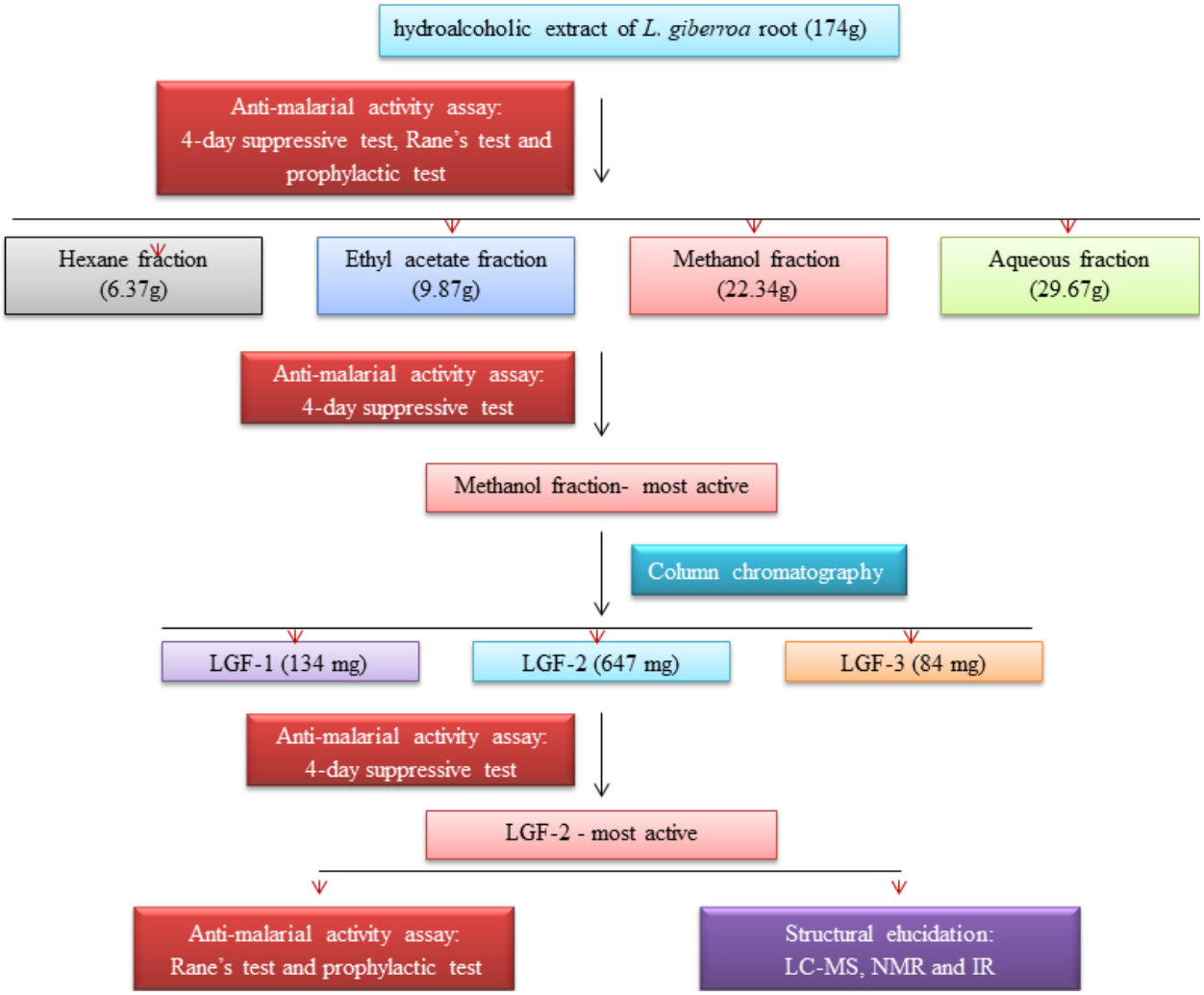


Figure 4: Bioassay guided isolation of an active compound from hydroalcoholic extract of *L. giberroa* root.

The optical rotation was measured by using automatic polarimeter (cell length 100 mm; sample temperature 19.8$^{o}$C; measuring time 10 second; concentration 0.54 %; observed rotation; -0.056$^{o}$Arc) to determine the rotation of the compound in the plane polarized light.

### 3.2.5. Acute oral toxicity study

Acute oral toxicity test for the hydroalcoholic extract, solvent fractions and sub-fractions (LGF-1, LGF-2 and LGF-3) of the roots of *L. giberroa* was conducted following the organization for

economic co-operation and development (OECD) guideline 425 (OECD, 2008). Forty healthy, female albino mice of 6–8 weeks of weighing 24-28 g were used to test the acute oral toxicity of the hydroalcoholic extract, each fraction or sub-fraction. All mice were fasted for 4 h before and 2 h after administration of the test samples. For each test sample, first, a single mouse was used for the study to determine the starting dose. For this, a single female mouse was given a single dose of 2000 mg/kg of the hydroalcoholic extract, a fraction or a sub fraction using oral gavage. Since neither death nor gross changes such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhea, mortality and other signs of toxicity were observed within 24 h after administering the test samples, additional four mice for each sample were used, and the same dose of the hydroalcoholic extract, fraction and sub-fraction was administered. The animals were observed continuously for 4 h with 30 min interval and then for 14 consecutive days with an interval of 24 h for the general signs and symptoms of toxicity, appetite and mortality.

### 3.2.6. Inoculation of mice

Blood smear was prepared on microscope slides from blood films taken from the donor (infected) mouse tail. The smear was fixed with absolute methanol and stained with Giemsa to determine parasitemia level of the donor mouse under a microscope. Mice with a parasitemia level of approximately 30% were used as a donor of chloroquine sensitive strain of *P. berghei* (ANKA) throughout the experiment. These mice were then sacrificed by cervical dislocation, and blood was collected in a Petri dish with an anticoagulant (0.5% trisodium citrate) by severing the jugular vein. The blood was then diluted with physiological saline (0.9%) based on parasitemia of the donor mice and the red blood cells (RBC) count of normal mice in such a way

that 1 ml blood contained 5 x $10^7$ infected erythrocytes. Each mouse then received 0.2 ml of diluted blood containing 1 x $10^7$ *P. berghei* infected erythrocytes by intraperitoneal (ip) route.

### 3.2.7. Animal grouping and dosing

During investigation of the hydroalcoholic extract, *P. berghei* infected mice were randomly divided into five groups of 5 mice each. Group I and II served as negative and positive controls, and 0.2 ml distilled water and chloroquine 25 mg/kg/day (CQ25) were administered to the mice, respectively. The remaining groups (III, IV and V) were treated with the hydroalcoholic extract of *L. giberroa* at 100 mg/kg/day (LGCE100), 200 mg/kg/day (LGCE200) and 400 mg/kg/day (LGCE400), respectively.

The study on the fractions was conducted using twenty-five mice for each fraction. Mice were randomly assigned into three treatment groups and two controls, five mice per group for each fraction. The first group received the vehicle used for reconstitution (Tween 80, 2% v/v in water for the hexane, ethyl acetate and methanol fractions, 0.2ml distilled water for aqueous fraction) and the second group was treated with the standard, CQ25 mg/kg/day. Treatment groups were given the fractions at a dose of 100 mg/kg/day, 200 mg/kg/day and 400 mg/kg/day dissolved in the respective vehicle.

Similarly, during the investigation of the column sub-fractions (LGF-1, LGF-2 and LGF-3), twenty-five *P. berghei* inoculated mice were randomly divided into five groups for each sub-fraction from column, each group containing five mice. The first group served as a negative control (Tween 80, 2% v/v in water) and the second group as positive control, CQ25 mg/kg/day. The other three served as treatment groups and received sub-fractions at a dose of 25, 50 and 100 mg/kg/day. All the test substances were administered orally using oral gavage.

Treatment was started 2 h post-infection on day 0 and continued daily for 3 days (i.e., from day 0 to day 3). On the fifth day (or day 4), Giemsa-stained thin blood film was prepared from the tail of each mouse to count the number of parasites under the microscope with an oil immersion objective of 100 x magnification power.

### 3.2.8. *In vivo* antimalarial activity test

#### 3.2.8.1. Four-day suppressive test

The four-day suppressive test on mice infected with chloroquine sensitive *P. berghei* was employed according to the method described by Peters (1975). For testing each sample, 25 mice were injected with inoculum of 1x $10^7$*P. berghei* as described in section 3.2.6. Two-hour post-infection, the mice were randomly distributed into five groups (for hydroalcoholic extract, each solvent fraction and each sub-fraction) and treated as indicated in section 3.2.7. Dosing was continued for additional three consecutive days 24, 48 and 72 h post-infection (until day 3). On day 4 of the experiment (at 96 h post-infection), blood was collected from the tail of each mouse and thin smear was prepared on a microscope slide to determine level of parasitemia. In addition, weight, temperature and packed cell volume (PCV) were measured just before infection and at the end of the experiment. Afterwards, mice were followed for 28 (day 0-day 27) days to determine the mean survival time (MST) for each group.

#### 3.2.8.2. Rane's test

Curative potential of the hydroalcoholic extract at doses of 100, 200 and 400 mg/kg/day or LGF-2 at doses of 25, 50 and 100 mg/kg/day were also evaluated using the method described by Ryley and Peters (1995). In this experiment, 25 mice were IP inoculated with an inoculum of 1 x $10^7$*P. berghei* infected erythrocyte. After 72 hours (day 3), the animals were randomly assigned into

five groups with five mice in each group. Treatment was continued for further 3 days (i.e., 96, 120, 144 h post-infection) with hydroalcoholic extract at doses of 100 mg/kg/day, 200 mg/kg/day or 400 mg/kg/day and LGF-2 at 25 mg/kg/day, 50 mg/kg/day and 100 mg/kg/day. Geimsa stained thin blood film was prepared using blood from the tail of each mouse daily for 5 days to monitor parasitemia level. Body weight, packed cellular volume (PCV), and temperature of the mice were also recorded on the $4^{th}$ (before the first dose) and $8^{th}$ days (24 hours after the last dose).

#### 3.2.8.3. Peter's repository test

Evaluation of the prophylactic potential of the hydroalcoholic extract and the most active sub-fraction (LGF-2 was carried out following the method described by Peters (1975). Accordingly, 25 mice were randomly distributed into five groups for testing each sample of hydroalcoholic extract and LGF-2. The mice were treated with LGCE at 100 mg/kg/day, 200 mg/kg/day and 400 mg/kg/day and LGF-2 at 25 mg/kg/day, 50 mg/kg/day and 100 mg/kg/day for four consecutive days. On the $5^{th}$ day, the mice in all groups were infected with inoculums of 1 x $10^7$ *P. berghei* infected erythrocyte. On the $8^{th}$ day, blood smears were prepared from each mouse and the parasite level was determined. Body weight, temperature, and PCV were recorded at day 5 before infection and at the end of treatment.

#### 3.2.8.4. Determination of parasitemia and survival time

A thin smear of blood from each mouse was applied on a different microscopic slide. The smear was fixed with absolute methanol and stained with 10% Geimsa stain for 15 min. Then, the slides were taken out, washed with gentle passage of tap water and dried at room temperature. With little drop of oil immersion, the number of parasite infected RBC were counted using light

microscope with the objective lens of 100 × magnification power. The parasitemia was determined by counting minimum of three fields per slide (Zucker, 1993). Percent parasitemia and percent parasitemia inhibition was calculated using the modified Peters and Robinson formula (Peters, 1992):

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

$$\% \text{ Suppression} = \frac{\% \text{ Parasitemia in negative control} - \% \text{ Parasitemia of treated group}}{\% \text{ Parasitemia in negative control}} \times 100$$

The dose that cured 50% of infected animals in four-day suppressive test had been determined as effective dose (ED50) using a nonlinear regression logistic dose-response model for isolated compound in four-day suppressive model.

