Antiprotozoal Activity of Extracts and a Major Compound from the Leaves of *Ranunculus multifidus* Forrsk.

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This is to certify that the thesis prepared by Betelhem Sirak, entitled: **"Antiprotozoal Activity of Extracts and a Major Compound from the Leaves of *Ranunculus multifidus* Forrsk."** 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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Abstract

Antiprotozoal Activity of Extracts and a Major Compound from the Leaves of *Ranunculus multifidus* Forrsk.

Betelhem Sirak

Addis Ababa University, 2021

Protozoal diseases caused by the genera *Leishmania*, *Plasmodium* and *Trypanosoma* have devastating impact mostly on the poorest countries of the world due to their prevalence, virulence and drug resistance ability. This calls for the development and discovery of new effective and safe chemotherapeutic agents from alternative sources such as medicinal plants. This study aimed at investigating the antiprotozoal activity of *Ranunculus multifidus* Forrsk. (Ranunculaceae), a medicinal plant used to treat leishmaniasis, malaria and trypanosomiasis in several African countries. Fresh leaves of *R. multifidus* were subjected to maceration using 80% methanol and hydrodistillation to obtain hydroalcoholic extract (RM-M) and hydrodistilled extract (RM-H), respectively. Antileishmanial activities of the extracts were studied on clinical isolates of the promastigote and amastigote forms of *Leishmania aethiopica* and *Leishmania donovani*, whilst 4-day-suppressive, Rane's and prophylactic tests were employed to investigate *in vivo* antimalarial activity against chloroquine sensitive *Plasmodium berghei* infected mice. *In vitro* and *in vivo* antitrypanosomal effects of the extracts were also investigated against *Trypanosoma congolense* field isolate. Results of the study revealed that both extracts were active against the studied protozoans, although RM-H demonstrated superior activity ($p < 0.001$) compared the RM-M. The IC_{50} values of RM-H were 0.490 and 0.984 μg/ml against promastigotes and 1.49 and 1.84 μg/ml against macrophage amastigotes of *L. aethiopica* and *L.*

donovani, respectively. At a dose of 35.00 mg/kg/day, RM-H demonstrated a chemosuppression value of 70% in 4-day-suppressive test. Furthermore, at a concentration of 4 mg/ml, RM-H ceased motility of *T. congolense* within 20 min. Further phytochemical analysis of RM-H using preparative thin layer chromatography (PTLC) led to the isolation of an α,β-unsaturated dilactone characterized as anemonin on the basis of FT-IR, APCI-MS, 1D- and 2D-NMR spectral assignments and also by comparison with the reported spectroscopic data of the same compound. Anemonin displayed significant ($p < 0.001$) antileishmanial activity with IC_{50} values of 1.33 nM and 1.58 nM against promastigotes and 1.24 nM and 1.91 nM against amastigotes of *L. aethiopica* and *L. donovani*, respectively. Anemonin was found to be relatively less toxic to macrophage cells with selectivity indices (macrophage cytotoxic/antileishmanial ratios) of 22 and 14, respectively. In a 4-day suppressive, Rane's and prophylactic antimalarial tests, anemonin showed median effective doses (ED_{50}s) of 2.17, 2.78 and 2.70 μM, respectively. At a concentration of 4 mg/ml, anemonin completely immobilized trypanosomes within 5 min of incubation, while the standard dug diminazene diaceturate immobilized the parasites within 10 min. In the *in vivo* antitrypanosomal assay, anemonin eliminates parasites at all the tested doses (8.75, 17.00 and 35.00 mg/kg/day) and prevented relapse. Results of the present study provided evidence that the leaves of *R. multifidus* possess genuine antileishmanial, antiplasmodial and antitrypanosomal activities, which may be attributed to the presence of anemonin. Thus, use of the plant in traditional medicine for the treatment of protozoal diseases seems to be well founded.

Keywords: *Ranunculus multifidus*, Traditional medicine, Antiplasmodial, Antileishmanial and cytotoxic/antiprotozoal ratios, Antitrypanosomal activities, Anemonin.

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List of acronyms and abbreviations

^{13}C NMR	Carbon thirteen Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
2% TW80	2% Tween 80
AAU	Addis Ababa University
ANOVA	Analysis of Variance
APCI	Atmospheric Pressure Chemical Ionization
CC_{50}	Concentration that reduced Cell viability by 50%
CHS	College of Health Sciences
CL	Cutaneous leishmaniasis
DA	Diminazene Diaceurate
DEPT	Distortionless Enhancement by Polarization Transfer
DMIP	Department of Microbiology, Immunology and Parasitology
DMSO	Dimethyl sulfoxide
DoP	Department of Pharmacology
ED_{50}	Effective dose that suppresses 50 % of parasitaemia
GSH	Glutathione
HINBCS	Heat Inactivated New Born Calf Serum
IC_{50}	Inhibitory concentration that suppresses 50 % of parasite
ip	Intraperitoneal
LC_{50}	Concentration causing 50% Lysis
LD_{50}	Lethal Dose which cause death to 50% of mice
MEM	Minimum Essential Medium
MS	Mass Spectrometry
MST	Mean Survival Time
MTT	Methyl Thiazolyl Tetrazolium
NMR	Nuclear Magnetic Resonance
NNN	Novy-MacNeal-Nicolle
OECD	Organization of Economic Cooperation and Development
PBS	Phosphate Buffer Saline

PCV	Packed Cell Volume
RBCs	Red Blood Cells
Rf	Retention Factor
rpm	Revolution Per Minute
RPMI-1640	Roswell Park Memorial Institute-1640
SEM	Standard Error of Mean
SoM	School of Medicine
SoP	School of Pharmacy
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
UV	Ultraviolet
VL	Visceral Leishmaniasis

1 Introduction

1.1 Protozoal diseases

Protozoans are unicellular eucaryotic parasitic organisms causing serious tropical diseases in both humans and animals (Spicer, 2008). Malaria, trypanosomiasis and leishmaniasis are among the major parasitic diseases distributed throughout the world, classified as blood and tissue protozoan infections (Paniker and Ghosh, 2017; Jawetz *et al*., 2001). These protozoal diseases are vector borne diseases (arthropod transmission) (Chacon-Cruz and Mitchell, 2003). Leishmaniasis and trypanosomiasis are caused by related kinetoplastid hemoflagellates protozoan pathogens, whereas malaria is caused by sporozoan parasite (Bruckner and Labarca, 2006). Protozoan diseases threaten almost one-third of the world's population mainly the poor and people living in tropical countries (Witschel *et al*., 2012).

1.1.1 Leishmaniasis

Leishmaniasis is caused by an obligate intracellular parasite of the genus *Leishmania*, transmitted by phlebotomine sand flies (CDC, 2020). Leishmaniasis causes illness to 1.5 to 2 million people worldwide (Torres-guerrero *et al*., 2017). The symptoms of leishmaniasis range from, self-healing skin ulcers cutaneous leishmaniasis (CL), more severe chronic mucocutaneous infections mucocutaneous leishmaniasis to severe life-threatening visceral disease visceral leishmaniasis (VL) (Torres-guerrero *et al*., 2017). Leishmaniasis is the ninth largest disease burden among individual infectious diseases (Alvar *et al*., 2012) with VL being a major opportunistic infection associated with HIV (Alvar *et al*., 2008; Molina *et al*., 2003). Leishmaniasis is highly prevalent in Ethiopia; people in the highlands are at higher risk of CL while VL is prevalent mostly in lowland arid areas of Ethiopia (Lemma *et al*., 2017; Bsrat *et al*.,

2015; Gadisa *et al*., 2015; Seid *et al*., 2014; Alvar *et al*., 2008). In Ethiopia *Leishmania aethiopica, L. major* and *L. tropica* are causative agents of CL while *L. donovani* is responsible for VL (Claborn, 2010; Ashford *et al*., 1973).

Leishmaniasis is diagnosed by observing the parasite under microscope from a culture and serological tests called direct agglutination test (Alvar *et al*., 2008; Boelaert *et al*., 1999). Efficacy, toxicity, cost and availability of drug are among factors determining the choice of drug to treat leishmaniasis (Bryceson, 2001). Pentavalent antimonials are the first antileishmanial agents introduced, which have been the mainstay of treatment for more than 60 years (Chulay *et al*., 1988). They are administered parenterally for 30 days which led to intolerance and toxicity and reduced compliance due to long term high dose therapy (Cruz *et al*., 2007). Amphotericin B is also a drug of choice for leishmaniasis, but the conventional amphotericin B has been reported for secondary resistance in HIV co-infected patients (Di Giorgio *et al.*, 1999). The new liposomal amphotericin B (Ambisome®) is less toxic and most efficient of the lipid-associated preparations, but the price restricts its use to wealthy countries (Bryceson, 2001). Currently available drugs for leishmaniasis are toxic and cause over 20% morbidity which led to poor patient compliance (Deep *et al*., 2017). Challenges also arise due to loss of immunity, parasite and human population heterogeneities, which play major role for the revival of drug resistance (Selvapandiyan *et al*., 2019; Croft *et al*., 2006; Boelaert *et al*., 2002). Although development of antileishmanial vaccine is feasible and progress has been made on the development of a vaccine, an effective one is not available yet (Okwor *et al*., 2016; Mutiso *et al*., 2013; Launois *et al*., 2008; Mauël, 2002). Prevention of leishmaniasis mainly focus on vector control (control of adult sand flies, interior residual sprays and insecticide-treated bed nets), augmented by other measures such as get rid of reservoir, screening and personal protection (Claborn, 2010).

1.1.2 Malaria

Malaria is a protozoan disease caused by *Plasmodium* species which is transmitted by the bite of female *Anopheles* mosquito through injection of sporozoites at the time of blood meal (Sriwichai *et al*., 2016). Malaria is one of the most pressing public health issues in developing countries (Hay *et al*., 2004). Globally, there are an estimated 229 million malaria cases in 87 malaria endemic countries worldwide in 2019, in which 94% of the cases (215 million cases) occur in African Region (WHO, 2020). In Ethiopia, 904,495 malarial cases occurred (738,155 cases due to *Plasmodium falciparum* and the rest due to *Plasmodium vivax*) in 2019 (WHO 2020). In Africa, particularly in sub-Saharan regions, malaria is one of the most serious medical issues with significant economic burden (Hay *et al*., 2004).

Malaria is diagnosed by clinical signs and symptoms and parasitological investigations (Roestenberg *et al*., 2012). Commonly used antimalarial drugs are from the following classes of compounds: the quinolines (chloroquine, quinine, mefloquine, amodiaquine and primaquine), the antifolates (pyrimethamine, proguanil and sulfadoxine), the artemisinin derivatives (artemisinin, artesunate, artemether, arteether) and hydroxynaphthaquinones (atovaquine) (Joseph *et al*., 2011; Na-Bangchang and Karbwang, 2009). Currently, the artemisinin-based combination therapies are first line treatments for uncomplicated falciparum malaria in all endemic countries (Mullard, 2018). Prevention and control of malaria include vector control with long-lasting insecticidal nets and indoor residual spraying (Afrane *et al*., 2014). Emergence and fast spread of multi-drug resistant *Plasmodium* parasites, development of insecticide resistance mosquito, and absence of effective vaccine are some of the challenges in the process of eradication and control of malaria (Afrane *et al*., 2014; Hay *et al*., 2004).

1.1.3 Trypanosomiasis

Trypanosomiasis is a potentially fatal human and animal disease transmitted by bite of an insect (tsetse fly) (Büscher *et al*., 2017; Brun, *et al*., 2010). Disease causing *Trypanosoma* species include: *Trypanosoma brucei* complex– causes African trypanosomiasis (sleeping sickness); *Trypanosoma brucei* with 3 sub-species: *Trypanosoma brucei rhodesiense*- causes acute sleeping sickness in East Africa (Rhodesian sleeping sickness), *Trypanosoma brucei gambiense*- causes chronic sleeping sickness in West Africa (Gambian sleeping sickness) and *Trypanosoma brucei brucei*- infects livestock (cattle, horse, dog, mule and donkey) and causes nagana disease in animals (Mwiinde *et al*., 2017; Franco *et al*., 2014; Bouteille and Buguet, 2012). *Trypanosoma cruzi* causes American trypanosomiasis (Chagas' disease) (Rassi and De Rezende, 2012; Rassi *et al*., 2010). *Trypanosoma congolense, T. evansi, T. simiae, T.b. brucei* and *T. vivax* are responsible for animal disease infecting cattle, sheep, camels, goats, horses, and many other domestic and wild mammals (Cayla *et al*., 2019).