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

### 3.2.8.5. Determination of packed cell volume, rectal temperature and body weight

Blood was collected from tail of each mouse in heparinized microhematocrit capillary tubes to 3/4$^{th}$ of their original height and sealed at their dry end with sealing clay. The tubes were then placed in a microhematocrit centrifuge with the sealed ends out wards. The blood was centrifuged at 12,000 rpm for 5 minutes. PCV was determined using the following relation indicated by Wote *et al* (2014).

$$\text{PCV} = \frac{\text{Volume of erythrocytes in a given volume of blood}}{\text{Total blood volume}} \times 100$$

Each mouse in a group was weighed using sensitive digital weighing balance and rectal temperature was measured using digital rectal thermometer. The percentage changes of their mean values that occurred before and after treatment were then calculated.

### 3.2.9. Data analysis

Data were entered into Statistical Package for Social Sciences (SPSS) version 25. The results were summarized as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to compare differences in mean among the groups. *P* value of less than 0.05 was considered statistically significant.

# 4. Results and Discussions

## 4.1. Percentage yields of the hydroalcoholic extract and fractions

Although, water extract of the root of *L. giberroa* is used by the community and traditional healers (Chekole, 2017), initial extraction was performed (1000 g powdered roots) using 80% methanol for this study. Alcoholic (methanol or ethanol) or hydroalcoholic extracts of plant materials contain a wide variety of polar and moderately polar compounds than a pure solvent (Otsuka, 2006). In this study 80 % methanol was used for extraction. Fractionation of the hydroalcoholic extract (78g) was made based on differences in polarity of the solvents that are immiscible with water.

The hydroalcoholic extract of *L. giberroa* afforded a reddish brown amorphous solid, while the hexane and ethyl acetate fractions were yellow brown colored semi solids. The aqueous fraction yielded a light brown solid material while the methanol fraction yielded a light brown semi-solid material. The percentage yields of the hydroalcoholic extract and solvent fractions are shown in Table 2.

**Table 2:** Actual and percentage yields of the hydroalcoholic extract and solvent fractions obtained from the roots of *L. giberroa*.

| Extract | Actual yield (g) | Percentage yield (w/w) |
|---|---|---|
| 80% hydroalcoholic extract | 174 | 17.4 |
| Hexane fraction | 6..37 | 8.17 |
| Ethyl acetate fraction | 9.87 | 12.65 |
| Methanol fraction | 22.34 | 28.64 |
| Water fraction | 29.67 | 38.04 |

## 4.2. Acute oral toxicity test

Toxicity is the main concern of therapeutic preparations from medicinal plants (Ahmed *et al*., 2006). Evaluation of the acute oral toxicity test of the hydroalcoholic extract, fractions and the compound of the roots of *L. giberroa* indicated that none of the tested substances caused gross behavioral changes and mortality within the next 14 days of follow up. This implies that the $LD_{50}$ value of the extract, fractions and the compound was greater than 2000 mg/kg in mice. Therefore, the constituents of the roots of *L. giberroa* are safe. The lack of toxicity by the plant extract fulfils the criteria set by OECD (2001). However, more through toxicity studies including chronic toxicity studies should be conducted for further development of the extract of the roots of *L. giberroa*.

## 4.3. *In vivo* antimalarial activity

### 4.3.1. *In vivo* antimalarial activity of hydroalcoholic extract

#### 4.3.1.1. Four-day suppressive test

The 4-day suppressive test provides evidence for the potential schizontocidal activity of plant extracts in early infection (Peters, 1975), indicating reduction of the overall pathogenic effect of the parasite on test groups in standard screening studies (Basir *et al.*, 2012).

The results of the 4-day suppressive test for the hydroalcoholic extract of roots of *L. giberroa* showed a significant ($p<0.001$) level of parasite suppression in a dose dependent manner. The extract had a chemo suppression value of 41.72, 58.95 and 73.05% at doses 100, 200 and 400 mg/kg, respectively (Table 3). The hydroalcoholic extract at all dose levels significantly prolonged the mean survival time. However, the extract exhibited lower activity ($p<0.001$) compared to the positive control, chloroquine.

**Table 3**: Parasitemia and survival time of infected mice treated with hydroalcoholic extract of the roots of *L. giberroa* in the 4-day suppressive test.

| Animal group | Dose in mg/kg | Parasitemia level | % Suppression | Survival (days) |
|---|---|---|---|---|
| DW | | 30.06±0.78 | - | 7.40±0.51 |
| CQ 25 | 25 | 0.0 | $100^{a3}$ | $29.20\pm0.66^{a3}$ |
| LGCE | 100 | 17.52±0.43 | $41.72^{a3b3d3e3}$ | $10.80\pm0.92^{a1b3e3}$ |
| | 200 | 12.34±0.39 | $58.95^{a3b3e3}$ | $14.00\pm0.7^{a3b3e3}$ |
| | 400 | 8.1000±0.43 | $73.05^{a3b3}$ | $19.60\pm0.93^{a3b3}$ |

Data are expressed as mean ± SEM; n = 5; compared to a, negative control; b, CQ25 mg/kg; c, 100 mg/kg; d, 200 mg/kg; e, 400 mg/kg; 1, $p< 0.05$; 2, $p<0.01$; 3, $p< 0.001$; DW, distilled water; CQ, chloroquine; LGCE, crude extract of the roots of *L. giberroa*.

Anemia, body weight loss and body temperature reduction are the general features of malaria infected mice (Lamikanra *et al.*, 2007). *P. berghei* causes reduction in rectal temperature of mice due to reduction in metabolic rates that occur because of increased parasitemia (Dikasso *et al.*, 2006). Therefore, ideal antimalarial agents obtained from plants are expected to prevent anemia, prevent body weight loss and stabilize body temperature in infected mice due to the rise in parasitemia level. The hydroalcoholic extract of *L. giberroa* significantly prevented weight loss, rectal temperature reduction and PCV reduction associated with an increase in parasitemia level. Again, the standard drug chloroquine showed a better effect than the extract in terms of these three parameters (Table 4).

**Table 4**: Packed cell volume, rectal temperature and body weight of infected mice treated with hydroalcoholic extract of the roots of *L. giberroa* in four-day suppressive test.

| Group | Dose | Weight | | | Temperature | | | Packed cell volume | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | (mg/kg) | D0 | D4 | %Change | D0 | D4 | %Change | D0 | D4 | % Change |
| DW | | 26.92±0.29 | 21.18±0.30 | -21.3±1.16 | 36.9±0.09 | 33.72±0.28 | -8.61±0.9 | 56.3±0.62 | 47.18±0.69 | -16.18±1.11 |
| CQ | 25 | 26.7±0.45 | 26..86±0.42 | 0.61±0.16$^{a3}$ | 36.96±0.13 | 36.58±0.23 | -1.03±0.37$^{a3}$ | 55.42±0.92 | 54.00±1.09 | -2.58±0.59$^{a3}$ |
| LGCE | 100 | 26.76±0.59 | 22.42±0.77 | -16.24±1.86$^{b3d1e3}$ | 36.82±0.19 | 34.78±0.17 | -5.5±0.58$^{a2b3d2e3}$ | 54.64±0.88 | 48.00±0.63 | -12.12±0.86$^{a1b3e2}$ |
| | 200 | 25.14±0.76 | 22.72±0.66 | -9.47±2.44$^{a3b2e1}$ | 36.84±0.05 | 36.04±0.12 | -2.17±0.36$^{a3}$ | 54.48±0.82 | 48.16±1.03 | -11.63±0.61$^{a2b3e2}$ |
| | 400 | 26.26±0.56 | 24.57±0.52 | -6.44±0.64$^{a3}$ | 36.94±0.09 | 36.64±0.09 | -0.81±0.12$^{a3}$ | 54.66±0.96 | 50.08±1.09 | -7.08±0.71$^{a3}$ |

Data are expressed as mean ±SEM; n=5; compared to a, negative control; b, CQ25 mg/kg; c, 100 mg/kg; d, 200 mg/kg; e, 400 mg/kg; 1, $p<0.05$; 2, $p<0.01$; 3, $p<0.001$; DW, distilled water; CQ, chloroquine; D0, pre-treatment value on day 0; D4, post- treatment value on day 4; LGCE, crude extract of the roots of *L. giberroa*

### 4.3.1.2. Rane's test

Rane's test evaluates the curative potential of the extracts on established infections (Ryley and Peter's, 1995). There was significant parasitemia suppression in all test doses used during the course of treatment indicating the curative potential of the hydroalcoholic extract of *L. giberroa*. Even though there was a gradual increment of parasitemia in the course of treatment, the extract had significant dose-dependent suppressive effect ($p<0.001$) (Figure 5A). The mean survival time indicated that the extract at all doses tested significantly prolonged survival days (Figure 5B). Nevertheless, the standard drug chloroquine was found to have curative effectiveness ($p<0.001$) supremely higher than the extract.

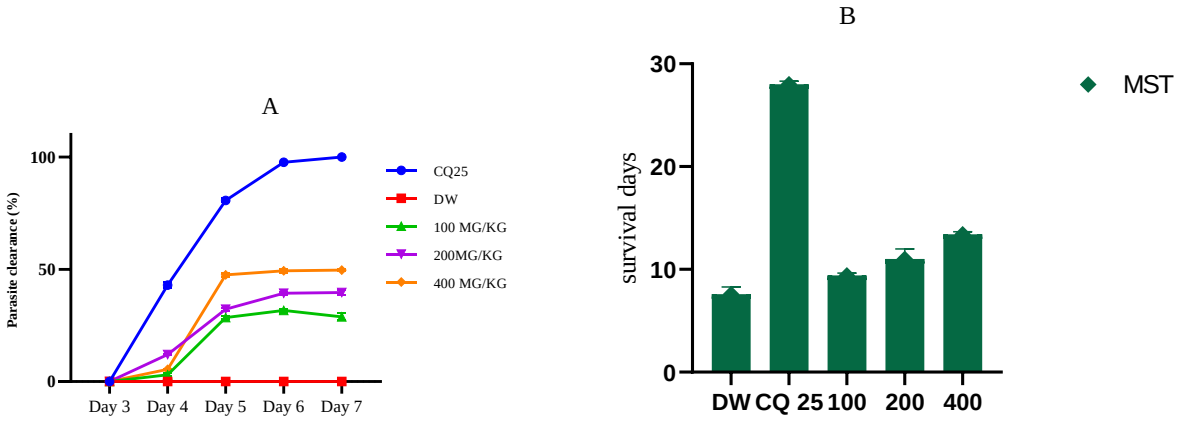


**Figure 5**: Parasitemia Suppression (A) and survival time (B) of infected mice treatment with hydroalcoholic extract of the roots of *L. giberroa* using Rane's model.

The extract showed a significant ($p<0.001$) suppression of body weight loss, rectal temperature and PCV reduction after an established infection in mice (Table 5). Again, the standard drug chloroquine had superior effects on body weight, rectal temperature and PCV. The overall lower curative (Figure 5) potential than suppressive (Table 3) effect could possibly be due to short duration of action of the constituent(s) to cover the exponentially growing parasites in established infection (Basir *et al.*, 2012).

Table 5:Rectal temperature, body weight and packed cell volume of infected animals treated with hydroalcoholic extract of roots of *L. giberroa* in the Rane's test model.

| Group | Dose | WIEGHT | | | RECTAL $T^0$ | | | PCV | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | (mg/kg | WD3 | WD7 | % Change | RT3 | RT7 | RT CHANGE | PCV3 | PCV7 | PCV Change |
| DW | | 27.19±0.43 | 22.40±0.22 | -17.61±0.72 | 36.90±0.10 | 32.66±0.22 | -11.49±0.53 | 54.68±0.33 | 45.92±0.35 | -16.02±0.37 |
| CQ | 25 | 26.02±0.65 | 26.66±0.90 | $2.38±1.16^{a3}$ | 36.96±0.14 | 36.74±0.11 | $-0.59±0.09^{a3}$ | 54.42±0.79 | 53.79±0.79 | $-1.16±0.18^{a3}$ |
| LGCE | 100 | 26.09±0.45 | 22.94±0.53 | $-12.09±0.95^{a3b3d1e3}$ | 36.80±0.16 | 34.06±0.33 | $-7.44±0.84^{a3b3d1e3}$ | 53.10±0.71 | 48.60±0.40 | $-8.43±1.14^{a3b3e2}$ |
| | 200 | 25.94±0.57 | 23.76±0.52 | $-8.39±0.24^{a3b3e1}$ | 36.74±0.17 | 34.82±0.29 | $-5.23±0.43^{a3b3}$ | 53.85±0.49 | 50.08±0.32 | $-6.98±0.65^{a3b3e1}$ |
| | 400 | 26.80±0.75 | 25.44±0.61 | $-5.03±0.41^{a3b3}$ | 36.92±0.15 | 35.60±0.23 | $-3.57±0.40^{a3b3}$ | 52.11±0.69 | 50.02±0.71 | $-4.01±0.63^{a3}$ |

Data are expressed as mean ±SEM; n=5; a, compared to negative control; b, to CQ25 mg/kg; c, to 100 mg/kg; d, to 200 mg/kg; e, to 400 mg/kg; 1, $p<0.05$; 2, $p<0.01$; 3, $p<0.001$; DW, distilled water; CQ, chloroquine; LGCE, *L. giberroa* crude extract; D3, pre-treatment value on day 3; D7, post- treatment value on day 7.