Animal trypanosomiasis is prevalent in the southern and western regions of Ethiopia where the primary vector tsetse-fly (*Glossina pallidipes)* exist (Lejebo *et al*., 2020). In Ethiopia bovine trypanosomiasis is caused by *T. congolense*, the dominant trypanosome species, followed by *T. vivax* and *T.b. brucei* (Amante and Tesgera, 2020; Gelaye and Fesseha, 2020; Abayneh and Tadesse, 2019). Trypanosomiasis is a major livestock problem due to its socioeconomic impact such as: mortality, morbidity, reduction in milk and meat production, abortion, and costs associated with treatment (Amante and Tesgera, 2020; Abera *et al*., 2016). Animals bitten by tsetse fly develop fever, progressive weight loss, anemia, frequent recumbent position and progressively become weak and unproductive (Chanie *et al*., 2012). Approaches to control trypanosomiasis in Ethiopia include control or elimination of tsetse flies by control of breeding

sites of vector and use of insecticides and sterile male techniques (Gelaye and Fesseha, 2020; Lejebo *et al*., 2019). The other is prevention or treatment of animals using trypanocidal drugs. Currently used chemoprophylaxis and therapeutic trypanocides include: diminazene aceturate, homidium, isometamidium, quinapyramine, suramin and, melarsen oxide cysteamine (cymelarsan) (Chaka and Abebe, 2003). Diminazene aceturate is the most commonly used therapeutic agent while isometamidium chloride is mostly used as a prophylactic agent (Al-Badrani, 2012). These approaches, however, have many drawbacks such as the high cost of drugs and insecticides, possibilities of undesirable environmental pollution by insecticides, and increasing development of resistance in the parasites to the existing drugs and toxicity (Mergia *et al.*, 2016; Ibrahim *et al*., 2012). The development of drug resistance and absence of vaccine against trypanosomes calls for the urgent need of new drugs for the control of trypanosomiasis (Anene *et al*., 2003, Chaka and Abebe, 2003).

1.2 The genus *Ranunculus*

The genus *Ranunculus* belongs to the family Ranunculaceae commonly known as a buttercup family (Evans, 2009). Members of the family are dicotyledonous flowering plants widely distributed in the world (Evans, 2009). Most species grow in mesic and wet environments (Srivastava, 2010). *Ranunculus* consists of 600 species of annual herbs distributed widely in the temperate regions of north hemisphere and in the tropics limited to higher altitude (Aslam *et al*., 2012; Evans, 2009).

1.2.1 Ethnobotanical uses

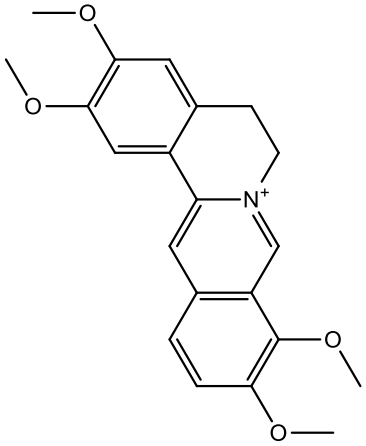
Ranunculus species are mostly used for the treatment of asthma, rheumatism and fever in a decoction form. Uses of the most important members of the genus are depicted in Table 1.

Table 1. Ethnobotanical uses of *Ranunculus* species in traditional medicine

Plant name	Plant part	Use	Reference
R. aquatilis	Whole plant	Fever, asthma, rheumatism	Dogra *et al*., 2015; Dobriyal *et al*., 1997
	Leaves	Helminthiasis	Hassan *et al*., 2017
R. arvensis	Leaves	Fever, asthma, gout	Dogra *et al*., 2015; Dobriyal *et al*., 1997;
R. bulbosus	Whole plant	Gout, arthritic and neuralgic pains	Leporatti and Ghedira, 2009
R. chinensis	Whole plant	Livestock diarrhea and parasite	Shen *et al*., 2010
R. diffusus	Leaves and stems	Rheumatism	Paulsamy *et al*., 2007
R. hirtellus	Roots	Swelling, wound healing	Sekar and Rawat, 2011; Uniyal *et al*., 2006;
	Arial parts	Livestock fever	Sharma and Singh, 1989
R. laetus	Leaves and flowers	Conjunctivitis	Dobriyal *et al*., 1997
	Roots	Tympany	Paulsamy *et al*., 2007
R. muricatus	Whole plant	Fever, gout, asthma, helminthiasis	Fatima *et al*., 2019; Hassan *et al*., 2017; Dogra *et al*., 2015; Dobriyal *et al*., 1997
R. pulchellus	Whole plant	Wound healing	Juyal and Ghildiyal, 2013
R. repens	Leaves	Hemorrhage	Mantle *et al*., 2000
R. sceleratus	Whole plant	Internal abscess, malaria, scrofula, snake or scorpion venom, pain, cold, rheumatism and acute icteric hepatitis, asthma, muscular pain	Fatima *et al*., 2019; Kumar *et al*., 2018; Ikram *et al*., 2014; Mei *et al*., 2012
R. stagnalis		Eczema	Chekole, 2017

1.2.2 Phytochemistry

Ranunculus species are rich sources of bioactive constituents that belong to various classes of secondary metabolites (Aslam *et al*., 2012). The protoberberine-type quaternary alkaloids such as palmatine **(1)** are common in the genus (Zhang *et al*., 2007; Bonora *et al*., 1990). Most members of the genus are also adept in the biosynthetic elaboration of coumarins like esculetin **(2)** (Hussain *et al*., 2009; Yun-xue *et al*., 2004) and phenolic compounds such as protocatechuyl aldehyde **(3)** (Deng *et al*., 2013). O-Methylated flavonol glycosides such as jacein **(4)** have been reported from several members of the genus (Khan *et al*., 2016; Bhatti *et al*., 2015; Hussain *et al*., 2009; Zou *et al.*, 2007; Li *et al*., 2005; Markham *et al*., 1997; Wagner *et al*., 1977). In addition, phytosterols, saponins and triterpenes are known to occur in different species *Ranunculus* (Nazir *et al*., 2013; Louaar *et al*., 2012; Hussain *et al*., 2009). Most members of the genus biosynthesize the toxic glycoside ranunculin **(5)**, a useful chemical marker of the family Ranunculaceae (Neag *et al*., 2018; Hill and van Heyningen, 1951). Enzymatic hydrolysis of ranunculin results in the formation of protoanemonin **(6)** which cyclodimerizes to give the water insoluble crystalline dimer anemonin **(7)** (Southwell and Tucker, 1993; Mahran *et al*., 1968; Hill and van Heyningen, 1951).



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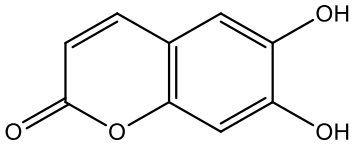

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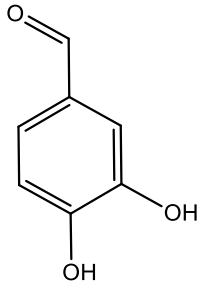

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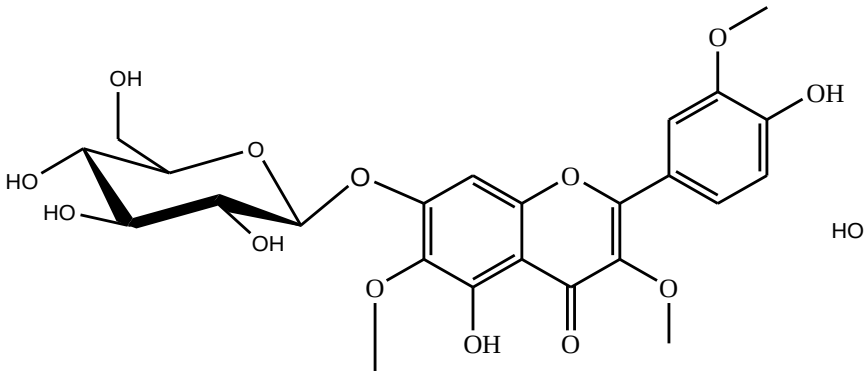

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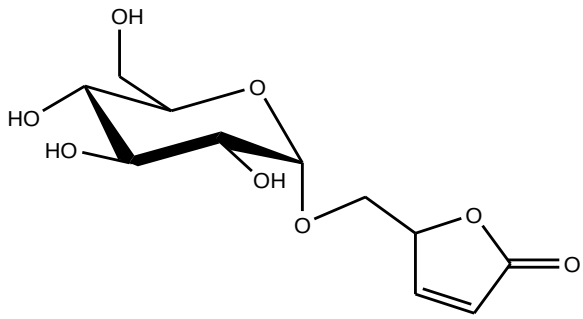

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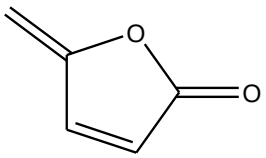

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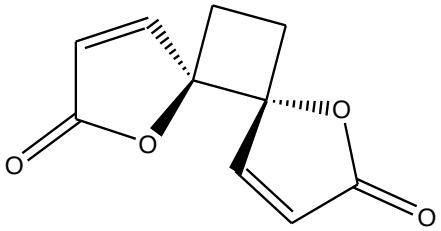


(7)

1.2.2 Pharmacological activities

Pharmacological studies done on the genus *Ranunculus* show that most members of the genus possess antibacterial (Hakan and Karagöz, 2018; Shahid *et al*., 2015; Hussain *et al*., 2009; Bonjar, 2004; Mares, 1987), antifungal (Hakan and Karagöz, 2018; Khan *et al*., 2016; Hachelaf *et al*., 2013), antiviral (Li *et al*., 2005), anti-inflammatory (Akkol *et al*., 2012; Prieto *et al*., 2003) and antioxidant (Shahid *et al*., 2015; Bhatti *et al*., 2015; Kaya *et al*., 2010; Mantle *et al*., 2000) activities. Azam *et al*. (2018) reported that the methanol and dichloromethane extracts of the aerial part of *R. muricatus* have *in vivo* leishmanicidal activity with an IC_{50} value of 0.29 µg/ml. Similarly, Orhan *et al*. (2006) have reported that the ethanolic extract of *R. trichophyllus* possesses antiprotozoal activity against *Trypanosoma b. rhodesiense* and *Leishmania donovani* with IC_{50} values of 27.8 and 52.3 µg/ml, respectively.

1.3 *Ranunculus multifidus* Forssk.

1.3.1 Botanical description

Ranunculus multifidus Forssk. (Ranunculaceae) is a perennial herb, which grows in moist and open grassy places, near rivers, streams and lakes, on wet slopes and in open mountain forest mostly considered as weed in meadows (Abebe, 2016; Teketay, 1993). The plant is an erect herb up to 1 m tall, the leaves are basal, cauline, hairiness and variable in shape and size (Teketay, 1993). The flowers are bright yellow cup-shaped solitary and axillary, with a five (sometimes six) obovate and glabrous petals which have a yellow stamen (Teketay, 1993). The plant is indigenous to Ethiopia and it is also found throughout much of the sub-Saharan African countries (Kasali *et al*., 2014; Teketay, 1993; Hedberg, 1987). In Ethiopia it is commonly known with vernacular names such as '*Etse siol*' (Geez), 'Gundi', (Amharic), '*Tuche* or *Aysmamata*' (Gamo), '*Abba warqe*' (Afaan Oromo) and '*Hogioo*' (Kaffa) (Teklehaymanot *et al*., 2007; Teketay, 1993). '*Etse siol*' in Geez literally means "a plant of hell" to indicate the burning sensation that is felt by applying it (Hedberg, 1987).

Figure 1. Areial part of *Ranunculus multifidus* Frossk. (Photographed by Betelhem Sirak around Dorze village South Ethiopia- August, 2019)

1.3.2 Ethnobotanical uses

Traditionally the fresh of leaves of *R. multifidus* are squeezed and applied topically to treat leishmaniasis in different parts of Ethiopia (Aschale *et al*., 2018; Birhan *et al*., 2017; Wolde-Mariam *et al*., 2015). In Gamo zone, south Ethiopia fresh leaves *R. multifidus* are chopped, filtrated and the juice given to cattle by oral drenching to treat oedema and trypanosomiasis (Tadesse and Balcha, 2018). In the Democratic Republic of Congo, decoction of the fresh leaves is used for the treatment of malaria and abdominal pain (Kasali *et al*., 2014). In Ethiopia the leaves of the plant are used for the treatment various ailments such as: tonsillitis (Kidane *et al*., 2014), cataract and eye infection (Giday *et al*., 2010), asthma, amoebiasis, haemorrhoid, and intestinal worms (Yineger *et al*., 2008), external tumor (*nekersa*)/cancer (Ayele, 2018; Abebe, 2016; Teklehaymanot *et al*., 2007), dysentery, eye diseases, skin diseases and toothache (Awas and Demissew, 2009; Giday *et al*., 2009). In South Africa decoction of the root is taken orally or in the form of enema to treat sexually transmitted diseases, tuberculosis, genital sores, warts, haemorrhoid, infertility, to cleanse blood during pregnancy and to ease labour (De Wet and Ngubane, 2014; Naidoo *et al*., 2013).