### 4.3.1.3. Prophylactic test

The hydroalcoholic extract exhibited a mild prophylactic effect**.** At all doses, the extract produced a significant ($p<0.001$) dose dependent parasitemia suppression (Table 6). Besides, all the treatment groups significantly prolonged survival time.

Table 6: Parasitemia and survival time of infected mice treated with hydroalcoholic extract of the roots of *L. giberroa* in prophylactic test.

| Group | Dose mg/kg | % Parasitemia | % Suppression | Survival (days) |
|---|---|---|---|---|
| DW | | 26.74±0.83 | - | 6.33±0.21 |
| CQ | 25 mg/kg | 5.68±0.26 | 78.76$^{a3}$ | 17.50±0.22$^{a3}$ |
| LGCE | 100 mg/kg | 22.08±0.56 | 17.43$^{a3b3d2e3}$ | 7.33±0.21$^{a3b3d3e3}$ |
| | 200 mg/kg | 18.74±0.37 | 29.91$^{a3b3e3}$ | 8.67±0.21$^{a3b3e3}$ |
| | 400 mg/kg | 15.20±0.20 | 43.16$^{a3b3}$ | 9.17±0.17$^{a3b3}$ |

Data are expressed as mean ± SEM; n = 5; a, compared to negative control; b, to CQ25 mg/kg; c, to 100 mg/kg; d, to 200 mg/kg; e, to 400 mg/kg; 1, $p < 0.05$; 2, $p <0.01$; 3, $p < 0.001$; DW, distilled water; CQ, chloroquine.

As shown in Table 7, the extract prevented body weight loss, rectal temperature and PCV reduction at all dose levels tested with prophylactic model.

Table 7: Packed cell volume, rectal temperature and body weight of infected mice treated with hydroalcoholic extract of the roots of *L. giberroa* in prophylactic test.

| | Dose | Body weight | | | Rectal temperature | | | Packed cell volume | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Groups | (mg/kg) | D0 | D4 | % Change | D0 | D4 | % Change | D0 | D4 | % Change |
| DW | | 26.18±0.63 | 22.28±0.53 | -14.89±0.32 | 36.88±0.12 | 32.48±0.09 | -11.93±0.13 | 55.70±0.37 | 48.60±0.24 | -12.74±0.37 |
| CQ25 | 25 | 25.54±0.81 | 26.10±0.78 | 2.22±0.65$^{a3}$ | 36.78±0.15 | 36.54±±0.13 | -.65±0.11$^{a3}$ | 54.30±0.66 | 53.84±0.79 | -.86±0.28$^{a3}$ |
| LGCE | 100 | 25.13±0.61 | 22.26±0.62 | -11.45±0.55$^{a3b3d3e3}$ | 36.56±0.22 | 34.62±0.59 | -6.31±0.39$^{a3b3e1}$ | 53.32±0.66 | 48.40±0.73 | -9.24±0.28$^{a2b3e2}$ |
| | 200 | 25.96±0.12 | 23.98±0.99 | -7.59±0.31$^{a3b3e3}$ | 37.06±0.09 | 34.72±0.10 | -5.32±0.27a3b3 | 54.26±0.37 | 50.46±0.37 | -7.00±0.29$^{a2b3e2}$ |
| | 400 | 27.46±0.47 | 26.30±0.50 | -4.24±0.24$^{a3b3}$ | 36.84±0.13 | 35.62±0.13 | -3.31±0.13$^{a3b1}$ | 52.28±0.93 | 50.50±0.80 | -3.39±0.37$^{a3}$ |

Data are expressed as mean ±SEM; n=5; a, compared to negative control; b, to CQ25 mg/kg; c, to 100 mg/kg; d, to 200 mg/kg; e, to 400 mg/kg; 1, $p<0.05$; 2, $p<0.01$; 3, $p<0.001$; CQ, chloroquine; LGCE, *L. giberroa* crude extract; D0, pre-treatment value on day 0; D4, post- treatment value on day 4; number refers to dose mg/kg

In the prophylactic test, the hydroalcoholic extract showed the lowest percentage suppression of parasitemia in comparison with its effect in the 4-day suppressive and Rane's tests. But, all the doses of the extract indicated significant suppressive effect on the level of parasitemia compared to the negative control. The lower chemo-suppressive effect of the hydroalcoholic extract in prophylactic test might have happened from fast metabolism that inactivates the active constituents of the extract responsible for antimalarial activity (Salawu *et al.*, 2010, Alli *et al.*, 2011). And also the other possible reason could be that the extract would have acted by metabolic activation of the immune system (Metodiewa *et al.*, 1999). Due that parasite clearance might not be total.

### 4.3.2. Antimalarial activity of the Soxhlet fractions

For further evaluation of the *in vivo* antimalarial activity of the hydroalcoholic root extract of *L. giberroa*, the extract was further fractionated by solvents having different polarity. The *in vivo* antimalarial activity of the fractions was evaluated using the 4-day suppressive test, the standard test used for antimalarial screening (Peter and Anatoli, 1998).

The results indicated that the methanol fraction showed the highest antimalarial activity with a maximum chemo suppression value of 64.37% at dose of 400 mg/kg, followed by the aqueous (47.92%), ethyl acetate (44.80%) and hexane (37.21%) fractions (Figure 6A). The methanol fraction prolonged mean survival time better than the rest of the Soxhlet fractions (Figure 6B. Compared to the extract it however had lesser chemo-suppressive and mean survival effect.

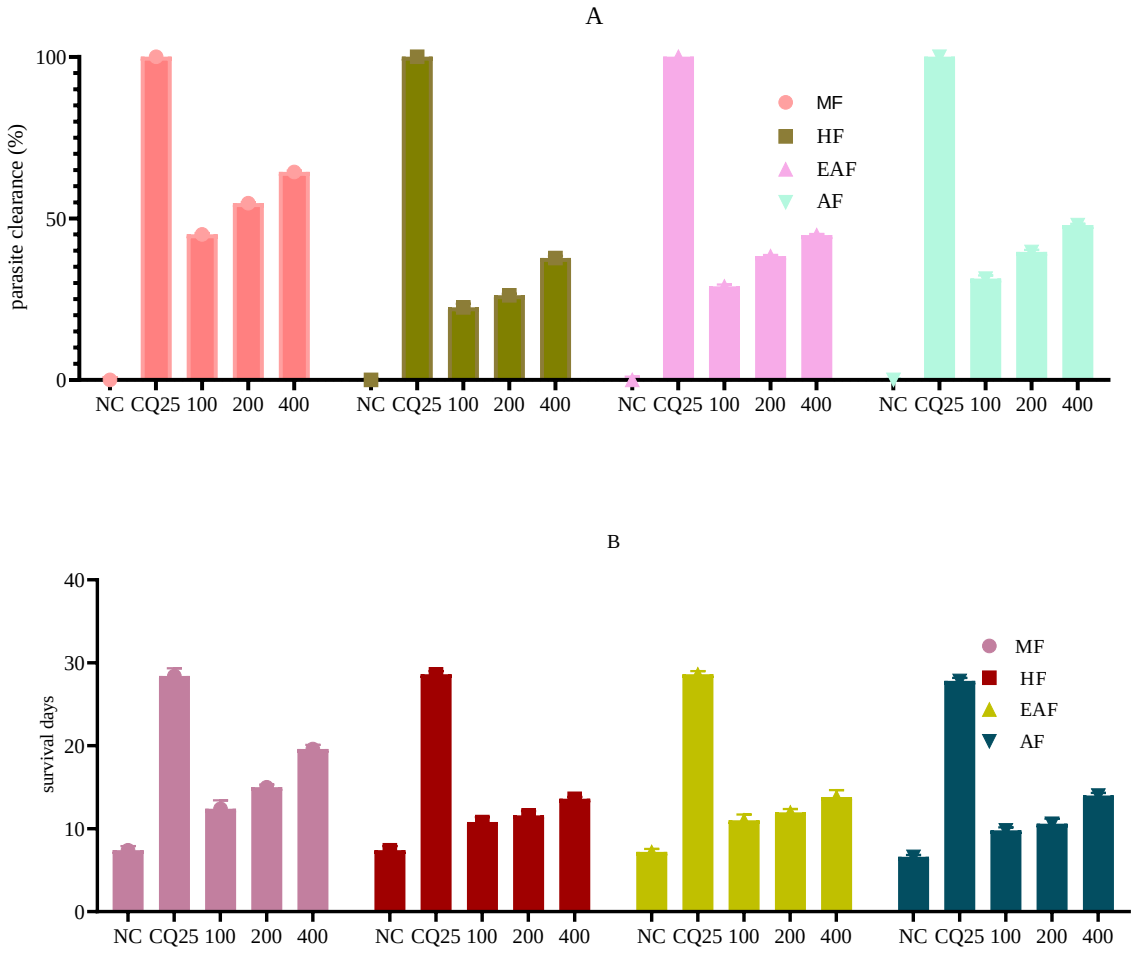


Figure 6: % suppression (A) and MST (B) of solvent fraction of *L. giberroa* root extract with four-day suppression test.

All the four fractions significantly ($P < 0.001$) suppressed infection-induced reduction of body weight, rectal temperature and PCV. The positive control chloroquine on its part showed pronounced stabilization of body weight, rectal temperature and PCV (Table 8).

Table 8: Temperature, weight and packed cell volume of infected animals treated with solvent fractions of *L. giberroa* in the 4day suppressive test.