1.4 Statement of the problem

Protozoan diseases are major health problems worldwide (Gubler, 2018). The effectiveness of current antiprotozoal therapies is significantly declining due to increased drug resistance, emerging cross resistance, requirements for parenteral administration and/or length of treatment, lack of new drugs with novel mechanisms of action and unavailability of effective vaccine (Capela *et al*., 2019; Chunge *et al*., 1985). Therefore, there is an urgent need of new, affordable, safe, effective and easily administered drugs (Andrews *et al*., 2014). Medicinal plants represent a

potential source of new drugs (Rout *et al*., 2009; Fournet and Muñoz, 2002; Tyler, 1999; Phillipson and Wright, 1991). This is because natural products have been known to be good sources of pharmacologically active compounds against several ailments, including protozoal infections (Simoben *et al*., 2018; Fournet and Muñoz, 2002). Despite the antiprotozoal claims of *R. multifidus*, to date, there appears is report in the literature concerning the antiprotozoal activity of the plant except the *in vitro* antimalarial activity on the crude extract (Clarkson *et al*., 2004). The aim of the present research is to investigate the antileishmanial, antimalarial and antitrypanosomal activities of the fresh leaves of *R. multifidus.*

2 Objectives

2.1 General objective

- To investigate the antileishmanial, antimalarial and antitrypanosomal activities of the fresh leaf extracts of *Ranunculus multifidus* and its major constituent(s)

2.2 Specific objectives

- To prepare 80% methanol extract from the fresh leaves of *R. multifidus;*
- To prepare hydrodistilled extract from the fresh leaves of *R. multifidus;*
- To carry out acute oral toxicity test on the leaf extracts of *R. multifidus*;
- To investigate the *in vitro* antileishmanial activity of the extracts against the promastigote and amastigote forms of *Leishmania aethiopica* and *L. donovani*;
- To study the *in vivo* antimalarial activity of the extracts against chloroquine sensitive *Plasmodium berghei*;
- To examine the *in vitro* and *in vivo* antitrypanosomal activity of the extracts against *Trypanosoma congolense* field isolate;
- To isolate major compound(s) from the most active extract;
- To elucidate structure(s) of the isolated compound(s);
- To determine acute oral toxicity of the isolated compound(s); and
- To determine the *in vitro* antileishmanial, *in vivo* antimalarial and *in vitro* and *in vivo* antitrypanosomal activities of the isolated compound(s)

3. Materials and Methods

3.1 Materials

3.1.1 Plant material

Fresh leaves of *R. multifidus* were collected from Dorze village located in Chencha woreda, Gamo zone, (520 km southwest of Addis Ababa, Ethiopia) located in the Rift Valley above the west shore of Lake Abaya at 6°11'36" N and 37°34'13" E. The plant material was authenticated by Ato Melaku Wondafrash, National Herbarium, Department of Biology, College of Natural and Computational Sciences, Addis Ababa University (AAU), where a botanical specimen was deposited (collection number BS-001) for future reference.

3.1.2 Chemicals

n-Hexane, chloroform, ethyl acetate and methanol (LOBA-Chemie, India), trisodium citrate (BDH Chemicals Ltd, England), Giemsa (ESJAY Chemicals, Maharashtra, India), resazurin sodium salt, dimethyl sulfoxide (DMSO), triton X-114 and potato starch powder (Sigma-Aldrich Laborchemikaien GmbH, Germany), phosphate buffer saline (PBS) (Gibco, USA), Roswell Park Memorial Institute-1640 (RPMI-1640) (Sigma-Aldrich, UK), minimum essential medium (MEM), heat inactivated new born calf serum (HINBCS), penicillin-streptomycin solution and (Sigma-Aldrich, Co., MO, USA) were all used as received.

3.1.3 Instruments

Rotavapor (Heidolph Instruments GmbH and Co., Germany), freeze dryer (Alpha 1-2LD plus Christ Co. Ltd., Germany).

Olympus inverted type light microscope (Shinjuku, Tokyo, Japan), Primo Star normal light microscope (Carl Zeiss, Germany) Gerhardt thermoshake (C. Gerhardt GmbH and Co. KG, Germany), Timo autoclave (AMGC, Autoclave PBI International benchtop, Pleinlaan, 27G.411, Belgium), biological safety cabinet class IIA with UV (Laboculture ESCO 903, USA), microplate fluorometer and luminometer (Thermo Scientific Flouroscan Ascent FL, Thermo Fisher Scientific, USA), Thermo Electron carbon dioxide CO_2 incubator (Fischer Scientific, USA), Lambda 9 spectrometer (Perkin/Elmer Corp., Germany) and microhematocrit centrifuge (Hettich haematokrit, Germany) were used for biological assay.

Atmospheric Pressure Chemical Ionization (APCI) mass spectra were measured on an Advion Expression spectrometer (AstraZeneca, United Kingdom), FT-IR spectra were run in Spectrum 65 FTIR (PerkinElmer, Waltham, MA, USA) and Nuclear Magnetic Resonance (NMR) spectra were recorded using VNMRS 400 MHz spectrometer (Agilent Technologies, USA).

3.1.4 Test organisms

3.1.4.1 *Leishmania*

Clinical isolates of *L. aethiopica* (306/17) and *L. donovani* (139/19) were obtained from Leishmaniasis Research and Diagnostic Laboratory (LRDL), at the Department of Microbiology, Immunology and Parasitology (DMIP), College of Health Sciences (CHS), School of Medicine (SoM), AAU. *L. aethiopica* strain (306/17) was clinically isolated from active cutaneous lesion of a 60-year-old female farmer who resides in Ocholo, Gamo zone, South Ethiopia. The *L. donovani* strain (139/19) was isolated from splenic aspirates of a 27-year-old male patient with visceral leishmaniasis, residing in Konso village, South Ethiopia. All antileishmanial assays were carried out at LRDL, SoM, CHS, AAU.

3.1.4.2 *Plasmodium berghei*

Chloroquine sensitive *Plasmodium berghei* ANKA strain was obtained from Mekelle University, Ethiopia. The parasites were subsequently maintained in the laboratory by serial blood passage from infected mice to the non-infected ones on weekly basis.

3.1.4.3 *Trypanosoma congolense*

Trypanosoma congolense was obtained from naturally infected cattle in Abulo Kebele, Arba Minch, Gamo zone, South Ethiopia. Infected blood was collected from an ear vein of cattle in heparinized microhaematocrit capillary tubes to 3/4th of original height and sealed at dry end with sealing clay. The blood was centrifuged at 12,000 rpm for 10 min. Then, the capillary tube was cut and the contents of the capillary tube at the buffy coat zone were gently expressed on to a microscope slide, mixed and covered with a coverslip (22 x 22 mm) (Murray *et al.*, 1977). The slide was examined under a 40x objective microscope for observation of the parasite motility (Murray *et al.*, 1977). Giemsa stain was employed for morphological conformation of *T. congolense* and examined under a microscope using an oil immersion 100x objective (Uilenberg, 1998). The infected blood was collected from the jugular vein of the cattle using ethylenediaminetetraaceticacid (EDTA) cotted tubes and 0.2 ml of infected blood was injected intraperitonially to 5 healthy mice (Woo, 1970). The parasites were maintained in the laboratory by serial blood passage from infected mice to the non-infected ones.

3.1.5 Reference drugs

Amphotericin B (Laborchemikaien GmbH, Germany), diminazene diaceurate BP Vet [4,4-(diazoamino)dibenzamindine diaceturate] + 1.31 g phenazone BP (Ashish, India) and pure

chloroquine phosphate supplied by Ethiopian Pharmaceutical Manufacturing Factory (EPHARM, Ethiopia) were used as reference drugs.

3.1.6 Experimental animals

White Swiss albino mice of either sex weighing 22 - 30 g and age 5 - 6 weeks were employed throughout the experiment. The mice were obtained from Department of Pharmacology (DoP), School of Pharmacy (SoP), CHS, AAU. The animals were held in stainless steel cages at room temperature and a 12 h light/12 h dark cycle. They were provided with water and food pellets *ad libitum* in the animal house of the DoP, SoP, CHS, AAU. All the experiments were conducted in accordance with the internationally accepted laboratory animal use and care guideline (ILAR, 1996) and were approved by the Institutional Review Board of the SoP, AAU.

3.2 Methods

3.2.1 Extraction

3.2.1.1 Maceration

Fresh leaves of *R. multifidus* (2 kg) were rinsed with water to remove dirt, placed in a mortar and crushed with a pestle. The crushed plant material was then macerated with sufficient amount of 80% methanol at room temperature for 72 h with continuous agitation and filtered first with sterile gauze and then using Whatman no. 1 filter paper. This was repeated twice and the combined filtrate was concentrated in a rotavapor at a 40 °C temperature. The remaining aqueous solution was dried in a lyophilizer. The dried extract labeled as RM-M was transferred to an amber-coloured bottle and stored in a refrigerator at 4 °C until use.

3.2.1.2 Hydrodistillation

Fresh leaves of *R. multifidus* (1 kg) were chopped into small pieces and subjected to hydrodistillation for 3 h using a Clevenger type apparatus. The condensate was collected and extracted with 100 ml chloroform (3x) using a separatory funnel. To the combined organic solvent extract, about 5 g of ahydrous sodium sulfate was added to remove moisture and then filtered using Whatman no 1. filter paper. The organic solvent was concentrated in a rotavapor at a temperature not exceeding 35 ºC to yield a pungent oil designated RM-H. RM-H was transferred into an amber-coloured vial and stored in a refrigerator at 4 ℃ for further experiment.

3.2.3 Isolation of a compound

RM-H was subjected to preparative thin layer chromatography (PTLC) and the chromatograms were developed using a mixture of *n*-hexane and ethyl acetate (5:1) as a mobile phase. The chromatograms were visualized using ultraviolet light (UV) of wave lengths 254 nm and 366 nm. The major band was carefully scrapped off from the plates, washed with a mixture of chloroform and methanol (1:1), filtered using Whatman no. 1 filter paper and concentrated to dryness under reduced pressure. The white powder (designated RM-H1) obtained was further purified by PTLC using ethyl acetate: hexane (3:2) as a solvent system, weighed, transferred into an amber-coloured vial and stored in a refrigerator at 4℃ until use.

3.2.4 Spectroscopic analysis

FT-IR spectra were recorded in the region between 4000 - 400 cm^{-1} in KBr pellets, with a resolution of 4 cm^{-1}. Mass measurement was carried out using APCI. NMR spectral data were obtained at room temperature on FT-NMR spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C using deuterated dimethyl sulfoxide. A region from 0 to 12 ppm for ^{1}H and 0 to 205

ppm for ^{13}C was employed for scanning. Signals were referred to an internal standard tetramethylsilane (TMS). Chemical shifts are reported in δ units and coupling constants (*J*) in Hz. Multiplicities of ^{1}H NMR signals are indicated as *d* (doublet) and *m* (multiplet).

3.2.5 Acute oral toxicity test

Acute oral toxicity study was conducted as per the internationally accepted protocol of OECD Guideline 425 (OECD, 2008). Fifteen healthy female non-pregnant and nulliparous mice 6 - 8 weeks, weighing 22 - 28 g were randomly grouped into 3 each having 5 mice. All mice were fasted (food only) for 4 h before and 2 h after administration of the test substances. The test substances were dissolved in 2% Tween 80 (2% TW80). One mouse from each group was orally administered 2000 mg/kg of RM-M (Group 1), RM-H (Group 2) and RM-H1 (Group 3), consecutively. Then the mice were observed for general signs and symptoms of toxicity and mortality within 24 h. The mouse receiving 2000 mg/kg of the RM-M (Group 1) survived but the mice in Group 2 and 3 died after 30 min of administration. Since no death was observed within 24 h, 2000 mg/kg of RM-M was administered to remaining 4 mice in Group 1. Then they were observed individually for general signs and symptoms of toxicity, physical or behavioral changes such as loss of appetite, ruffled fur, lacrimation, mortality, and other signs of toxicity. The observation was carried out for 4 h with 30 min interval and then for 14 consecutive days with an interval of 24 h (OECD, 2008).

Main test was conducted for acute oral toxicity study of RM-H and RM-H1. Since there was no information regarding the 50% lethal dose (LD_{50}) and the slope of the dose-response curve for both test substances, dosing was initiated at 175 mg/kg (OECD, 2008). Twelve mice were grouped into two groups of six mice each and one mouse from each group received 175 mg/kg of

the test substances [RM-H (Group 1) and RM-H1 (Group 2)]. Since the experimental animals survived for 48 h, doses of the test substances were increased by a factor of 3.2 to 550 mg/kg. Following this, 550 mg/kg of RM-H and RM-H1 were given to the second mouse of Group 1 and Group 2, respectively, which resulted in the death of both mice 4 h after administration. Therefore, the remaining 4 mice in each group received 175 mg/kg of RM-H, while those in Group 2 were given 175 mg/kg/mouse of RM-H1. The mice were observed individually for general signs and symptoms of toxicity, physical or behavioral changes such as loss of appetite, ruffled fur, lacrimation, mortality, and other signs of toxicity continuously for 4 h with 30 min interval and then for 14 consecutive days with an interval of 24 h (OECD, 2008).

3.2.6 Antileishmanial assay

3.2.6.1 *Leishmania* culture

Clinically isolated *L. aethiopica* and *L. donovani* parasites were cultured in Lock's treated Novy-MacNeal-Nicolle (NNN) medium containing antibiotic solution (penicillin 100 IU/ml and streptomycin 100 µg/ml) (Tegazzini *et al*., 2016). The preparation of NNN medium and Lock's solution is shown in Appendix 1. The logarithmic stage parasites were transferred from NNN media into tissue culture flasks containing complete RPMI-1640 medium (RPMI-1640 medium supplemented with 10% HIFCS and 100 IU penicillin/ml-100µg/ml streptomycin solution) at 22℃ for *L. aethiopica* and 26℃ for *L. donovani* (Habtemariam, 2003; Ephros *et al*., 1999). The culture was monitored every day for two weeks to ensure healthy growth of parasites (Castilla *et al*., 1995). The logarithmic stage of the parasites was used for antipromastigote assay, while stationary phase (metacyclic phase) was used for macrophage infection (antiamastigote assay) (Tegazzini *et al*., 2016).

3.2.6.2 Antipromastigotes assay

To 96-well plates filled with 100 µl of complete RPMI-1640 medium, 100 µl of test substance (300 µg/ml) dissolved in 3% DMSO was added on the first well. Then, 100 µl was taken into subsequent wells then the last 100 µl was discarded, to achieve three-fold serial dilution. A total of 12 dilutions ranging from 100 µg/ml up to 0.000565 µg/ml were made to establish a full dose titration and determine IC_{50} values. All dilutions were done carefully by avoiding bubble formation. DMSO <1% was used as a negative control (<1% to prevent its negative outcome in cell viability), and amphotericin B was used as a positive control (same serial dilution as the test substances to calculate IC_{50}) (Abeje *et al*., 2014). Then, 100 µl of suspension of parasites (1 x 10^6 promastigotes/ml of *L. aethiopica* or L. *donovani*) were added to each well and contents of the plates were incubated at 22 ℃ for *L. aethiopica* and 26 ℃ for *L. donovani* (Castilla *et al*., 1995). After 68 h of incubation 2 µl fluorochrome resazurin solution (12.5 mg AlamarBlue dissolved in 100 ml of PBS) were added and incubated accordingly for 4 h for a total of 72 h (Rampersad, 2012). The fluorescence intensity was then measured by microplate fluorometer and luminometer at excitation wavelength of 530 nm and emission wavelength of 590 nm (Rampersad, 2012). The background fluorescence intensity of the complete media, samples, and reference drug was measured and subtracted for the corresponding wells. The IC_{50} values for each test substance was evaluated from sigmoidal dose–response curves using computer software Graphpad prism 8.0 and values were expressed as mean ± standard error of mean (SEM) of triplicate experiments with each test concentration in duplicate (Maleki *et al*., 2017).

3.2.6.3 Antiamastigote assay

Macrophage was collected from mice first by disinfecting skin around the peritoneum with 70% ethanol and a mouse was injected 1 ml of freshly prepared 2% starch intraperitoneally (Cote *et al*., 2006). After four days, the mice were sacrificed by spinal dislocation and the skin underlying the peritoneal cavity was shaved aseptically to expose the intact peritoneum. Then 10 ml of sterile ice-cold PBS with 3% HINBCS were injected into the peritoneal cavity and the peritoneal wall was massaged carefully to dislodge the attached macrophages (Welkos *et al*., 1989). Macrophages were then collected by drawing 6 - 8 ml exudates of the PBS, the exudates transferred into sterile 15 ml test tube, and centrifuged at 3500 rpm for 10 min at 4 ℃. Portion of the resulting pellet was cultured in 96-well plates containing 1×10^4 macrophage suspended in complete RPMI-1640 medium and kept for macrophage cytotoxicity assay (Dutra *et al*., 2016). The remaining portion of macrophages was suspended in complete MEM media containing 10% HINBCS, 2 mM L-glutamine, 100 IU penicillin and 100 µg streptomycin/ml. The host cells (macrophage) were counted and adjusted accordingly using haemocytometer to 1×10^6 cells per ml in complete MEM media. Then 300 µl (3×10^5 macrophages) were seeded in every well of 8-well plate containing removable microscopic slides. The cultured macrophage cells were allowed for adherence for at least 12 h at 37 ℃ in 5% CO_2. Non-adherent cells were washed twice with pre-warmed complete MEM media and incubated overnight in fresh media (Montrieux *et al*., 2014). Following overnight incubation, adherent cells were infected separately with late stationary stage *L. aethiopica* and *L. donovani* promastigotes with a parasite-to-cell ratio of 10:1 and incubated further for 12 h. After removal of non-internalized promastigotes by extensive washing with complete MEM media, cultures were left to rest for about 4 h (Borborema *et al*., 2011).

Amphotericin B was used as a reference drug to check sensitivity of the parasites. Samples were serially diluted in 8-well plates as indicated in Section 3.2.6.2. The culture prepared above was incubated with or without 100 µl of test substance for three days at 31 ℃ (*L. aethiopica*) and 37 ℃ (*L. donovani*), 5% CO_2 and 95% relative humidity. Following 72 h of incubation, the media was discarded, slides washed with PBS (prewarmed at 37 ℃), methanol was used for fixation for 15 min, then the slides were stained with 10% Giemsa for 15 min and washed with distilled water. The slides were then observed under microscope with oil immersion (100x objective). The number of amastigotes was determined by counting amastigotes in at least 50 macrophages in duplicate cultures (Borborema *et al*., 2011). Antiamastigote activity was determined by assessing the infection rate and parasitic load in both treated and untreated groups. Infection was considered adequate if more than 70% of the macrophages present in negative control were infected. The total actual parasite burdens were calculated using the infection index shown below (Chanmol *et al*., 2019).

$$\% \text{ Infection index} = \frac{\text{No of infected macrophage}}{\text{Total macrophage counted}} \times \frac{\text{No of amastigotes in 50 infected macrophages}}{\text{Total macrophage counted}}$$

The first half of the equation gives the percentage of infected macrophages and the second gives average parasite load found in infected macrophages (Paladi *et al*., 2012). The IC_{50} is inhibitory concentration of test substance that reduces 50% amastigotes density. The IC_{50} values for the samples were obtained from sigmoidal dose–response curves using Graphpad prism 8.0. The values were expressed as mean ± SEM of triplicate experiments with each test concentration carried out in duplicate (Poorrajab *et al*., 2009).

3.2.7 *In vitro* macrophage cytotoxicity assay

Macrophage cells (Section 3.2.6.3) were cultured in complete RPMI-1640 media in humidified 5% CO_2 and incubated at 37 °C for 24 h (Dutra *et al*., 2016). In 96-well plates containing a pre-cultured 100 µl suspensions of 1 x 10^4 macrophage, the medium was replaced by 100 µl of serially diluted test substances. A total of 12 dilutions (1000 µg/ml - 0.00565 µg/ml) dissolved in 1% DMSO was made (Borborema *et al*., 2011). Then contents of the plates were incubated at 37 °C in 5% CO_2. After 48 h of incubation, 5 µl (1/20, v/v) fluorochrome resazurin solution was added into each well and the fluorescence intensity was measured after 3 h using microplate fluorometer and luminometer at excitation wavelength of 530 nm and emission wavelength of 590 nm (Dutta *et al*., 2005). The concentration which kills 50% of the cells for each test substance (CC_{50}) was calculated from sigmoidal dose response curves using Graphpad prism 8.0 as mean ± SEM of triplicate experiments with each test concentration in duplicate (Poorrajab *et al*., 2009).

3.2.8 Selectivity index

Selectivity index (SI) was determined using CC_{50} of the normal macrophage and the IC_{50} of amastigotes. The selectivity of the test substance in killing the parasites as opposed to mice macrophage cells was assessed by the following formula: (Dutra *et al*., 2016).

$$\text{Selectivity index (SI)} = \frac{CC_{50}\ \text{macrophage}}{IC_{50}\ \text{amastigote}}$$

3.2.9 *In vitro* haemolysis test

Haemolytic activity was determined by using RBCs prepared from freshly collected O^+ human blood (2 ml) added to of 48 ml of PBS and centrifuged at 3500 rpm for 10 min at 4 °C. The

supernatant was washed off (3x) with PBS resulting in the formation of approximately 1 ml of RBC pellets (Löfgren *et al*., 2008). The resulting pellet was then re-suspended in 49 ml of PBS to make 2% blood suspension and the concentration adjusted to 1.9×10^9 RBC/ml. Then, 200 µl of the blood suspension was pipetted into eppendorf tubes containing each test substance at concentrations of 3.7, 11.11, 33.33, 100, 300, and 900 µg/ml to give a final volume of 1500 µl (Malagoli, 2007). The suspensions containing 2.5×10^8 RBS/ml was carefully mixed and incubated at 37 ℃ for 2 h. The membrane destabilizing activity of each test substance was determined in terms of its capability to rupture the cell membranes of RBC letting the release of haemoglobin into the solution. The mixture was centrifuged at 3500 rpm for 10 min resulting in intact and ruptured RBC to pellet liberating the haemoglobin in the supernatant solution and 75 µl from the supernatant of each tube was collected in 96-well plates and absorbance was measured at 540 nm using a Lambda 9 spectrometer (Dayeh *et al*., 2004). Triton X-114 (5 µl/ml) was used as a positive control which was prepared by adding 50 µl of blood to 100 µl Triton X-114 and incubated at 37 ℃ for 30 min (Somboonwiwat *et al*., 2005). RBC suspension with 1% DMSO was used as a negative control. Haemolytic effects were expressed as percentage of the absorbance of the positive control (100%) and the 50% lytic concentrations (LC_{50}) (Dayeh *et al*., 2004; Dathe *et al*., 1996). LC_{50} were determined non-linear regression analysis from sigmoidal dose-response curves with Graphpad prism 8.0 software, expressed as mean ± SEM, from triplicate experiments with each test concentration done in duplicate (Valadares *et al*., 2011).

3.2.10 Antimalarial assay

3.2.10.1 Inoculation of mice

Chloroquine sensitive strain of *P. berghei* (ANKA) was used for the antimalarial assay. Parasitaemia of the donor mice was first determined by preparing blood smear on microscope slides from blood film taken from infected mice tail (Tang *et al*., 2007). The smear was fixed with methanol and stained with Giemsa to determine parasitaemia level of the donor under a microscope. When the parasitaemia level was 30 - 40%, parasitized erythrocytes were collected from the donor mouse by cardiac puncture using a sterile syringe and placed in a Petri dish containing an anticoagulant (0.5% trisodium citrate) and then immediately diluted with uninfected mice blood and normal saline (0.9%) in such way that the final volume contains 5×10^7 infected erythrocytes/ml of blood (Fidock *et al*., 2004). The diluted blood (0.2 ml) was then injected into all the experimental mice intraperitoneally (ip) (Hilou *et al*., 2006).

3.2.10.2 Four-day suppressive test (Peter's test)

A four-day suppressive test against mice infected with chloroquine sensitive *P. berghei* was employed according to the method described by Peters (1975). Fifty-five mice were injected with inoculum of 1×10^7 *P. berghei* infected erythrocytes ip on the first day (day 0) (Waako *et al*., 2005). Two h post-infection, the mice were randomly distributed into eleven groups each containing five mice. Group 1 served as negative control (received vehicle 2% TW80, 10 ml/kg/day) and Group 2 as positive control (received chloroquine, 25 mg/kg/day). The remaining nine groups were treatment groups. Groups 3 - 5 received 100, 200 and 400 mg/kg/day of RM-M, respectively, while Groups 6 - 8 and Groups 9 - 11 received 8.75, 17.50 and 35.00 mg/kg/day RM-H and RMH-1, respectively. All the test substances were administered orally using oral

gavage and the doses were determined based on the acute oral toxicity test results. The middle dose was one tenth of the safe dose (~2000 mg/kg for RM-M and ~175 mg/kg for RM-H and RM-H1). The higher dose was twice the middle dose, and the lower dose was half of the middle dose (Mergia *et al*., 2016; Tamru *et al*., 2012). Treatment was started 3 h post-infection on day 0 and continued for additional three consecutive days at 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 parasitaemia (Misganaw *et al*., 2019). In addition, body weight, rectal temperature and packed cell volume (PCV) were measured just before infection and at the end of the experiment (Ancelin *et al*., 2003). Afterwards, mice were followed for 28 days (day 0 - day 27) so as to determine the mean survival time (MST) for each group (Fentahun *et al*., 2017).