| Group | Dose | Weight | | | Temperature | | | Packed cell volume | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | (mg/kg) | D0 | D4 | %Change | D0 | D4 | %Change | D0 | D4 | %Change |
| TW80 | | 26.96±0.24 | 21.10±0.29 | -21.73±0.91 | 37.10±0.13 | 32.72±0.15 | -11.80±0.55 | 56.5±0.63 | 45.70±0.83 | -19.13±0.66 |
| CQ | 25 | 26.68±0.59 | 26.98±0.64 | 1.11±0.18$^{a3}$ | 37.08±0.06 | 36.90±0.07 | -0.48±010$^{a3}$ | 54.28±0.99 | 53.41±0.89 | -1.59±0.41$^{a3}$ |
| HF | 100 | 27.14±0.75 | 22.46±0.59 | -17.22±0.52$^{a3b3d3e3}$ | 37.02±0.08 | 33.44±0.19 | -9.7±0.41$^{a1b3e3}$ | 55.50±0.83 | 47.94±0.41 | -13.58±0.90$^{a3b3d3e3}$ |
| | 200 | 26.18±0.73 | 22.82±0.68 | -12.84±0.35$^{a3b3e2}$ | 37.14±0.11 | 33.90±0.17 | -8.72±0.33$^{a3b3e2}$ | 55.06±1.06 | 50.32±0.86 | -8.59±0.23$^{a3b3e3}$ |
| | 400 | 25.50±0.86 | 23.10±0.0.78 | -9.40±0.42$^{a3b3}$ | 36.94±0.11 | 34.76±0.24 | -5.90±0.60$^{a3b3}$ | 55.88±0.82 | 53.36±0.73 | -4.5±0.14$^{a3b2}$ |
| Tw80 | | 26.96±0.24 | 22.54±0.67 | -14.34±1.34 | 36.9±0.13 | 33.46±0.33 | -9.32±0.86 | 53.92±1.04 | 47.34±0.68 | -1 2.20±0.75 |
| CQ | 25 | 26.68±0.59 | 28.26±0.62 | 5.93±0.4$^{a3}$ | 36.94±0.1 | 36.78±0.12 | -4.34±0.07$^{a3}$ | 53.16±0.49 | 52.97±0.49 | -0.35±0.07$^{a3}$ |
| EAF | 100 | 26.41±0.62 | 24.36±0.88 | -7.84±1.54$^{a2b3}$ | 36.86±0.13 | 35.22±0.16 | -4.45±0.4$^{a3b3}$ | 56.34±0.71 | 52.19±0.91 | -7.37±0.59$^{a3b3e3d1}$ |
| | 200 | 26.3±0.59 | 23.8±0.72 | -.9.53±1.24$^{b3e1}$ | 36.9±0.14 | 35.22±0.31 | -4.56±0.59$^{a3b3}$ | 55.06±0.49 | 52.42±0.70 | -4.79±0.57$^{a3b3}$ |
| | 400 | 26.12±1.00 | 24.9±0.77 | -4.54±0.87$^{a3b3}$ | 36.74±0.15 | 35±0.28 | -4.73±0.64$^{a3b3}$ | 54.62±0.55 | 53.02±0.49 | -2.92±0.41$^{a3b1}$ |
| TW80 | | 26.52±0.39 | 21.82±0.42 | -17.72±1.09 | 37.08±0.73 | 33.42±0.92 | -9.87±0.23 | 56.18±0.82 | 49.92±0.70 | -11.13±0.49 |
| CQ | 25 | 26.92±0.64 | 27.30±0.69 | 1.41±0.42$^{a3}$ | 37.14±0.07 | 36.90±0.07 | -0.64±0.06$^{a3}$ | 55.40±0.93 | 55.08±0.92 | -0.58±0.13$^{a3}$ |
| MF | 100 | 26.68±0.65 | 22.80±0.58 | -14.54±0.38$^{b3e3d1}$ | 36.80±0.08 | 34.31 ±0.16 | -7.52±0.78$^{a3b3d2e3}$ | 55.50±1.07 | 51.36±1.40 | -7.52±0.78$^{a3b3e3}$ |
| | 200 | 26.40±0.78 | 23.60±0.80 | -10.63±0.89$^{a3b3e3}$ | 37.04±0.08 | 35.36±0.19 | -5.59±0.15$^{a3b3e3}$ | 55.35±0.79 | 52.26±0.83 | -5.59±0.15$^{a3b3e1}$ |
| | 400 | 27.28±0.71 | 25.94±0.98 | -4.91±0.99$^{a3b3}$ | 36.88±0.12 | 36.26±0.28 | -3.16±0.54$^{a3}$ | 54.48±1.15 | 52.76±1.14 | -3.16±0.54$^{a3b1}$ |
| DW | | 26.82±0.36 | 21.30±0.42 | -20.59±0.91 | 37.1±0.08 | 32.88±0.24 | -11.37±0.58 | 56.86±0.58 | 49.54±1.17 | -12.92±1.23 |
| CQ | 25 | 26.70±0.45 | 26.82±0.44 | 0.45±0.08$^{a3}$ | 36.88±0.12 | 36.68±0.12 | -0.54±0.09$^{a3}$ | 53.30±1.00 | 52.86±0.95 | -0.81±0.10$^{a3}$ |
| AF | 100 | 26.44±0.64 | 21.78±0.48 | -17.6±0.71$^{b3d3e3}$ | 37.02±0.80 | 33.88±0.17 | -8.48±0.46$^{a3b3e3d1}$ | 55.90±0.63 | 51.69±0.73 | -7.53±0.68$^{a3b3e2}$ |
| | 200 | 25.78±0.73 | 22.8±0.77 | -11.58±1.10$^{a3b3c3e3}$ | 37.08±0.07 | 34.88±0.59 | -5.93±0.27$^{a3b3e1}$ | 54.38±0.82 | 51.75±0.91 | -4.84±0.59$^{a3}$ |
| | 400 | 26.26±0.56 | 25.36±0.48 | -3.4±0.44$^{a3b1}$ | 36.92±0.15 | 35.42±0.19 | -4.06±0.35$^{a3b3}$ | 55.32±0.57 | 53.72±0.76 | -2.89±0.76$^{a3}$ |

Data are expressed as mean ± SEM; n = 5; a, compared to negative control; b, to CQ25 mg/kg; c, to 100 mg/kg; d, to 200 mg/kg; e, to 400 mg/kg; 1, $p < 0.05$; 2, $p < 0.01$; 3, $p < 0.001$; TW80, 2% Tween80; CQ, chloroquine; HF, Hexane fraction; EAF, ethyl acetate fraction; MF, methanol fraction; AF, aqueous fraction; DW, distilled water as a vehicle; D0, pre-treatment value on day 0; D4, post- treatment value on day 4.

### 4.3.3. Effect of sub-fraction in the four-day suppressive test

As mentioned in 4.3.2 (Figure 6A), column fractionations of the most active fraction the methanol fraction led to the collection of three column sub-fractions, LGF-1, LGF-2 and LGF-3; of which LGF-2 was found to be a pure compound. The column fractions and the compound displayed a significant dose dependent antimalarial activity in the 4-day suppressive test (Figure 7A). Moreover, the compound, LGF-2, (68.21%) showed higher parasite suppression effect than the column fractions, LGF-1 (42.31%) and LGF-3 (36.98%). The compound (LGF-2) treated groups had a better mean survival time than other column fractions treated groups (Figure 7B). Interestingly, the compound (LGF-2) prolonged the mean survival days of the 100 mg/kg/day treated groups to 18 days. The antimalarial activity of LGF-2 was considered very good; at the dose of 100 mg/kg/day due to its percentage growth inhibition was greater than 50% (Munoz *et al*., 2000).

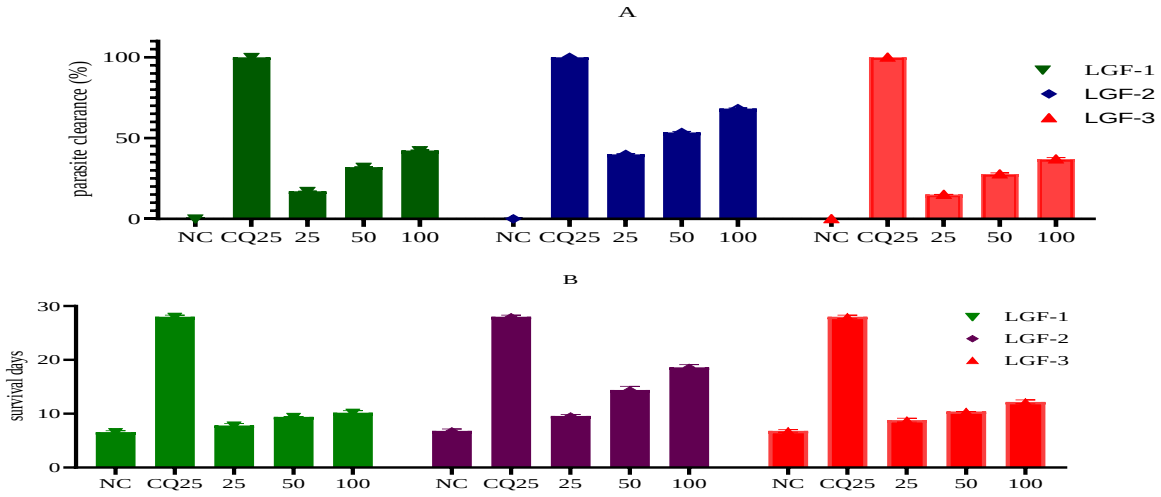


Figure 7: Antimalarial activity (A) and mean survival time (B) of the column fractions of *L. giberroa* and the compound (LGF-2) against mice infected with *Plasmodium berghei* in the 4-day suppressive test.

The column fractions and the compound produced a significant effect on the reduction of body weight loss, PCV and rectal temperature drop. However, chloroquine treated groups had a much-pronounced effect that column fractions and compound (LGF-2) treated groups (Table 9).

Table 9: Body weight, rectal temperature and packed cell volume of *Plasmodium berghei* infected mice before and after administration of the column fractions of *L. giberroa* and the compound (LGF-2) in the four-day suppressive test.

| Group | Dose | Weight | | | Temperature | | | Packed cell volume | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | mg/kg | D0 | D4 | %Change | D0 | D4 | %Change | D0 | D4 | %Change |
| 2%TW80 | | 26.60±0.46 | 23.52±0.50 | -11.57±0.18 | 36.86±0.07 | 33.56±0.29 | -8.95±0.78 | 54.80±0.72 | 47.78±0.51 | -12.79±0.36 |
| CQ | 25 | 26.63±0.64 | 26.75±0.65 | 0.43±0.09$^{a3}$ | 36.92±0.12 | 36.74±0.13 | -0.49±0.13$^{a2}$ | 56.21±0.51 | 56.03±0.52 | -0.32±0.16$^{a3b3}$ |
| LGF-1 | 25 | 26.73±0.55 | 24.12±0.64 | -9.83±0.76$^{b3}$ | 37.04±0.08 | 34.38±0.37 | -7.19±0.83$^{b3}$ | 56.07±0.20 | 51.30±0.37 | -8.50±0.43$^{a3b3}$ |
| | 50 | 25.16±0.61 | 23.13±0.55 | -8.07±0.61$^{b3a1}$ | 36.84±0.09 | 34.22±0.39 | -7.11±0.01$^{b3}$ | 55.89±0.75 | 51.54±0.95 | -7.81±0.75$^{a3b3}$ |
| | 100 | 26.87±0.36 | 24.88±0.39 | -7.44±0.40$^{b3a2}$ | 37.12±0.09 | 35.72±0.49 | -3.77±0.37 | 55.18±0.51 | 51.36±0.39 | -6.89±0.04$^{a3b3}$ |
| 2%TW80 | | 25.86±0.49 | 21.50±0.44 | -16.86±0.71 | 36.70±0.19 | 32.84±0.17 | -10.51±0.40 | 54.76±0.41 | 46.88±0.50 | -14.38±0.96 |
| CQ | 25 | 25.60±0.52 | 26.36±0.59 | 2.95±0.50$^{a3}$ | 36.72±0.09 | 36.54±0.09 | -0.49±0.13$^{a3}$ | 54.04±0.79 | 53.70±0.82 | -0.6321±0.12$^{a3}$ |
| LGF-2 | 25 | 26.14 ±0.99 | 23.12±0.97 | -11.61±0.39$^{a3b3d3e3}$ | 37.02±0.14 | 34.00±0.14 | -8.16±0.15$^{a3b3e3}$ | 54.20±0.98 | 48.32±0.84 | -10.84±0.19$^{a2b3d1e3}$ |
| | 50 | 24.88±0.79 | 23.10±0.65 | -7.11±0.59$^{a3b3e3}$ | 36.72±0.10 | 34.20±0.20 | -6.86±0.41$^{a3b3e3}$ | 54.98±0.83 | 50.57±0.79 | -8.02±0.32$^{a3b3e3}$ |
| | 100 | 26.90±0.35 | 26.14±0.39 | -2.83±0.61$^{a3b3}$ | 36.98±0.11 | 35.64±0.07 | -3.62±0.33$^{a3b3}$ | 54.10±0.60 | 51.84±0.79 | -4.19±0.59$^{a3b3}$ |
| 2%TW80 | | 26.82±0.36 | 21.30±0.42 | -20.59±0.91 | 37.1±0.08 | 32.88±0.24 | -11.37±0.58 | 56.86±0.58 | 49.54±1.17 | -12.92±1.23 |
| CQ | 25 | 26.70±0.45 | 26.82±0.44 | 0.45±0.08$^{a3}$ | 36.88±0.12 | 36.68±0.12 | -0.54±0.09$^{a3}$ | 53.30±1.00 | 52.86±0.95 | -0.81±0.10$^{a3}$ |
| LGF-3 | 25 | 26.44±0.64 | 21.78±0.48 | -17.6±0.71$^{b3d3e3}$ | 37.02±0.80 | 33.88±0.17 | -8.48±0.46$^{a3b3e3d1}$ | 55.90±0.63 | 51.69±0.73 | -7.53±0.68$^{a3b3e2}$ |
| | 50 | 25.78±0.73 | 22.8±0.77 | -11.58±1.10$^{a3b3c3e3}$ | 37.08±0.07 | 34.88±0.59 | -5.93±0.27$^{a3b3e1}$ | 54.38±0.82 | 51.75±0.91 | -4.84±0.59$^{a3b3}$ |
| | 100 | 26.26±0.56 | 25.36±0.48 | -3.4±0.44$^{a3b1}$ | 36.92±0.15 | 35.42±0.19 | -4.06±0.35$^{a3b3}$ | 55.32±0.57 | 53.72±0.76 | -2.89±0.76$^{a3b3}$ |

Data are expressed as mean ±SEM; n=5; a, compared to negative control; b, to CQ25 mg/kg; c, to 25 mg/kg; d, to 50 mg/kg; e, to 100 mg/kg; 1, p<0.05; 2, p<0.01; 3, p<0.001; 2% TW80, 2% Tween 80; CQ, chloroquine; LGF-3, *L. giberroa* fraction 3 from column; D0, pre-treatment value on day 0; D4, post- treatment value on day 4.