3.2.10.3 Rane's test

Rane's test, which evaluates the curative potential of RM-H1, was carried as the method described by Ryley and Peters (1970). Twenty-five mice were injected ip with inoculum of 1 x 10^7 *P. berghei* infected erythrocytes on the first day (Day 0) (Mulaw *et al*., 2019). At day 3 the animals were randomly allocated into five groups with five mice in each group. Group 1 served as negative control received vehicle (2% TW80, 10 ml/kg/day) and Group 2 as positive control received chloroquine 25 mg/kg/day (Misganaw *et al*., 2019). Group 3 - 5 were treated with 8.75, 17.50 and 35.00 mg/kg/day RM-H1, respectively. Treatment continued for a further 3 days (i.e., 96, 120, 144 h post-infection) (Nureye *et al*., 2018). Parasitaemia level was recorded daily thru out the experiment starting from day 3 (Mekonnen, 2015; Bantie *et al*., 2014). PCV, rectal temperature and body weight were measured just before the first dose (day 3) and at the end of

the experiment (day 7). Thereafter, all groups were followed for 28 days and survival time was recorded (Mekuria *et al*., 2021).

3.2.10.4 Prophylactic test

Investigation of the prophylactic potential of RM-H1, was done as to the method described by Peters (1965). Twenty-five mice were randomly assigned into five groups of five mice each. Group 1, which served as negative control received vehicle (2% TW80, 10 ml/kg/day) and Group 2 (the positive control group) received chloroquine 25 mg/kg/day. Groups 3 - 5 were treated with 8.75, 17.50 and 35.00 mg/kg/day of RM-H1, respectively. Treatment was given orally for 3 days, 24 h after the last treatment (day 0), all mice were infected with inoculum of 1 x 10^7 *P. berghei* infected blood (Misganaw *et al*., 2019; Mulaw *et al*., 2019). Seventy-two h post-infection (day 3), blood smears were prepared from each mouse and the parasitaemia level was determined (Nureye *et al*., 2018). PCV rectal temperature and body weight were measured just before parasite inoculation (day 0) and at the end of the experiment (day 3) (Mekonnen, 2015; Bantie *et al*., 2014). Finally, the groups were followed for 28 days in order to record their survival time (Mekuria *et al*., 2021).

3.2.10.5 Determination of parasitaemia and survival time

Blood from each mouse was applied on different microscope slides and a thin smear was made. Then the smear was fixed with methanol for 15 min and stained with 10% Giemsa for 15 min. The slides were washed with tap water and dried at room temperature. The number of parasite infected RBC were counted using a light microscope with an oil immersion objective lens magnification power of 100x. Parasitaemia was determined by counting a minimum of three

fields per slide (Zucker and Campbell, 1993). Percent parasitaemia and percent inhibition were calculated by the following Peters and Robinson formula (1992)

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

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

MST was determined using the formula indicated below

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

3.2.10.6 Determination of packed cell volume, rectal temperature and body weight

Blood was collected from tail of each mouse in heparinized microhaematocrit capillary tubes to 3/4th of the tube and sealed at their dry end with sealing clay (Wernery *et al*., 2001). The tubes were then placed in a microhematocrit centrifuge with the sealed ends out wards (Wintrobe, 1935). The blood was centrifuged at 12,000 rpm for 10 min (Wintrobe, 1935). PCV was determined using the following relation (Gilmour and Sykes, 1951).

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

All mice were weighed using sensitive digital weighing balance and digital rectal thermometer was used to measure rectal temperature (Gudjonsson, 1932). The percentage changes of mean values of all the three parameters that occurred before and after treatment were then calculated.

$$\% \text{ Change} = \frac{\text{Mean on final day - Mean on intial day}}{\text{Mean on intial day}} \times 100$$

3.2.11 Antitrypanosomal assay

3.2.11.1 Determination of parasitaemia

Parasitaemia was monitored in blood obtained from the tail of mice. The number of parasites was counted by microscope at 40x magnification using the "Rapid Matching" method, which involves microscopic counting of parasites per field in infected blood and logarithm values of trypanosome number were obtained by matching with the table converted to Log number to provide absolute number of trypanosomes per ml of blood (Herbert and Lumsden, 1976).

3.2.11.2 *In vitro* assay

In 96-well plates, 200 µl of blood containing about 20 - 25 parasites per field was mixed with 50 µl of the test substances at concentrations of 20, 10, 2 and 0.5 mg/ml to produce effective test concentrations of 4, 2, 0.4 and 0.1 mg/ml, respectively. The dose was determined from previous *in vitro* studies (Mergia *et al*., 2015; Tewabe *et* al., 2014; Yusuf *et al*., 2012). Parasites suspended in 50 µl of 1% DMSO were used as a negative control. Similar doses of diminazene diaceurate (DA) were used as positive control (Mergia *et al*., 2015; Tewabe *et* al., 2014). The 96-well plates were incubated at 37 ℃ for 5 min, then about 2 µl of test mixtures was placed on separate microscope slides and covered with a coverslip (22 x 22 mm) and the parasites were observed every 5 min for a total duration of 1 h (Maikai *et al*., 2008; Nok, 2002; Freiburghaus *et al*., 1996). Cessation or drop in motility of the parasites in test sample-treated blood compared to the negative control was taken as a measure of trypanocidal activity (Atawodi and Ogunbusola,

2009; Paveto *et al*., 2004). *In vitro* trypanocidal activity was performed in triplicate experiments with each test concentration done in duplicate (Abdeta *et* al., 2020; Adeiza *et al*., 2009).

3.2.11.2.1 *In vivo* infectivity test

Infectivity test was performed in order to know if there are any remaining infective parasites after *in vitro* tests (Feyera *et al.*, 2011). The remaining incubation mixtures (0.2 ml) from Section 3.2.11.2 were inoculated ip into healthy mice (5 mice per dose) then the mice were monitored for development of parasitaemia for 30 days (Mergia *et al*., 2015; Tewabe *et al*., 2014).

3.2.11.3 *In vivo* assay

Fifty-five mice were injected ip with 0.2 ml of *T. congolense* infected blood diluted with PBS containing approximately 10^5 trypanosome cells collected from donor mouse by cardiac puncture (Tesfaye *et al*., 2015). The mice were left to develop parasitaemia for 2 weeks so that the average parasitaemia became approximately 7.20 (Log number) ~$10^{7.20}$/ml (Atawodi and Ogunbusola, 2009). After 2 weeks the mice were randomly divided into eleven groups with five mice in each group. Treatment started on the 14^{th} day post-infection (day 0 of treatment). Mice in Group 1 injected with vehicle (1% DMSO, 10 ml/kg/day) served as a negative control, while mice in Group 2 injected with a single dose of DA (28 mg/kg) served as a positive control (Mergia *et al*., 2016; Maikai, 2010). The remaining nine groups served as treatment groups. Animals in Groups 3 - 5 were injected with 100, 200 and 400 mg/kg/day of RM-M, respectively, while animals in Groups 6 - 8 and Groups 9 - 11 were injected 8.75, 17.50 and 35.00 mg/kg/day of RM-H and RM-H1, respectively. Dose selection for each group was based on oral acute toxicity results as discussed under Section 3.2.10.2 All test substances were dissolved in 1% DMSO and

administered ip every morning for seven days with continuous monitoring of parasitaemia every other day until the end of the experiment at 14th day (Mergia *et al*., 2016). For the assessment of antitrypanosomal effect of the test substance, the level of parasitaemia (expressed as Log number of parasites per ml of blood) in the treated animals was compared with those of the control animals (Mustapha *et al*., 2013). A thin smear was made from a drop of blood obtained from the tail of a mouse on microscope slides and monitorerd for parasitaemia every other day at 400x total magnification. Parasitaemia leavel was determined using the "Rapid Matching" technique of Herbert and Lumsden (1976). The wet blood smear was prepared in triplicate from mice, and the slide counts mean value was taken per sample. Body weight, rectal temperature and PCV were measured just before infecting the mice (pre-infection), just before starting treatment (day 0), at the end of the treatment (day 7) and at end of the experiment (day 14) as described in Section 3.2.10.6. Five healthy uninfected and untreated mice were used for comparison of the three parameters (body weight, rectal temperature and PCV) (Abdeta *et al*., 2020; Mergia *et al*., 2016).

3.2.12 Statistical analysis

Data analysis was carried out using IBM SPSS (Statistical Package for Social Sciences) Statistics for Windows, Version 25.0. Results were expressed as mean ± standard error of mean (M ± SEM). Statistical significance was determined by one-way ANOVA followed by Tukey post hoc test to compare different parameters among the treatment and control groups. $P < 0.05$ were considered significant.

4 Results and Discussion

4.1 Extraction

In traditional medicine, fresh leaves of *R. multifidus* are squeezed and applied topically (Aschale *et al*., 2018; Birhan *et al*., 2017; Wolde-Mariam *et al*., 2015) or taken orally (Tadesse and Balcha, 2018; Tekle, 2015; Kasali *et al*., 2014) for the treatment of protozoal diseases. In the current study, fresh leaves of *R. multifidus* were used to prepare extracts in order to mimic the form in which the plant is used in traditionally. As the bioactive compounds may vary from polar to nonpolar, two extraction methods were considered. Cold maceration with 80% methanol was used to extract heat-labile polar and moderately polar compounds, while hydrodistillation was employed to extract thermally stable relatively non-polar components. Maceration with hydroalcohol gave 6.4% (w/w) of brown powder, while hydrodistillation yielded 0.56% (w/w) yellow coloured irritating oil.

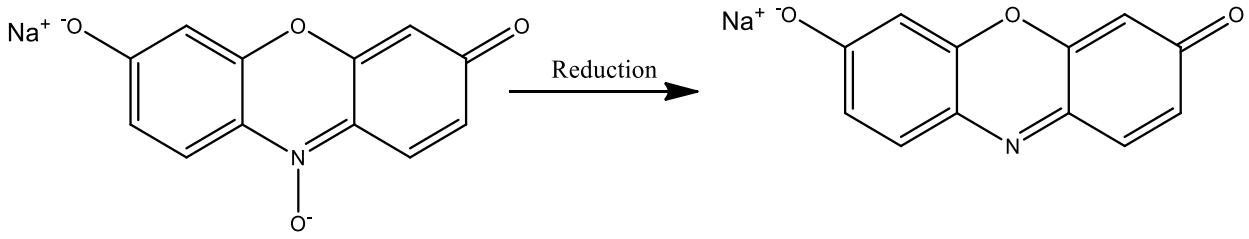
4.2 Acute oral toxicity of extracts

Acute toxicity test results of the present study documented that the hydroalcoholic extract (RM-M) was safe by oral route at a dose of 2000 mg/kg as there was no mortality and sign of toxicity within 14 days of observations which indicates that the 50% lethal dose (LD_{50}) of RM-M is above 2000 mg/kg (OECD, 2008). However, at the same dose, of the hydrodistilled extract (RM-H) showed severe toxicity leading to death of the experimental animals. Thus, main test was employed to determine the LD_{50} of RM-H, which revealed that RM-H has LD_{50} above 175 mg/kg but less than 550 mg/kg.

4.3 Antileishmanial activity of extracts

Traditional medicine practitioners and local people in different parts of Ethiopia widely use the leaves of *R. multifidus* for the treatment of leishmaniasis (Aschale *et al*., 2018; Birhan *et al*., 2017; Wolde-Mariam *et al*., 2015). It was on the basis of this ethnobotanical back-ground that the plant was selected for antileishmanial assay. In the current study, antileishmanial assay were done on both promastigote and amastigote forms of *L. aethiopica* and *L. donovani*, which are the major causes of CL and VL in Ethiopia, respectively (Lemma *et al*., 2017; Gadisa *et al*., 2015; Ashford *et al*., 1973).

AlamarBlue® or resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) reduction assay was used to determine the antileishmanial activity of the test substances as the technique permits a simple, rapid, reliable, sensitive, cost-effective method for continuous monitoring of cell cultures (Hoet *et al*., 2004). The assay technique is based on the principle that metabolically active cells (growing cells) in the culture medium have the capacity to reduce the oxidized, nonfluorescent and blue redox indicator resazurin **(8)** to the reduced, fluorescent and red form of the dye resorufin **(9)** (Figure 2) (Maes *et al*., 2004). Cell viability was determined by measuring fluorescent signal at excitation wavelength of 530 nm and emission wavelength of 590 nm. Fluorescence intensitie is proportional to the number of viable cells (Rampersad, 2012).



(8) **(9)**

Figure 2. Conversion of resazurin **(8)** to resorufin **(9)** by metabolically active cells

In the present study a positive linear correlation was established between viability of cell and concentrations of test substances (R^2 = 0.981 – 0.992) in both antileishmanial and cytotoxicity assays. As shown in Table 2, the tested extracts displayed antileishmanial activity on both the promastigote and amastigote forms of *L. aethiopica* and *L. donovani* with IC_{50} values ranging from 0.49 to 22.12 µg/ml. Though, RM-H was significantly ($p < 0.001$) more active than RM-M, although its activity was less than that of the positive control amphotericin B. Even though promastigotes and amastigotes evidently vary in their bioenergetics, morphology, gene expression, protein phosphorylation and expression of membrane proteins which result in difference in their susceptibility to test substances (Handman, E., 1999; Callahan *et al*., 1997; Glew *et al*., 1988), the extracts displayed similar activities against the promastigote and amastigotes forms of the tested parasites.