## 4.4. Isolation and characterization

### 4.4.1. Chromatography

Bioassay guided fractionation of the hydroalcoholic extract of the roots of *L. giberroa* gave a pure major active compound, LGF-2 (Figure 4). The TLC chromatograms of the compound, LGF-2, is shown in figure 8. The purity of the compound (LGF-2) was also checked by liquid chromatography and found to be >95% (Annex 5).

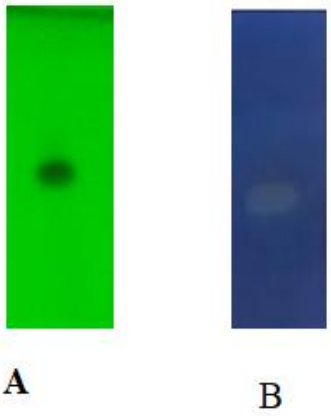


Figure 8: TLC chromatogram of LGF-2; viewed under UV at 254 (A) and 366 nm (B) (solvent system chloroform/methanol (4:1)); $R_f$ value 0.56.

## 4.4.2. Structural elucidation

The compound (LGF-2) was isolated as yellow solid. The $^{13}$C NMR spectrum (Table 10) of LGF-2 revealed a total of twenty carbons (Figure 9A) of which 16 of them were shown on the DEPT-135 spectrum (Figure 9C). The signals at δ 28.94, δ 32.55, δ 60.58 and δ 61.55 indicated the presence of four methylene groups in the compound as confirmed by the down ward signal on DEPT-135. From the 12 upward signals observed in the DEP-135 spectrum, only 11 signals were observed in DEPT-90 spectrum. This showed the presence of 11 CH group at δ 65.08, δ 70.61, δ 73.75, δ 77.37, δ 77.44, δ 80.02, δ 100.54, δ 109.66, δ 125.53, δ 136.61 and δ 145.48 (Figure 9B). The signal at δ 19.12 which was observed in $^{13}$C NMR and $^{13}$ C-DEPT 135 but not observed in the $^{13}$C-DEPT 90 confirmed the presence of one methyl group in the structure. The down ward signal at δ 60.58 and δ 61.55 indicates the presence of oxygenated methylene groups. The signals at δ 69.57, δ 72.46, δ 77.55 and δ 83.31 were not observed either in the DEPT-135 spectrum or in the DEPT-90 spectrum. These indicated the presence of four quaternary carbon signals. The $^{13}$C NMR data at δ 109.64, δ 145.47and δ 125.51, δ 136.61 also supports the presence of two double bonds. The signal at δ 100.54 is a typical signal for anomeric carbon

which confirms the presence of sugar moiety in the structure, and the signals at δ 61.55, δ 69.55, δ 73.75, δ 77.44 and δ 77.55, revealed five carbons of the sugar (Table 10). Thus, from the $^{13}C$ and DEPT experiments it can be concluded that LGF-2 has four methylene groups, 11 carbons of single proton, one methyl carbon and four quaternary carbons in its chemical structure of LGF-2. And also confirms the presence of alkenes, oxygenated carbons and glucose moiety in the structure.

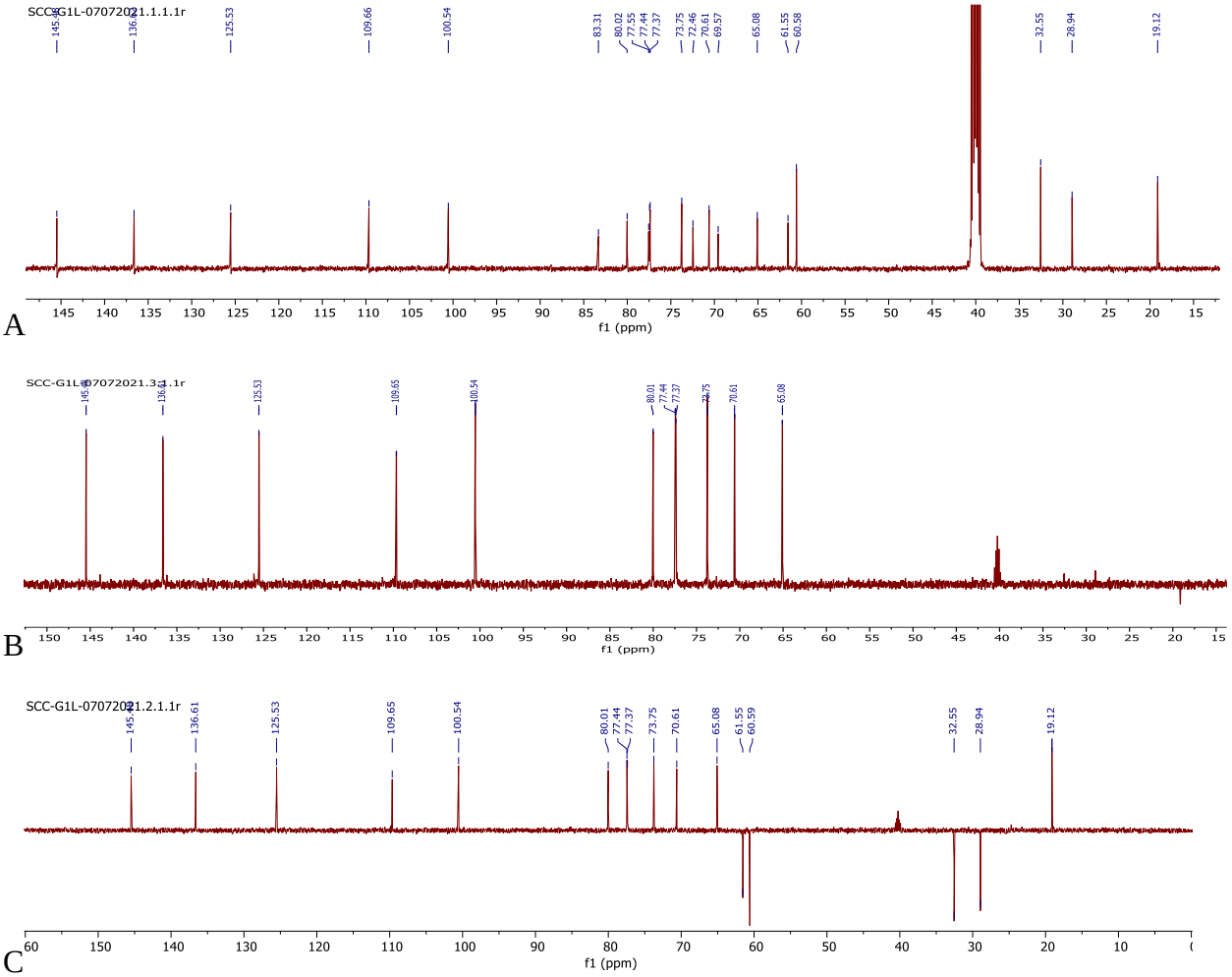


Figure 9: $^{13}C$ (A), DEPT-90 (B) and DEPT-135 (C) of LGF-2.

The $^{1}$H NMR spectrum of LGF-2 revealed (Annex 1) (Table 10) the presence of 28 hydrogens. An anomeric proton at δ 4.15 (d, *J* = 7.7 Hz, 1H) confirming the presence of a sugar unit in LGF-2. The signals at δ 1.50, δ 2.07 and δ 3.41 and each integrated to two protons are attributed to methylene hydrogens. The down field shifted methylene signal (δ 3.41) was attributed to an oxygenated methylene; three proton signals which is doublet of doublets at δ 1.80 provides evidence for the presence of a methyl group which is found in close proximity to olefinic carbons. The signals at δ 4.45 as doublets and δ 4.04 as triplets which are integrated to one proton each stood for the presence of two methine protons adjacent to two consecutive chiral carbons (C-8 and C-9). Two pairs of trans coupled (δ 5.37, δ 5.82 and δ 5.71, δ 6.39) (*J* = 16.9, 15.5 and 15.7 Hz) protons are attributed to the presence of two double bond carbons (Table 10).These double bonds were assigned as *E* due to the large vicinal coupling constant (*J*= 15.5 Hz and 14.5 Hz) between alkene protons C-10 and C-11, and (*J*=16.1 Hz and 15.9 Hz) between alkene protons of C-2 and C-3 (Chao *et al.*, 2015). Similar to $^{13}$C-NMR, $^{1}$H NMR provided evidence for the presence of sugar moiety, methylene protons, methyl group and alkene protons.

The HMBC spectrum of LGF-2 revealed the connection of methyl protons at carbon 1 to the olefinic carbon at carbon 2 and 3. A single proton at carbon 11 belonging to the olefinic carbon showed correlation with two methylene carbons of LGF-2 at carbon 12, 13 and methine carbon at position 9. The carbon located on carbon 12 of LGF-2 indicates the long-range correlation with the single proton located at olefinic carbon 10. The two hydrogens on the oxygenated methylene group of carbon at position 14 displayed connections to carbon 12 and 13. Correlation has also displayed long-range correlation of the methine proton at carbon 8 to the carbon 9 (Annex 2). The large coupling constant (*J*= 7.7 Hz) of H-l' revealed the β-configuration of this

anomeric centres (Ishimaru *et al*., 1991).

The $^{1}H$-$^{1}H$ COSY spectrum of LGF-2 exhibited correlation cross peaks between $CH_3$ (C-l) protons and CH(C-2) proton, CH(C-2) proton and CH(C-3) proton, CH(C-8) proton CH(C-9) and CH(C-l0) proton, CH(C-l0) proton and CH(C-11) proton, CH(C-11) proton and $CH_2$ (C-12) protons, $CH_2$ (C-12) protons and $CH_2$ (C-13) protons-CH and also $CH_2$ (C-13) protons and $CH_2OH$(C-14) protons (Annex 3).

In addition to NMR, the mass spectrum of the compound also revealed information regarding to the chemical structure of LGF-2 (Annex 6. Mass spectrum of LGF-2 gave a pseudomolocular ion peak at *m/z* 414.1 $[M+NH_4^+]^+$ and resembled with calculated value (Calc. m/z 414.46998$[M+NH_4^+]^+$). The mass spectrum fragment at 217 which is the base peaks of pyranosides (Sayed *et al.*, 2010) confirmed as the presence of glucose moiety in the structure of the compound. Also, the signal at 199 represents $[(M/2)+H^+]$ fragment. [M-OH] and [M-2OH] peaks at 379 and 361, respectively indicate that LGF-2 is an alcohol (Halket and Zaikin, 2003). The molecular ion peak of alcohols is small and sometimes undetectable due to incomplete conversion and unexpected reactions (Traeger and Morton, 2004). Based on the data, the molecular weight of LGF-2 is deduced to be 396 and its molecular formula $C_{20}H_{28}O_{8.}$

The IR spectrum of LGF-2 revealed the presence of O-H, C-O, C-H stretch of $CH_3$, C-H stretch of $CH_2$ and C≡C functional groups stretch. The broad bands at 3379 $cm^{-1}$ and 1070 $cm^{-1}$are attributed to the presence of hydroxyl group and C-O stretches respectively. The bands at 2922 $cm^{-1}$ and 2854$^{-cm}$ provided evidence for the existence of sp3 hybridized C-H stretch while the weak band at 2229 $cm^{-1}$is attributed to acetylenic carbons stretch (Annex 4).

Based on data from NMR, MS and IR, and comparison with a previously reported data (Ishimaru *et al*., 1991), the isolated compound is characterized as lobetyolin (LBT) (**27**) (Figure 10). Previously, LBT was reported from the methanol root extract of *Codonopsis tangshen* and *L. inflata (ref)*. Other polyacetylenes such as, lobetyolinin (**28**), isolobetyol (**29**), and lobetyol (**30**) were also reported from *L. chinensis*.

Table 10: $^{1}H$ and $^{13}C$ NMR data of compound LGF-2 measured in DMSO-D6.

| Present data | | | Reference (Ishimaru *et al*., 1991) | |
|---|---|---|---|---|
| Position | 1H NMR LGF-2 δ (ppm) | $^{13}C$ NMR of LGF-2 δ(ppm) | $^{1}H$ NMR δ (ppm) | $^{13}C$ NMR δ(ppm) |
| 1 | 1.80 (dd, *J* = 6.9, 1.9 Hz, 3H) | 19.12 | 1.73 (dd, *J*=6.8, 2.3 Hz, 3H) | 19.20 |
| 2 | 6.39 (dq, *J* = 16.1, 6.8 Hz, 1H) | 145.48 | 6.29 (dq, *J*=16.2, 6.8, 1H) | 144.70 |
| 3 | 5.71 (dd, *J* = 15.9, 2.1 Hz, 1H) | 109.66 | 5.55 (dd, *J*=16.2, 2.3, 1H) | 110.40 |
| 4 | - | 83.31 | - | 82.40 |
| 5 | - | 77.55 | - | 78.20 |
| 6 | - | 72.46 | - | 72.90 |
| 7 | - | 69.57 | - | 70.80 |
| 8 | 4.45 (d, *J* = 5.4 H z, 1H) | 65.08 | 4.40 (d, *J*=6.8 Hz,1H) | 65.60 |
| 9 | 4.04 (t, *J* = 7.1 Hz, 1H) | 80.02 | 4.20 (t, *J*= 6.8 Hz, 1H) | 81.80 |
| 10 | 5.37 (dd, *J* = 15.5, 7.4 Hz, 1H) | 125.53 | 5.4 (dd, *J*=16.2, 6.8 Hz, 1H) | 126.40 |
| 11 | 5.82 (dt, *J* = 14.5, 6.8 Hz, 1H) | 136.61 | 5.86 (dt, *J*= 16.2, 6.8 Hz, 1H) | 136.70 |
| 12 | 2.07 (quin, *J* = 7.2 Hz, 2H) | 28.94 | 2.09 (*br* dd, *J*=13.5, 6.8, 2H) | 28.90 |
| 13 | 1.50 (quin, *J* = 6.6, Hz, 2H) | 32.55 | 1.56(quin, *J*=6.8, 2H) | 33.00 |
| 14 | 3.41 (t, *J* = 6.9 Hz, 2H) | 60.58 | 3.49 (t, 2H, *J*= 6.8) | 61.90 |
| 1’ | 4.15 (d, *J* = 7.7 Hz, 1H) | 100.54 | 4.32 (d, *J*=7.5, lH*)* | 100.80 |
| 2’ | 3.0 (m, 1H) | 73.75 | 3.24(m, lH) | 74.70 |
| 3’ | 3.0 (m, 1H) | 77.44 | 3.24 (m, lH) | 77.90 |
| 4’ | 3.10 (t, *J* = 8.3 Hz, 1H). | 70.61 | 3.34 (t, *J*= 8.3, 1H) | 71.50 |
| 5’ | 3.0 (m, 1H) | 77.37 | 3.24 (m, 1H) | 77.80 |
| 6’ | 3.65 (d, *J* = 11.7 Hz, 2H) | 61.55 | 3.77 (dd, *J*=12.1, 6.8, 1H) | 62.00 |

The specific rotation of the isolated compound (lobetyolin) which was determined as [α] = -10.370.

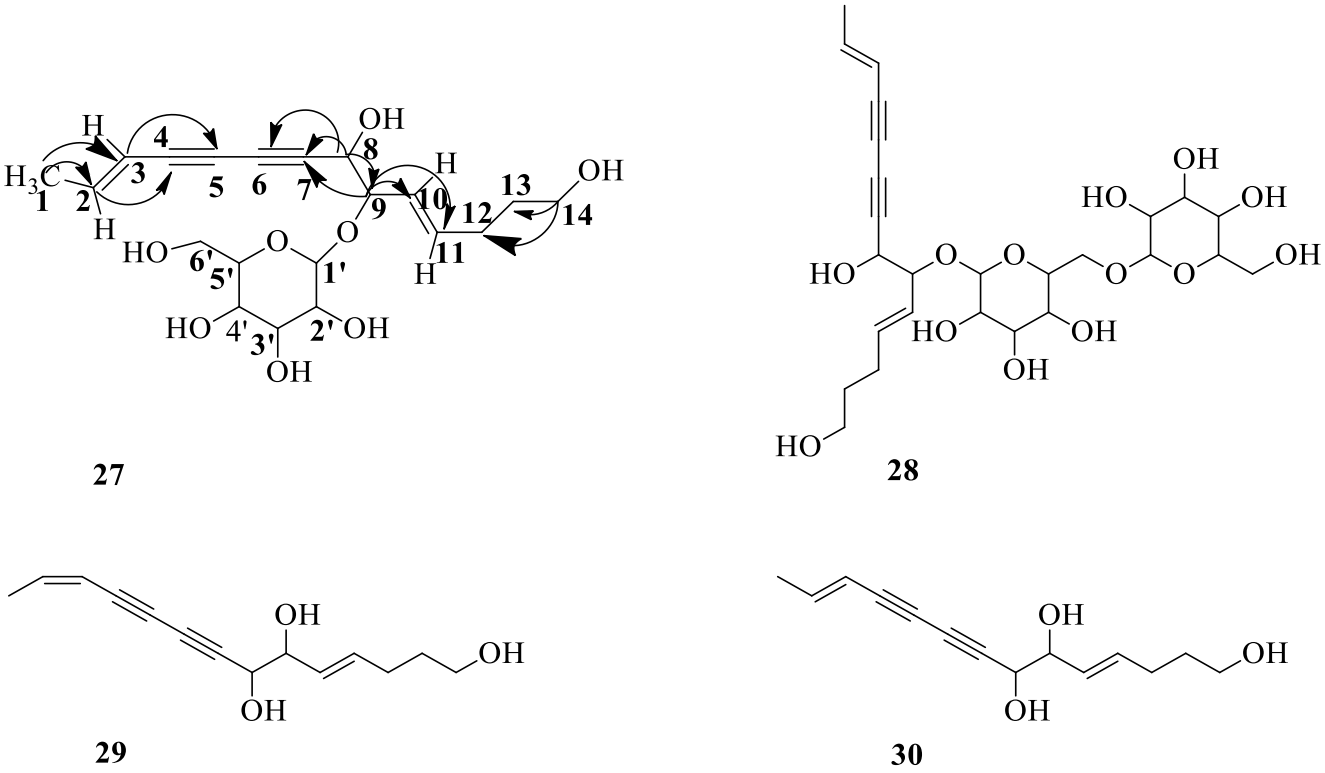


Figure 10: HMBC correlation of LBT isolated from *L. giberroa* Hemsl and other polyacetylenes isolated from *L. chinensis* Lour.

LGF-2 (LBT) tested by using four-day suppressive test against *P. berghei* in infected mice resulted in 68.21% parasite suppression (Figure 7A). Further investigation of LBT (lobetyolin) using Rane's test and prophylactic test was conducted and the results are described in section 4.5.

## 4.5. The curative and prophylactic activity of lobetyolin

The lobetyolin treated groups displayed a significant suppressive effect against established *P. berghei* infection in mice in a dose-dependent manner with Rane's test (Figure 11A). The compound significantly prolonged the mean survival days of the treated groups (maximum 13

days) showing its curative potential. But the standard drug was more effective ($p<0.001$) than all the test doses (Figure 11B).

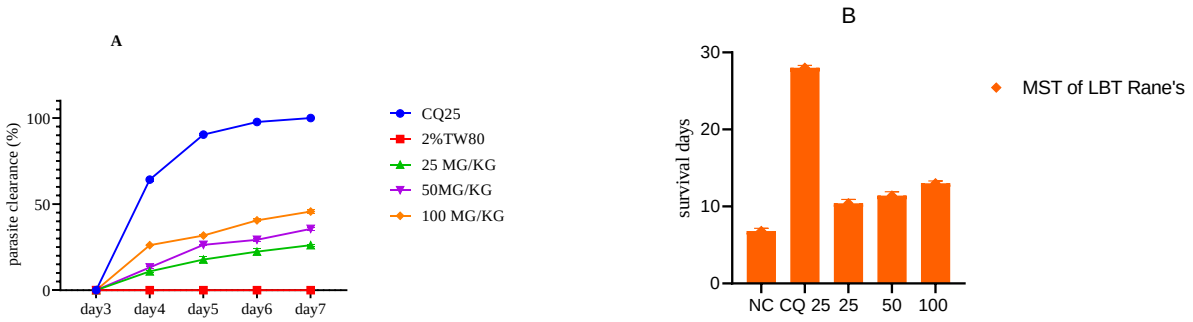


Figure 11: Antimalarial activity (A) and mean survival time (B) of lobetyolin against mice infected with *Plasmodium berghei* in the Rane's test.

All dose levels tested, lobetyolin exhibited suppression of body weight loss, rectal temperature and PCV reduction with Rane's model. However, the positive control had superior activity (Table 11).

Table 11: Body weight, rectal temperature and packed cell volume of *Plasmodium berghei* infected mice before and after administration of lobetyolin in the Rane's test.

| Group | Dose | Weight | | | Rectal temperature | | | Packed cell volume | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | (mg/kg) | D0 | D7 | % Change | D0 | D7 | % Change | D0 | D7 | % Change |
| 2% TW80 | | 26.56±0.57 | 23.42±0.48 | -11.79±0.75 | 36.80±0.17 | 33.62±0.19 | -8.64±0.28 | 54.33±0.62 | 48.92±0.33 | -9.94±0.53 |
| CQ | 25 | 25.80±0.64 | 26.18±0.60 | $1.49\pm0.12^{a3}$ | 37.02±0.09 | 36.74±0.11 | $-0.75\pm0.16^{a3}$ | 53.95±0.51 | 53.60±0.54 | $-0.65\pm0.08^{a3}$ |
| LBT | 25 | 25.94±0.36 | 24.02±0.41 | $-7.42\pm0.45^{a3b3d1e2}$ | 36.78±0.16 | 34.20±0.18 | $-7.01\pm0.22^{a1b3e3}$ | 53.10±0.71 | 49.60±0.48 | $-6.57\pm0.49^{a3b3e3}$ |
| | 50 | 25.52±0.69 | 24.23±0.73 | $-5.05\pm0.98^{a3b3}$ | 36.80±0.12 | 34.64±0.26 | $-5.87\pm0.54^{a3b3e2}$ | 53.85±0.49 | 50.36±0.43 | $-6.47\pm0.72^{a3b3e2}$ |
| | 100 | 27.30±0.33 | 26.24±0.17 | $-3.85\pm0.77^{a3b3}$ | 36.92±0.15 | 35.60±0.23 | $-3.58\pm0.40^{a3b3}$ | 52.11±0.69 | 50.34±0.63 | $-3.39\pm0.18^{a3b2}$ |

Data are expressed as mean ±SEM; n=5; a, compared to negative control; b, to CQ25 mg/kg; c, to 25 mg/kg; d, to 50 mg/kg; e, to 100 mg/kg; 1, $p<0.05$; 2, $p<0.01$; 3, $p<0.001$; 2% TW80, 2% Tween 80; CQ, chloroquine; LBT, *lobetyolin*; D0, pre-treatment value on day 0; D4, post- treatment value on day 4.

Lobetyolin produced a significant effect ($p<0.001$) in parasitemia suppression (Table 12) in Peter's repository test. The compound prolonged survival time, although less than the standard

drug chloroquine (Table 12). The compound significantly attenuated body weight loss and rectal temperature and PCV reduction.