From the results shown in Table 2, the *in vitro* macrophage cytotoxic activity of RM-M is much less than those of RM-H and amphotericin B. Haemolysis test also confirmed that RM-M has a very high LC_{50} value (>1000 µg/ml) indicating its relative safety to red blood cells. Both RM-H and amphotericin B were found to be toxic to macrophages and cause red blood lysis at much lower concentrations. Overall, the hydroalcoholic extract appeared to be less toxic than the hydrodistilled extract. This is congruent with previous studies that reported the aqueous and dichloromethane: methanol (1:1) extracts of *R. multifidus* do not have cytotoxic effect on methyl thiazolyl tetrazolium (MTT) cellular viability assay against the human embryonic kidney epithelial cell line (104% cell growth at 100 µg/ml) (Naidoo *et al*., 2013).

Table 2. Antileishmanial activity, macrophage cytotoxicity and haemolytic property of the fresh leaf extracts of *Ranunculus multifidus* against promastigote and amastigote forms *Leishmania aethiopica* and *Leishmania donovani in vitro*

Test substance	Antileishmanial activity IC_{50} (µg/ml)				Cytotoxicity (µg/ml)		Selectivity index	
	Promastigotes		Amastigotes		Macrophage CC_{50}	Haemolysis LC_{50}		
	La	Ld	La	Ld			La	Ld
RM-M	$14.92\pm0.554^{b3,d3}$	$22.12\pm0.564^{b3,c3}$	$17.487\pm0.298^{b3,c3}$	$19.325\pm0.24^{b3,c3}$	256.62±0.211	> 1000	14	13
RM-H	0.49 ± 0.004^{c3}	0.984 ± 0.028^{c3}	1.49 ± 0.004^{c3}	1.814 ± 0.028^{c3}	4.98±1.583	25.68±0.07	3	3
AMB	0.0157±0.08	0.0067±0.008	0.0095±0.004	0.0063±0.011	4.31±0.983	47.25±0.54	453	684

Data expressed as mean ± SEM; n = 3; a: compared to RM-M, b: compared to RM-H, c: compared to Amphotericin B; 1: $p < 0.05$, 2: $p < 0.01$, 3: $p < 0.001$; La: *Leishmania aethiopica*, Ld: *Leishmania donovani*, RM-M: 80% methanol extract of *R. multifidus*, RM-H: hydrodistilled extract of *R. multifidus*; CC_{50}: concentration causing 50% cytotoxicity, LC_{50}: concentration causing 50% lysis; AMB: amphotericin B.

4.4 Antimalarial activity of extracts

The leaves of *R. multifidus* are used for the treatment of malaria in the Democratic Republic of Congo (Kasali *et al*., 2014). According to Clarkson *et al*. (2004), the whole plant extracted with dichloromethane: methanol (1:1) possesses *in vitro* antimalarial activity against chloroquine-sensitive strain of *Plasmodium falciparum* with an IC_{50} value of 2.3 µg/ml. Therefore, investigation of the plant for its *in vivo* antimalarial effect is warranted.

In the current study the *in vivo* antimalarial activity of *R. multifidus* leaf extracts was examined using the classic 4-day suppressive test to evaluate the chemosuppressive effect of the plant during early infection. Percent inhibition of parasitaemia (percent suppression) and survival time were taken as parameters for determination of the activity of test substances throughout the assay. Moreover, as malarial infection causes anemia, change in body (rectal) temperature, and reduction in body weight (Kumatia *et al*., 2012), potential of the tested substances to reduce anemia, prevent body weight loss, and regulate temperature in the infected mice were also studied.

Experimental results showed that both RM-M and RM-H possess chemosuppressive effect on parasitaemia in non-dose dependent manner. These effects were statistically significant relative to the negative control ($p < 0.001$). The chemosuppression percentages ranged from 39.20 to 70.61%. As shown in Table 3, RM-H exhibited a significant inhibition ($p < 0.001$) of parasite multiplication (70.61%), at an oral dose of 35.00 mg/kg per day. All tested doses of the extracts exhibited significantly lower ($p < 0.001$) parasitaemia reduction compared to the positive control. Moreover, the test substances prolonged MST significantly ($p < 0.001$) compared to the negative control although the values were significantly ($p < 0.001$) smaller than the standard drug chloroquine.

Table 3. Antimalarial activity of the leaf extracts of *Ranunculus multifidus* in mice infected with *Plasmodium berghei* in 4-day suppressive test

Test substances (mg/kg/day)	% Parasitaemia	% Suppression	Mean survival time (in days)
2% TW80	26.61±0.76	-	9.1 ± 0.32
CQ 25	0.2±0.2^{a3}	99.24	28 ± 0.0^{a3}
RM-M 100	15.82±1.09^{a3,b3,d3,e1,f3,g3,h3}	39.20	12.4±0.4^{a3,b3,h1}
RM-M 200	12.64±0.24^{a3,b3,f3,g3,h3}	48.42	12.8±0.5^{a3,b3,h1}
RM-M 400	9.58±0.29^{a3,b3,g1,h3}	53.18	13.1±0.24^{a3,b3}
RM-H 8.75	10.95±0.54^{a3,b3}	58.85	13.8±0.86^{a3,b3}
RM-H 17.50	9.68±0.48$^{a3, b3}$	63.62	14.0±0.83^{a3,b3}
RM-H 35.00	7.82±0.16^{a3,b3}	70.61	14.4±0.50^{a3,b3}

Data are expressed as mean ± SEM; n = 5; a: compared to 2% TW80, b: compared to chloroquine 25, c: compared to RM-M 100, d: compared to RM-M 200, e: compared to RM-M 400, f: compared to RM-H 8.75, g: compared to RM-H 17.50, h: compared to RM-H 35.00; 1: $p < 0.05$, 2: $p < 0.01$, 3: $p < 0.001$; CQ: chloroquine, 2% TW80: 2% tween 80 vehicle, RM-M: 80% methanol extract of *R. multifidus*, RM-H: hydrodistilled extract of *R. multifidus*.

The 4-day suppressive test results demonstrated that the tested extracts prevent body weight reduction significantly ($p < 0.001$) in comparison with the negative control. Whilst the effects of

the lower (100 mg/kg/day) and middle (200 mg/kg/day) doses of RM-M were much less than that of the positive control, the higher dose of RM-M and all dose levels of RM-H significantly ($p < 0.001$) protected parasite-induced weight reduction in infected mice as compared to those in the negative control group (Table 4). All dose levels of the tested extracts significantly ($p < 0.001$) prevented reduction in rectal temperature as compared to the negative control. As shown in Table 4, except the lower (100 mg/kg/day) and middle (200 mg/kg/day) doses of RM-M, all the others displayed statistically comparable temperature stabilization effect with that of the standard drug. All dose levels of RM-M and RM-H exhibited a statistically significant ($p < 0.001$) effect in the circumvention of PCV decline in *P. berghei* infected mice compared to the negative control although their effect was lower ($p < 0.001$) than that of the standard drug.

Table 4. The effect of leaf extracts of *Ranunculus multifidus* on body weight, rectal temperature and packed cell volume of *Plasmodium berghei* infected mice in 4-day suppressive test

Test substances (mg/kg/day)	Body weight (g)			Rectal temperature (°C)			Packed cell volume (%)		
	Day 0	Day 4	% Change	Day 0	Day 4	% Change	Day 0	Day 4	% Change
2% TW80	26.01±0.20	22.18±0.62	-14.72	36.58±0.01	33.04±0.21	-9.67	59.2±0.24	48.6±0.96	-17.90
CQ 25	26.32±0.57	29.25±0.65	11.32^{a3}	36.61±0.18	36.58±0.16	-0.08^{a3}	57.2±0.58	58.8±0.73	2.79^{a3}
RM-M 100	26.64±0.91	26.84±0.96	0.7 ^{a3,b3}	36.46 ±0.20	35.12±0.27	-3.67^{a3,b3}	56.4±0.74	50.2±0.58	-10.99^{a1,b3}
RM-M 200	26.84±0.96	27.90±1.09	3.94 ^{a3,b3}	36.42±0.18	35.56± 0.19	-2.36^{a3,b1}	58.0±0.70	52.2±0.86	-10.00^{a3,b3}
RM-M 400	26.56±1.06	28.32±1.14	6.62 ^{a3}	36.62±0.03	36.22±0.28	-1.09^{a3}	57.4±0.92	52.2±0.54	-9.05^{a3,b3}
RM-H 8.75	25.37±0.92	26.64±0.91	5.00^{a3}	36.26±0.08	35.16±0.29	-2.03^{a3}	57.2±0.86	51.2±0.73	-10.48^{a3,b3}
RM-H 17.50	24.88±0.51	26.84±0.96	7.87^{a3}	36.5±0.16	35.8±0.18	-1.91^{a3}	55.6±0.50	50.0±0.54	-10.07^{a3,b3}
RM-H 35.00	26.1±0.63	28.72±1.05	10.03^{a3}	36.69±0.15	36.42±0.18	-0.76^{a3}	57.6±0.50	52.4±0.67	-9.02^{a3,b3}

Data are expressed as mean ± SEM; n = 5; a: compared to 2% TW80, b: compared to chloroquine, c: compared to RM-M 100, d: compared to RM-M 200, e: compared to RM-M 400, f: compared to RM-H 8.75, g: compared to RM-H 17.50, h: compared to RM-H 35.00; 1: $p < 0.05$, 2: $p < 0.01$, 3: $p < 0.001$; CQ: chloroquine, 2% TW80: 2% tween 80 vehicle, RM-M: 80% methanol extract of *R. multifidus*, RM-H: hydrodistilled extract of *R. multifidus*.

Analysis of the 4-day suppressive test results indicated that both leaf extracts of *R. multifidus* inhibited parasite multiplication in *P. berghei* infected mice confirming the potential of the plant to prevent or mitigate primary attack due to malaria. Furthermore, the extracts improved survival

time, prevented weight loss, maintain rectal temperature and ameliorated anemia of infected mice, indicating that the plant has the capacity to reduce the overall pathogenic outcome of the parasite on test groups (Basir *et al*., 2012; Krettli *et al*., 2009).

4.5 Antitrypanosomal activity of extracts

In Ethiopian traditional medicine fresh leaves of *R. multifidus* are used for the treatment of animal trypanosomiasis (Tadesse and Balcha, 2018; Tekle, 2015). In this study extracts of *R. multifidus* were tested against *T. congolense* field isolate obtained from infected cattle residence in Gamo, southern Ethiopia, where the parasite is the dominant bovine trypanosome species in the region as well as in Ethiopia (Amante and Tesgera, 2020; Gelaye and Fesseha, 2020; Abayneh and Tadesse, 2019).

4.5.1 *In vitro* activity

Preliminary antitrypanosomal activity screening was conducted by an *in vitro* method, which is relatively simple, cheap and reliable (Peter *et al*., 2012; Mikail, 2009). The assay is based on parasite motility since motility is a relatively reliable indicator of viability of most trypanosomes (Atawodi *et al*., 2003). A complete elimination or reduction in motility of trypanosomes when compared to the control was taken as index of trypanocidal activity (Atawodi *et al*., 2009). At a concentration of 4 mg/ml, RM-H displayed antitrypanosomal activity by ceasing motility of the trypanosomes within 20 min and drastically reduced motility within 25 and 45 min at concentrations of 2 and 1 mg/ml, respectively. RM-M reduced motility of the trypanosomes within 30, 45 and 55 min at 4, 2 and 1 mg/ml test concentrations, respectively. Diminazine aceturate, the positive control used in this study, immobilized motility of trypanosomes within 10, 15, 30 and 45 min at 4, 2, 1 and 0.1 mg/ml test concentrations, respectively, while the

negative control (1% DMSO) and the lower test concentration (0.1 mg/ml) of both RM-H and RM-M did not immobilize or reduce motility of trypanosomes (Table 5).

In vivo infectivity test results revealed that mice treated with the positive control diminazine aceturate and RM-H (4 mg/ml) were free form parasites (lost infectivity) during the study period (for 30 days), while mice treated with RM-M (1 and 0.1 mg/ml), RM-H (0.1 mg/ml) and negative control (1% DMSO) developed infection at the 12^{th} day (Table 5).