Additionally, chloroquine treated group showed significant effect as compared to all extract treated groups.

Table 12: Parasitemia and survival time of infected mice treated with LBT from the roots of *L. giberroa* in prophylactic test

| Group | Dose (mg/kg) | % Parasitemia | %Suppression | Survival time |
|---|---|---|---|---|
| 2% TW80 | | 24.95±0.27 | - | 6.20±0.21 |
| CQ25 | 25 | 4.7±0.17 | 81.16$^{a3}$ | 14.60±0.22$^{a3}$ |
| LBT | 25 | 18.87±0.23 | 24.37$^{a3b3d3e3}$ | 6.40±0.21$^{a3b3d3e3}$ |
| | 50 | 16.17±0.23 | 35.19$^{a3b3}$ | 8.80±0.21$^{a3b3e3}$ |
| | 100 | 15.08±0.11 | 39.55$^{a3b3}$ | 10.20±0.17$^{a3b3}$ |

Data are expressed as mean ± SEM; n = 5; a, compared to negative control; b, to CQ25 mg/kg; c, to 25 mg/kg; d, to 50 mg/kg; e, to 100 mg/kg; 1, $p < 0.05$; 2, $p < 0.01$; 3, $p < 0.001$; 2% TW80, 2% Tween 80; CQ, chloroquine; LBT, lobetyolin

Related to body weight, significant effect was observed at 50 mg/kg ($p<0.05$) and 100 mg/kg ($p<0.01$) test doses in comparison with the negative control group. Additionally, chloroquine treated group showed significant effect as compared to all extract treated groups.

All the three doses of LBT attenuated the reduction of rectal temprature and PCV due to parasite infection significantly ($p<0.001$) in comparison with the negative control group. Similarly, the effect of standard drug was higher ($p<0.001$) than LBT treated groups on prevention of both rectal temprature and PCV reduction (Table 13).

Table 13: Body weight, rectal temperature and packed cell volume of *Plasmodium berghei* infected mice before and after administration of lobetyolin in the in prophylactic test.

| Groups | Dose | Body weight | | | Rectal temperature | | | packed cell volume | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | mg/kg | D0 | D3 | % Change | D0 | D3 | % Change | D0 | D3 | %Change |
| 2% TW80 | | 27.45±0.40 | 26.45±0.39 | -2.91 | 36.17±0.14 | 35.23±0.14 | -2.60 | 58.56±0.38 | 56.49±0.38 | -3.53 |
| CQ2 | 25 | 26.78±0.38 | 26.85±0.38 | $0.26^{a3}$ | 35.74±0.22 | 35.65±0.20 | -0.25 | 57.70±0.65 | 57.56±0.67 | $-0.24^{a3}$ |
| LBT | 25 | 25.83±0.38 | 25.19±0.38 | $-2.48^{b3}$ | 37.10±0.12 | 36.34±0.11 | $-2.48^{b3d2e2}$ | 57.65±0.49 | 56.30±0.48 | $-2.34^{a3b3d1e2}$ |
| | 50 | 26.01±0.34 | 25.47±0.35 | $-2.08^{a1b3}$ | 36.80±0.14 | 36.24±0.13 | $-1.52^{a3b3}$ | 56.10±0.49 | 54.93±0.47 | $-2.08^{a3b3}$ |
| | 100 | 27.86±0.55 | 27.00±0.53 | $-1.47^{a2b3}$ | 36.75±0.13 | 36.40±0.13 | $-0.95^{a3b3}$ | 56.73±0.66 | 55.66±0.67 | $-1.88^{a3b3}$ |

Data are expressed as mean ±SEM; n=5; a, compared to negative control; b, to CQ25 mg/kg; c, to 25 mg/kg; d, to 50 mg/kg; e, to 100 mg/kg; 1, $p<0.05$; 2, $p<0.01$; 3, $p<0.001$; 2% TW80, 2% Tween 80; CQ, chloroquine; LGF3, *L. giberroa* fraction 3 from column; D0, pre-treatment value on day 0; D4, post- treatment value on day 4.

The $ED_{50}$ estimated from a plot of Log dose against % parasitaemia of negative control (normalized) (Figure 12) of LBT is 36.8 mg/kg/day for four-day suppressive test. Previous studies reported LBT as having anti-inflammatory, anti-oxidative (Lu *et al.*, 2016) and xanthine oxidase inhibiting properties (Wang *et al.*, 2020). LBT also possess cytotoxic activities against lung and colon cancer (He *et al.*, 2020).

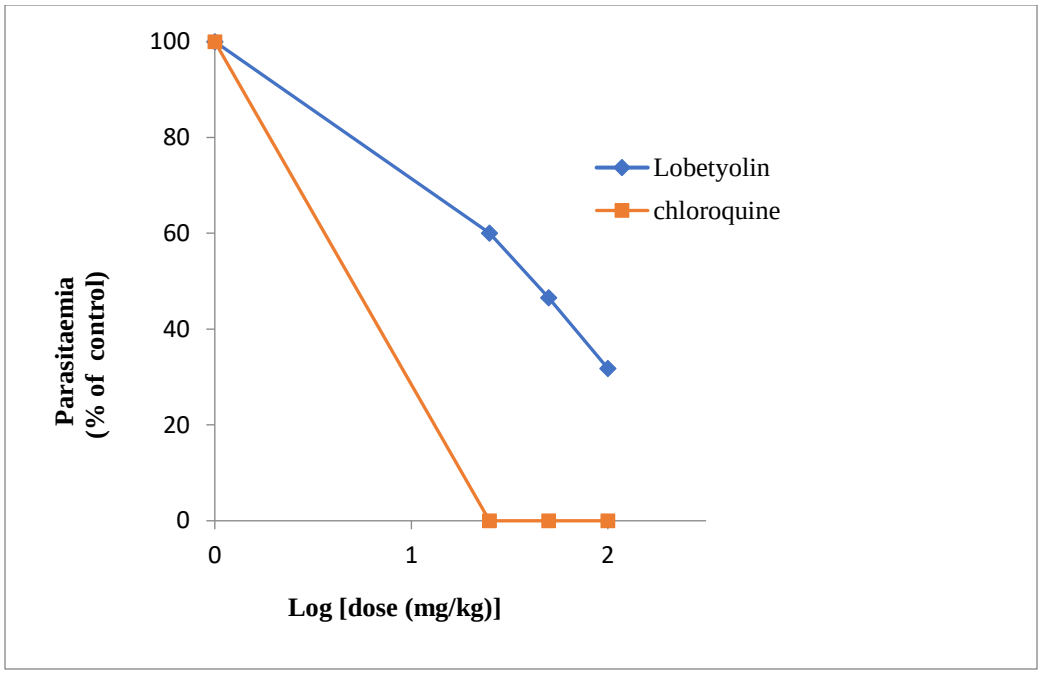


Figure 12: $ED_{50}$ of LBT in mice infected with *Plasmodium berghei* in four-day suppressive test; $ED_{50}$ was estimated from a plot of Log dose against % parasitaemia of negative control (normalized).

## Conclusion

The acute toxicity test of the hydroalcoholic extract, solvent fractions and LBT from *L. giberroa* roots resulted in $LD_{50}$ greater than 2000 mg/kg. *In vivo* antimalarial activity study against *P. berghei* infection in mice in four-day suppressive test showed chemo-suppression activity of the hydroalcoholic extract (73.05 %) at 400 mg/kg, methanol fraction (64.37%) at 400 mg/kg and LBT (68.21%) at 100 mg/kg. In the Rane's test for hydroalcoholic at 400 mg/kg and isolated compound at 100 mg/kg showed 49.35 % and 45.71 % parasite suppression respectively. The prophylactic test on the hydroalcoholic at 400 mg/kg and LBT at 100 mg/kg showed 43.16 % and 39.55 % parasite suppression respectively. The results of this study support the traditional use of the plant. LBT which is isolated from the roots of *L. giberroa* has a potential to serve as a lead compound for the development of new antimalarial drugs.

## Recommendation

Depending on the findings of the present study the following recommendations can be implied:

- The sub-acute and chronic toxicity study of the hydroalcoholic extract and isolated compounds need to be conducted;
- Antimalarial activity tests against other *Plasmodium* species must be carried out.

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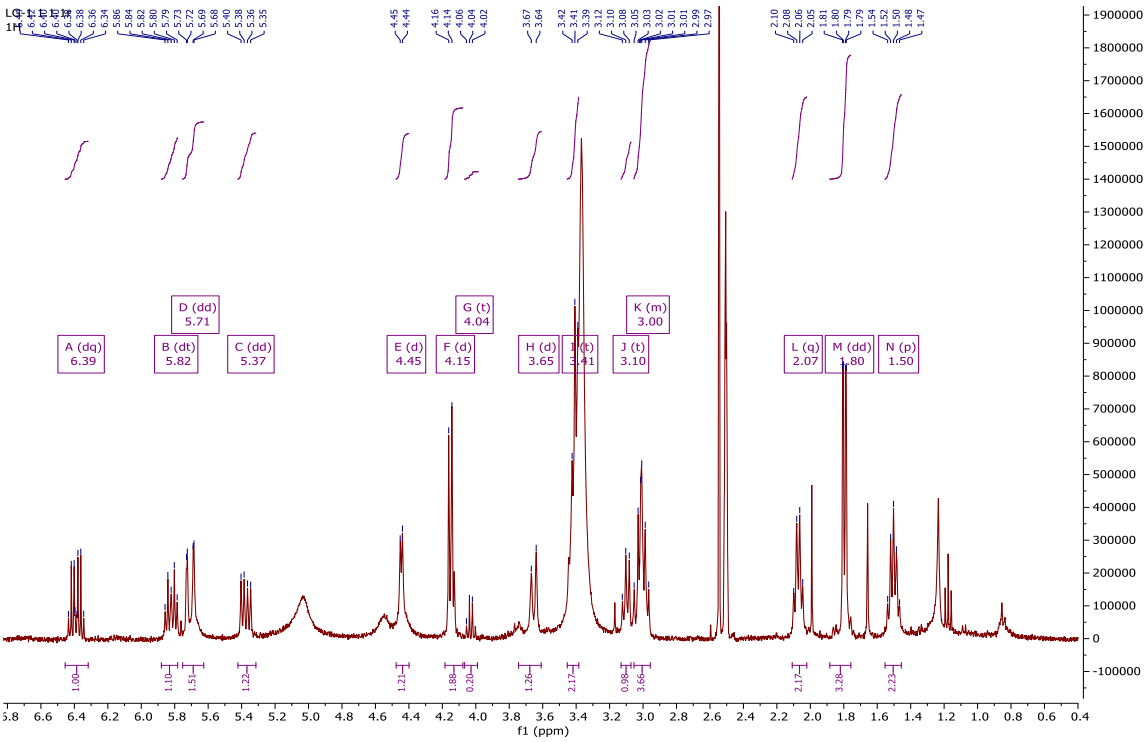
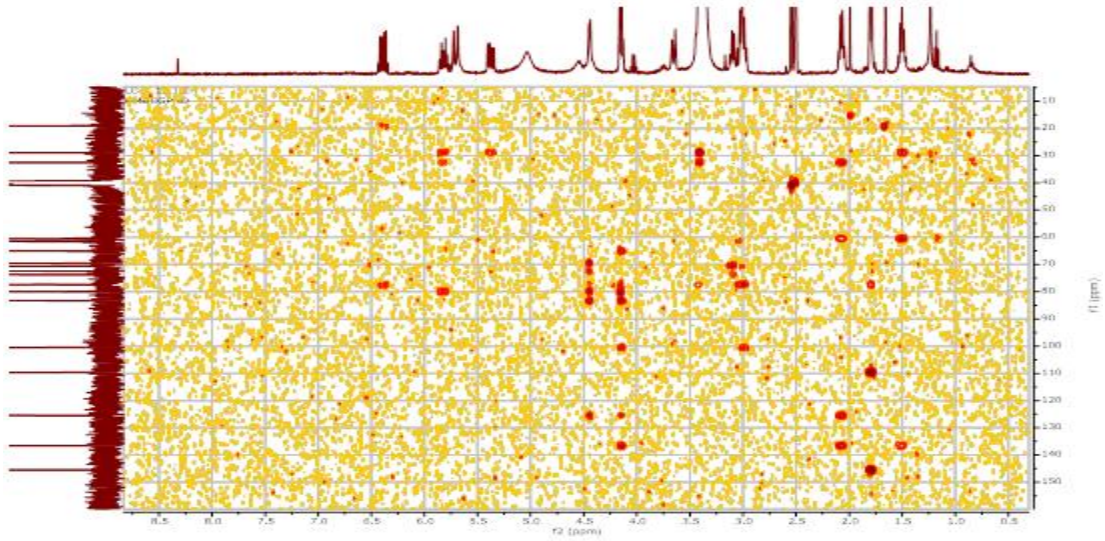
# Annex

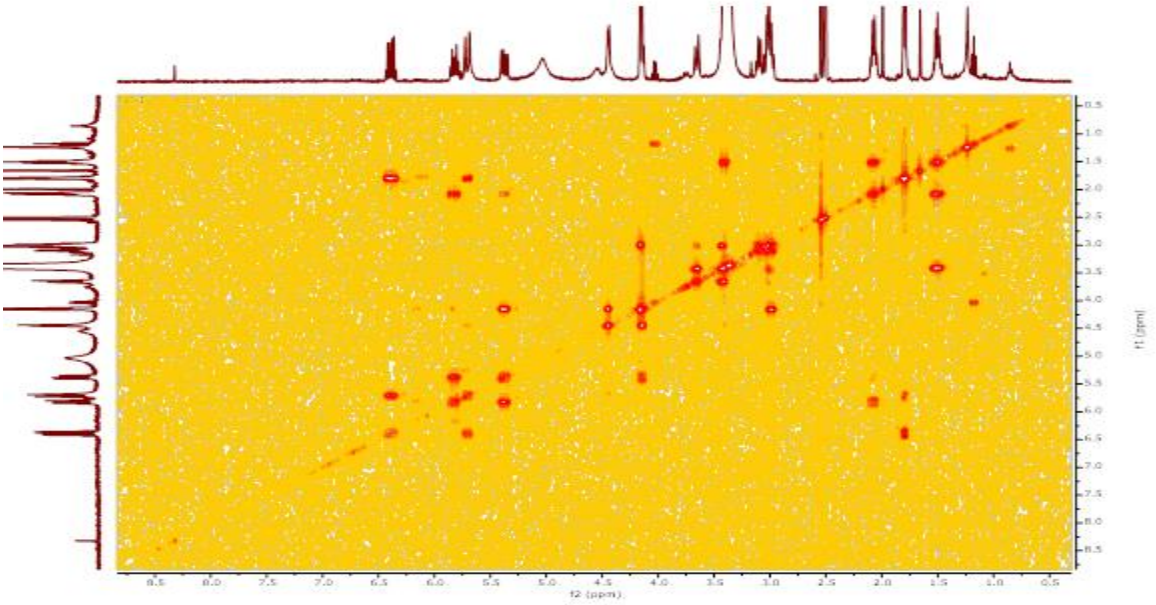
## Annex 1: $^{1}H$ NMR of LGF-2



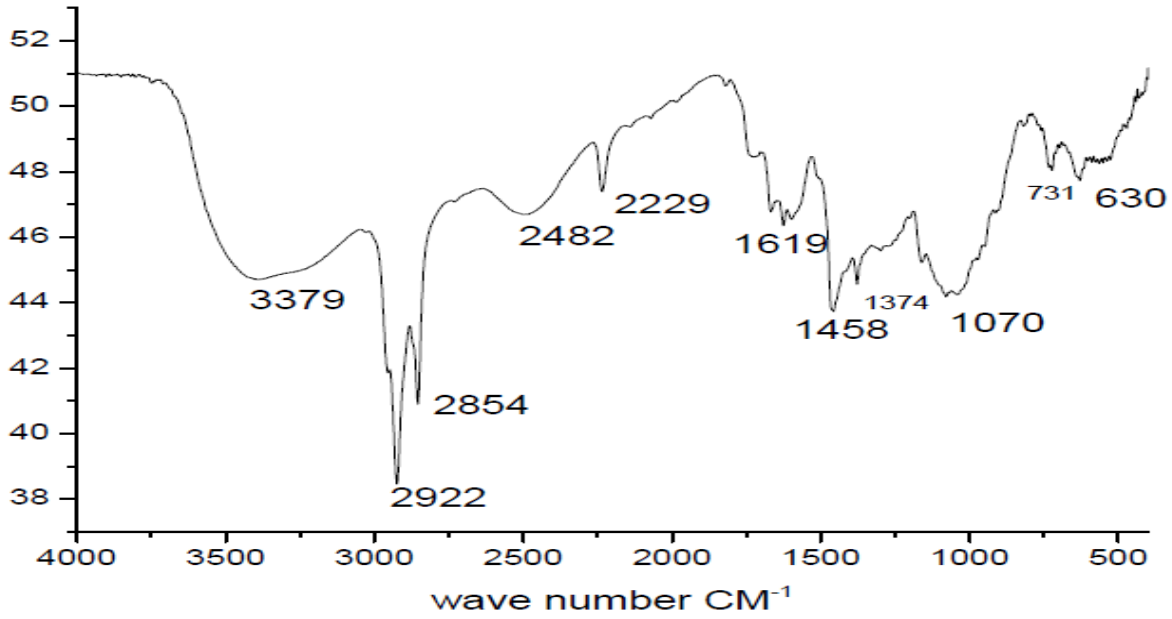

## Annex 2: HMBC of LGF-2



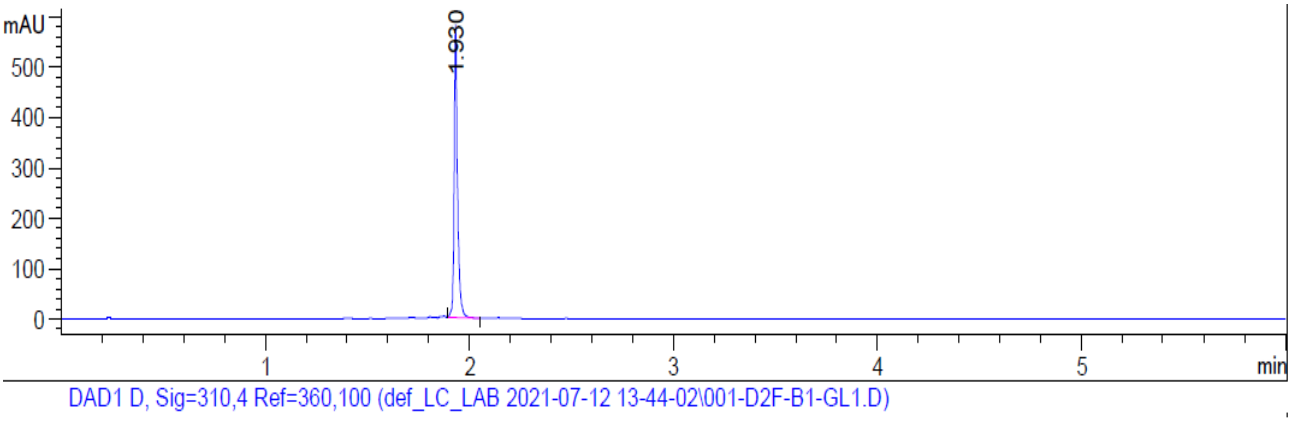
Annex 3: $^{1}$H HCOSY of LGF-2



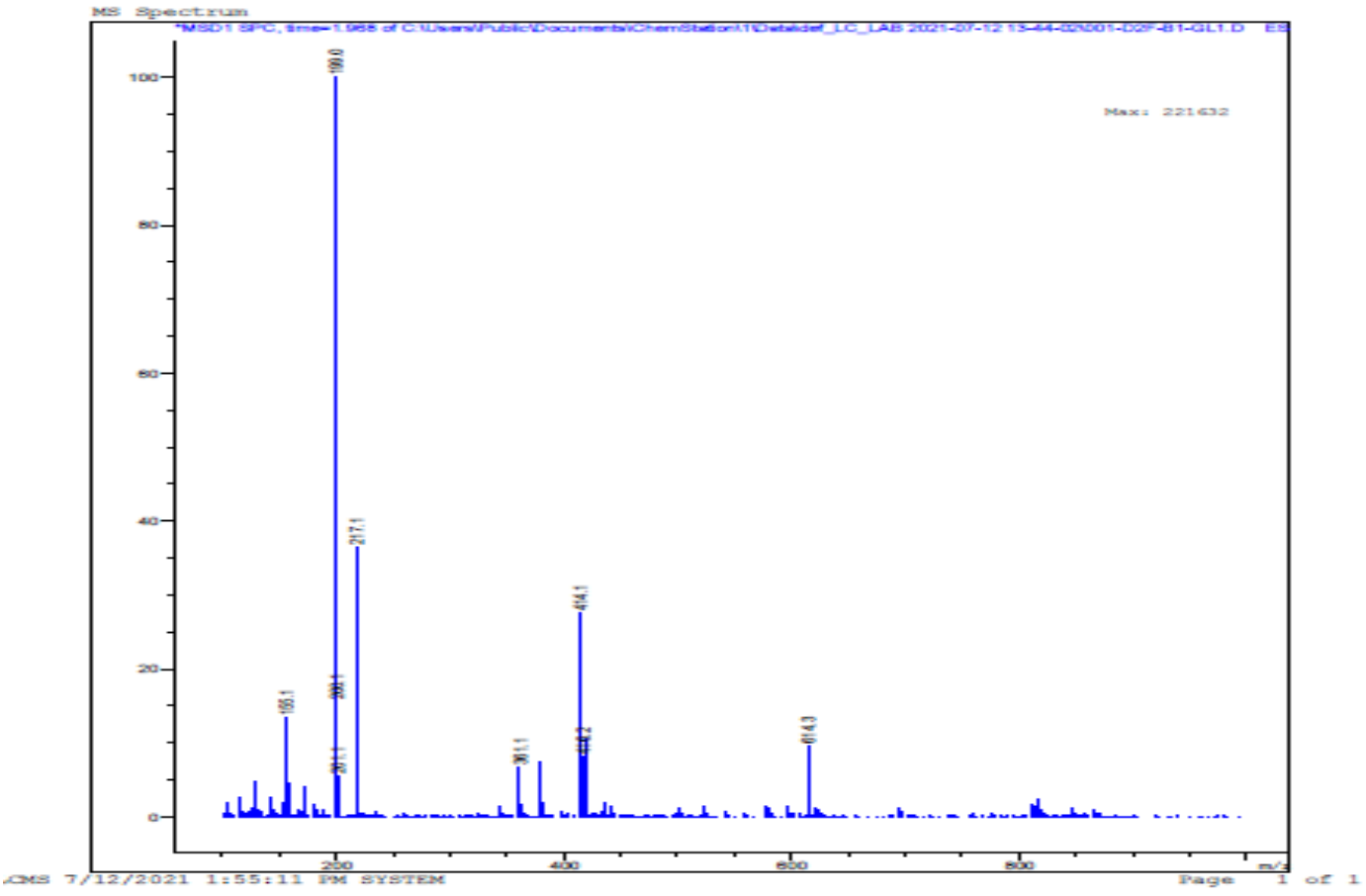
Annex 4: IR spectrum of LGF-2



Annex 5: LC spectrum of LGF-2




Appendix 6: MS of LGF-2



Annex 7: Ethical clearance paper

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Addis Ababa University

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School of Pharmacy
Ethical Review Committee

ቀን Date: September 8, 2021

ቁጥር Ref. No.: ERB/SOP/356/13/2021

To: **Getnet Tadege**
School of Pharmacy

**Re: Ethical Clearance**

It is to be recalled that you submitted a research proposal entitled "**Studies On *In-Vivo* Antimalarial Activity of the Root of *Lobelia giberroa* Hemsl. against *Plasmodium Berghei* in Mice.**" The committee thoroughly reviewed the proposal based on its operational guidelines and found that it fulfills all the ethical requirements stipulated in the guidelines. This is, therefore, to inform you that the proposal is ethically approved for implementation.

With best regards,

Shemsu Umer (PhD)
Chairperson, ERC
School of Pharmacy
College of Health Sciences
Addis Ababa University

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