Table 5. *In vitro* antitrypanosomal effect of the leaf extracts of *Ranunculus multifidus* on *Trypanosoma congolense* motility and *in vivo* infectivity test

Dose (mg/ml)	Effect	1% DMSO	DA	RM-M	RM-H
4 .0	Change in motility (in min)	NE	10*	30**	20*
	No of infectious mice / Total no of mice	5/5	0/5	2/5	0/5
	Infection interval (in days)	12±0.00	Ni	18±0.34	Ni
2.0	Change in motility (in min)	NE	15**	45**	25**
	No of infectious mice / Total no of mice	5/5	1/5	3/5	2/5
	Infection interval (in days)	12±0.00	22±0.58	16±0.48	18±0.56
0.4	Change in motility (in min)	NE	30**	50**	45**
	No of infectious mice / Total no of mice	5/5	3/5	5/5	4/5
	Infection interval (in days)	12±0.00	16±0.56	12±0.00	14±0.37
0.1	Change in motility (in min)	NE	45**	NE	NE
	No of infectious mice / Total no of mice	5/5	4/5	5/5	5/5
	Infection interval (in days)	12±0.00	14±0.48	12±0.00	12±0.00

Data are expressed as mean ± SEM; n = 5; Ni: no infection; DA: diminazine aceturate; 1% DMSO (dimethyl sulfoxide): vehicle; RM-M: 80% methanol extract of *R. multifidus*, RM-H: hydrodistilled extract of *R. multifidus*; * motility ceased; ** motility drastically reduced; NE: no effect on motility.

Results of the present study showed that RM-H possesses appreciable *in vitro* antitrypanosomal activity. Incubation of trypanosomes with 4 mg/ml of RM-H inhibited healthy mice from developing infection in the observation period. This is consistent with earlier studies which reported that some plant extracts have the capacity to cause trypanosomes lose their infectivity to mice (Mergia *et al*., 2016; Yusuf *et al.*, 2012). However, *in vitro* antitrypanosomal activities

displayed by the lower dose levels of RM-H could not be corroborated by blood incubation infectivity test suggesting that complete immobility of the parasites *in vitro* may not necessarily indicate that the parasites were dead (Yusuf *et al*., 2012). In that event, the lower dose levels might have only immobilized the parasite by causing unfavorable conditions, but not killed the parasites. As a consequence, when suitable physiological conditions are created the parasites might have recovered and become infective (Mergia *et al*., 2016).

4.5.2 *In vivo* activity

According to Atawodi *et al*. (2003), plants with *in vitro* antitrypanosomal activities must be tested *in vivo* before a definite statement can be made on their therapeutic potentials. Since both RM-M and RM-H showed appreciable *in vitro* antitrypanosomal activity they were further investigated by *in vivo* assay.

In the current study, effect of *R. multifidus* extracts on parasitaemia level of *T. congolense*-infected mice was assessed. The results demonstrated that mice treated with all dose levels of RM-H and RM-M showed low ($p < 0.001$) parasitaemia level, throughout the observation period as compared to the negative control group. However, neither of the extracts completely eliminated the parasites in the infected mice blood stream, but only reduced ($p < 0.001$) the level of parasitaemia compared to negative control (Table 6). At a dose of 35.00 mg/kg/day, RM-H exhibited statistically significant ($p < 0.001$) parasitaemia reduction compared to the RM-M and lower and middle doses of RM-H. Mice treated with diminazene aceturate, displayed no parasite growth from day 2 to 10, though relapse occurred in all mice approximately on days 12-14 of treatment. Previously, similar results have been reported by Mergia *et al*. (2016), Tewabe *et al*. (2014) and Ibrahim *et al*. (2012). The relapse seen in mice treated with diminazene aceturate

might be due to the emergence and spread of trypanocidal drug resistance in several African countries, including Ethiopia (Miruk *et al*., 2008; Assefa and Abebe, 2001; Afewerk *et al*., 2000).

Table 6. *In vivo* antitrypanosomal effect of the leaf extracts of *Ranunculus multifidus* on parastaemia (Log number trypanosomes/ml) on *Trypanosoma congolense* infected mice

Test substance (mg/kg/day)	Log number trypanosomes/ml							
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
1% DMSO	7.26±0.06	7.74±0.14	8.28 ±0.07	8.58±0.07	8.64±0.06	8.70±0.0	8.82±0.07	8.94±0.06
DA 28	7.32±0.07	2.16±1.32^{a3}	0.00±0.00^{a3}	0.00±0.00^{a3}	0.00±0.00^{a3}	0.00±0.00^{a3}	1.08±1.08^{a3}	2.16±1.32^{a3}
RM-M 100	7.26±0.06	6.90±0.09^{b3}	6.6 ±0.00^{a3,b3,h3}	6.18±0.20^{a3,b3,h3}	4.86±1.23^{b1}	6.30±0.09^{a3,b3,h3}	6.48±0.07$^{a3,\ b3}$	6.78±0.07^{a3,b3}
RM-M 200	7.32±0.07	6.78±0.12^{b3}	6.48±0.07^{a3,b3,h3}	5.88±0.20^{a3,b3,h3}	4.44±1.11	6.12±0.2^{a3,b3,h3}	6.42±0.07^{a3,b3}	6.60 ±0.00^{a3,b3}
RM-M 400	7.26±0.05	6.72±0.07^{b3}	6.18±0.07^{a3,b3,h3}	5.52±0.12^{a3,b3,h3}	2.16±1.32^{a3}	3.24±1.32^{a3,b3,h3}	4.32±1.08^{a3}	5.40±1.13^{a3}
RM-H 8.75	7.26±0.05	6.72±0.07^{b3}	6.18±0.07^{a3,b3,h3}	5.52±0.12^{a3,b3,h3}	2.16±1.32^{a3}	3.24±1.32^{a3,b3,h3}	4.32±1.08^{a3}	4.50±1.13^{a3}
RM-H 17.50	7.26±0.05	6.72±0.07^{b3}	6.18±0.07^{a3,b3,h3}	5.52±0.12^{a3,b3,h3}	1.08±1.08^{a3}	3.16±1.32^{a3,b3,h3}	4.32±1.08^{a3}	4.50±1.13^{a3}
RM-H 35.00	7.26±0.06	5.4 ±0.00^{b1}	2.16±1.32^{a3,b3}	1.08±1.08^{a3}	1.08±1.08^{a3}	2.24±1.32^{a3}	4.32±1.08^{a3}	4.50±0.00^{a3}

Data are expressed as mean ± SEM; n = 5; a: compared to 1% DMSO, b: compared to DA, c: compared to RM-M 100, d: compared to RM-M 200, e: compared to RM-M 400, f: compared to RM-H 8.75, g: compared to RM-H 17.50, h: compared to RM-H 35.00; 1: $p < 0.05$, 2: $p < 0.01$, 3: $p < 0.001$; 1% DMSO(dimethyl sulfoxide): vehicle, DA: diminazine aceturate, RM-M: 80% methanol extract of *R. multifidus*, RM-H: hydrodistilled extract of *R. multifidus.*

Treatment with the prepared extracts of *R. multifidus* prevented loss of body weight associated with parasitaemia compared to negative controls at day 0, day 7 and day 14. However, the effect of RM-H was far better than that of RM-M (Figure 3). Both extracts lowered rectal temperatures of the experimental animals compared to the negative control group ($p < 0.001$), although the effect of diminazine aceturate was superior ($p < 0.001$) to those of the extracts, with the exception of 35.00 mg/kg/day concentration of RM-H, which showed comparable activity with that of the positive control (Figure 4). As shown in Figure 5, administration of the extracts to the infected mice produced a marked difference in PCV when compared with the untreated uninfected animals. But neither the positive control nor the plant extracts were able to reverse PCV of the infected mice to normal values. Overall, the *in vivo* activity study revealed that the leaf extracts of *R. multifidus*, particularly RM-H, has potential antitrypanosomal activity.

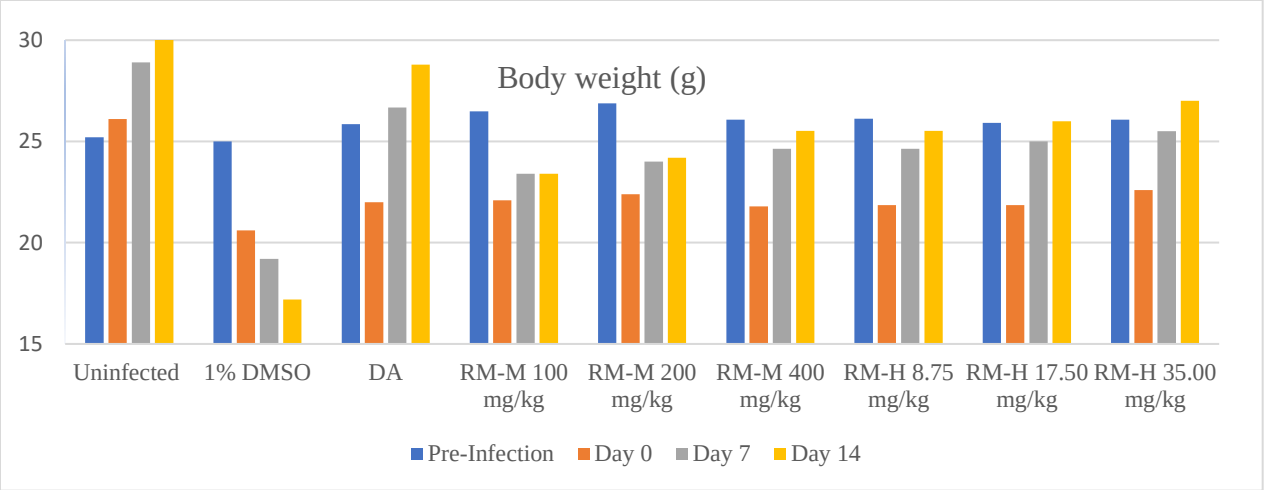


Figure 3. Body weight (g) of healthy uninfected and *Trypanosoma congolense*-infected mice treated with 80% methanol extract (RM-M) and hydrodistilled extract (RM-H) of the leaves of *Ranunculus multifidus* in *in vivo* antitrypanosomal assay

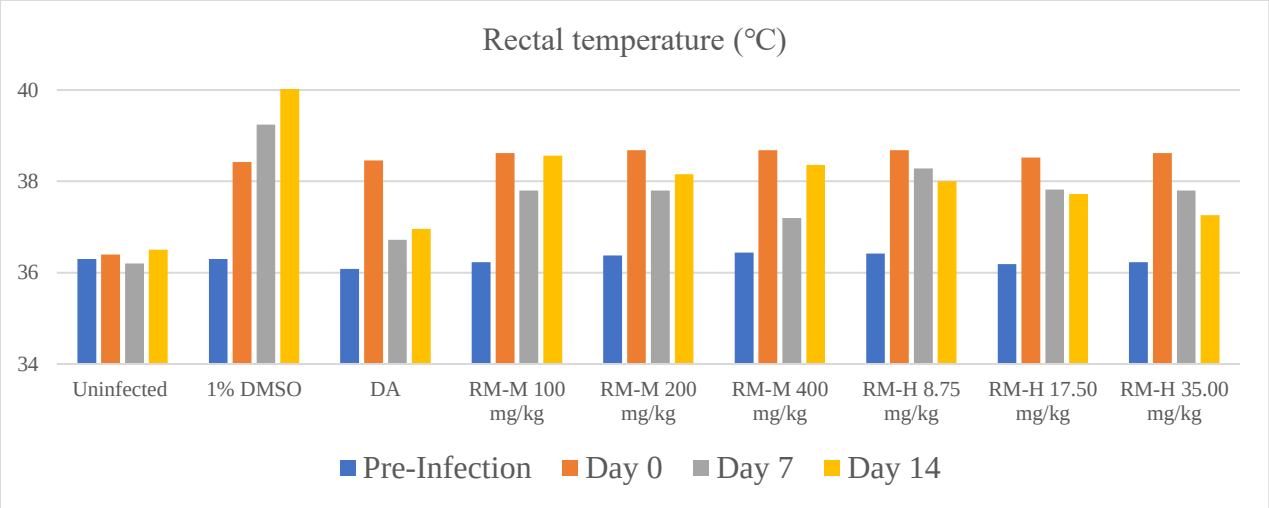


Figure 4. Rectal temperature (°C) of healthy uninfected and *Trypanosoma congolense*-infected mice treated with 80% methanol extract (RM-M) and hydrodistilled extract (RM-H) of the leaves of *Ranunculus multifidus* in *in vivo* antitrypanosomal assay

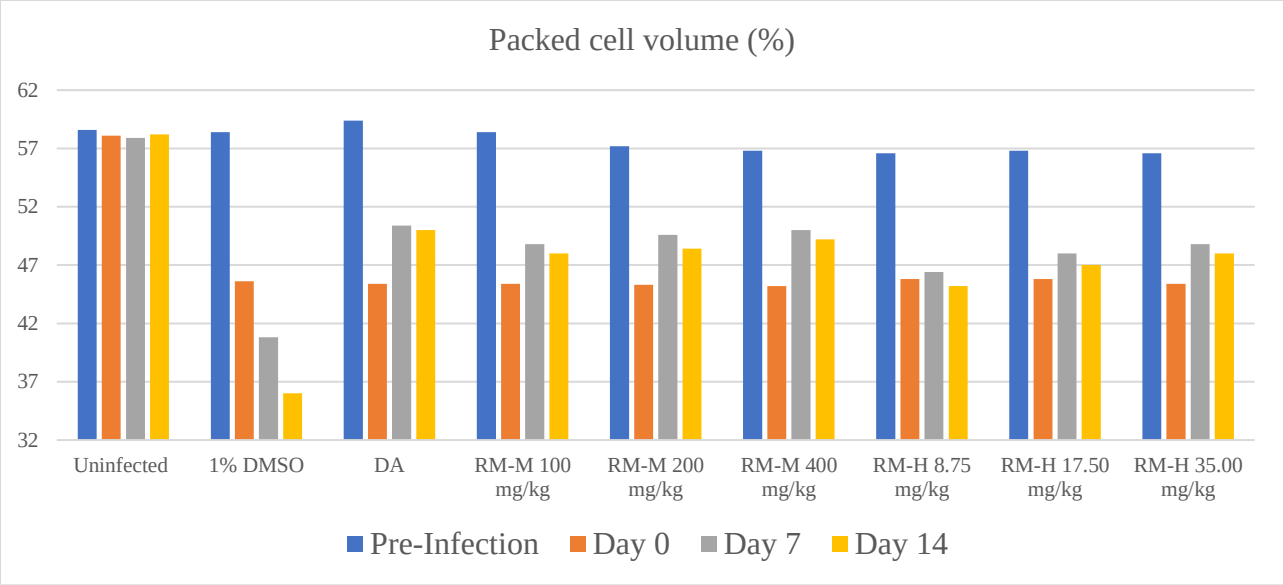


Figure 5. Packed cell volume (%) of healthy uninfected and *Trypanosoma congolense*-infected mice treated with 80% methanol extract (RM-M) and hydrodistilled extract (RM-H) of the leaves of *Ranunculus multifidus* in *in vivo* antitrypanosomal assay

4.6 Isolation of compound

In view of the strong antitryprotozoal activity demonstrated by the hydrodistilled extract of *R. multifidus*, RM-H was further subjected to phytochemical analysis to identify the compound(s) responsible for bioactivity. Analytical TLC of RM-H using hexane: ethyl acetate (5:1) solvent system showed the presence of at least 2 compounds (Figure 6a). The major compound designated RM-H1 appeared dark when viewed under 254-nm UV radiation, but was not visible at 366 nm. RM-H1 was isolated by repeated preparative TLC over silica gel as a pale-yellow irritating oil which solidified immediately at room temperature to a white-coloured crystalline powder. The white powder was further purified by preparative TLC over silica gel using a mixture of ethyl acetate and hexane in a ratio of 3:2 as a solvent system (Figure 6b).

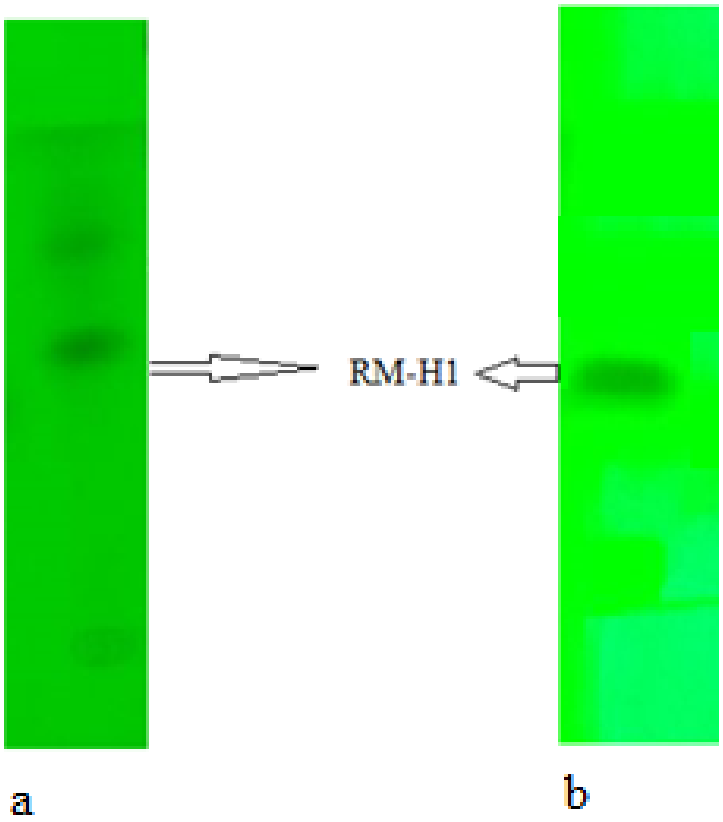


Figure 6. Silica gel TLC chromatograms of (a) the hydrodistilled extract (RM-H) of *Ranunculus multifidus* leaves in hexane: ethyl acetate (5:1) solvent system (b) purified RM-H1 in hexane: ethyl acetate (2:3) solvent system viewed under UV light of 254 nm

4.7 Structural elucidation of RM-H1

RM-H1 was obtained as a white crystalline powder with an R_f value of 0.59 in: hexane: ethyl acetate (2:3) solvent system. APCI-MS of RM-H1 showed MH^+ ion, a pseudo-molecular ion (M+1) peak, at 192.9 *m/z* (Figure 7). The exact calculated molecular mass was found to be 192.0422 amu which corresponds to a relative molecular formula of $C_{10}H_8O_4$.

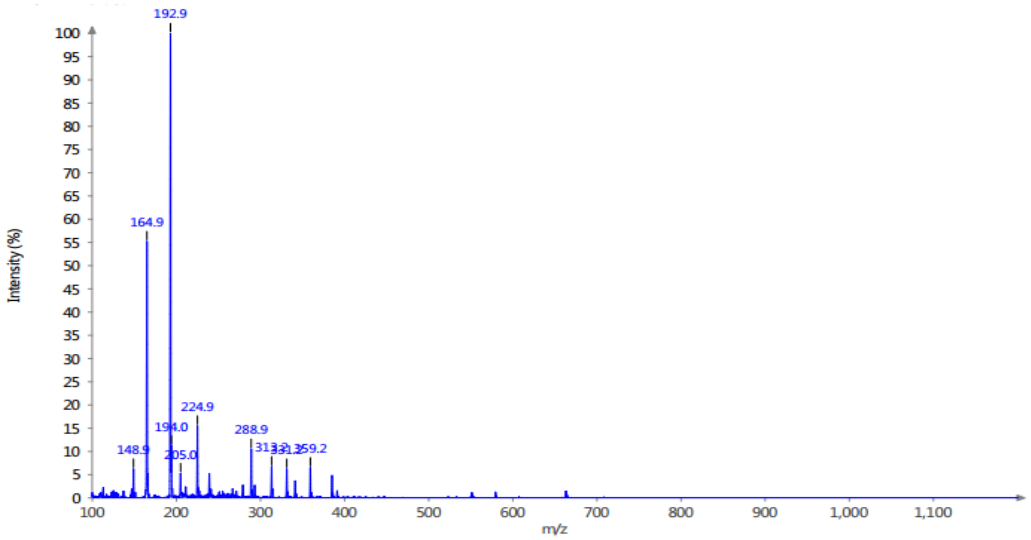


Figure 7. Atmospheric pressure chemical ionization mass spectrum of RM-H1

The FT-IR spectrum of RM-H1 exhibited sp^2 and sp^3 hybridized C-H stretch bands at 3100 and 2930 cm^{-1}, respectively. The presence of a conjugated ester carbonyl group was revealed by the strong sharp band at 1770 cm^{-1}. The spectrum also showed medium and strong bands at 1600 and 1110 cm^{-1} assigned to C=C and C-O stretch, respectively (Figure 8).

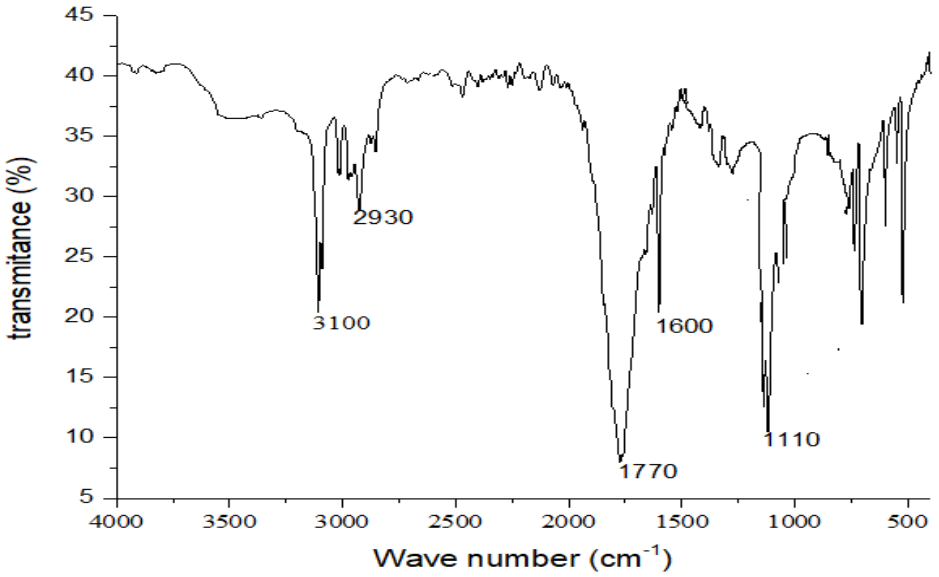


Figure 8. Fourier-transform infrared spectrum of RM-H1

As depicted in Figure 9, the ^{1}H-NMR spectrum of RM-H1 revealed the presence of 4 cylobutane protons assigned to H-6a, H-6a' (δ 2.64, 2H, m) and H-6b, H-6b' (δ 2.25, 2H, m). Four furanon protons were also observed and assigned to H-3, H-3' (δ 6.32, 2H, d, *J* = 5.7 Hz) and H-4, H-4' (δ 8.28, 2H, d, *J* = 5.7 Hz) (Figure 9). The ^{13}C-NMR spectrum (Figure 10) of RM-H1 displayed 5 signals representing 10 carbon atoms, an indication that the compound is a dimer. The DEPT-135 spectrum (Figure 11), revealed the presence of two CH_2 and four CH carbons. The signals at δ 171.8, 156.5, 121.1, and 90.44 were assigned to the lactone carbonyl (C-2 and C-2'), olefinic (C-4 and C-4'), (C-3 and C3') and quaternary (C-5 and C-5') carbons, respectively (Table 7). The upfield peak at *δ* 23.8 was assigned to the methylene carbons of the cyclobutane ring at C-6 and C-6' (Figure 10). The ^{1}H-^{1}H COSY spectrum clearly shows a cross peak between H-3, H-3' and H-4, H-4'. A further spin-spin coupling between H-6a, H-6a' and H-6b, H-6b' is also apparent in the spectrum (Appendix 2).

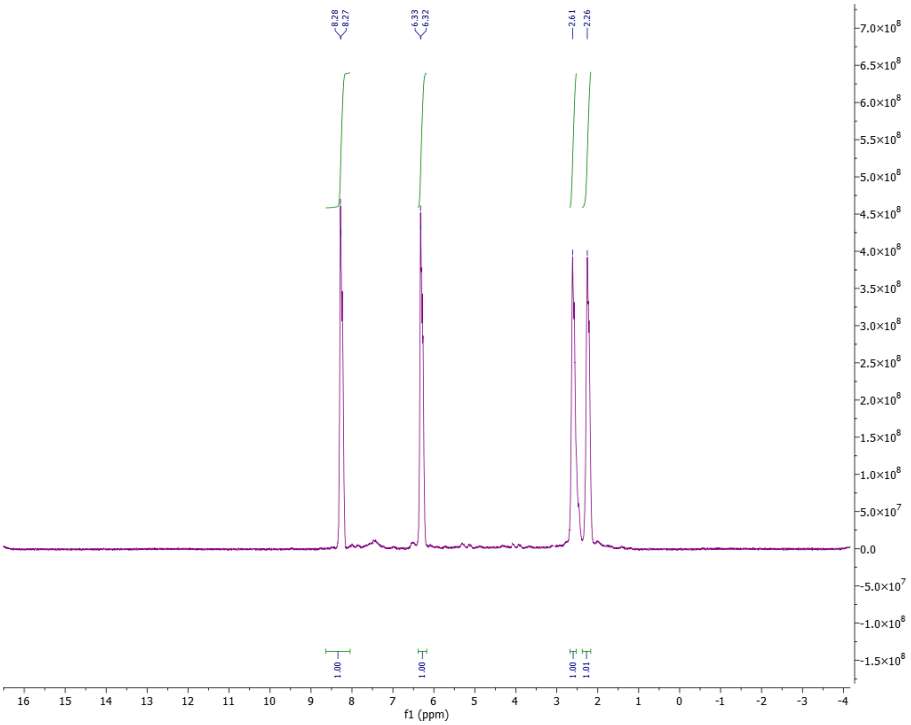


Figure 9. ^{1}H-NMR spectrum of RM-H1

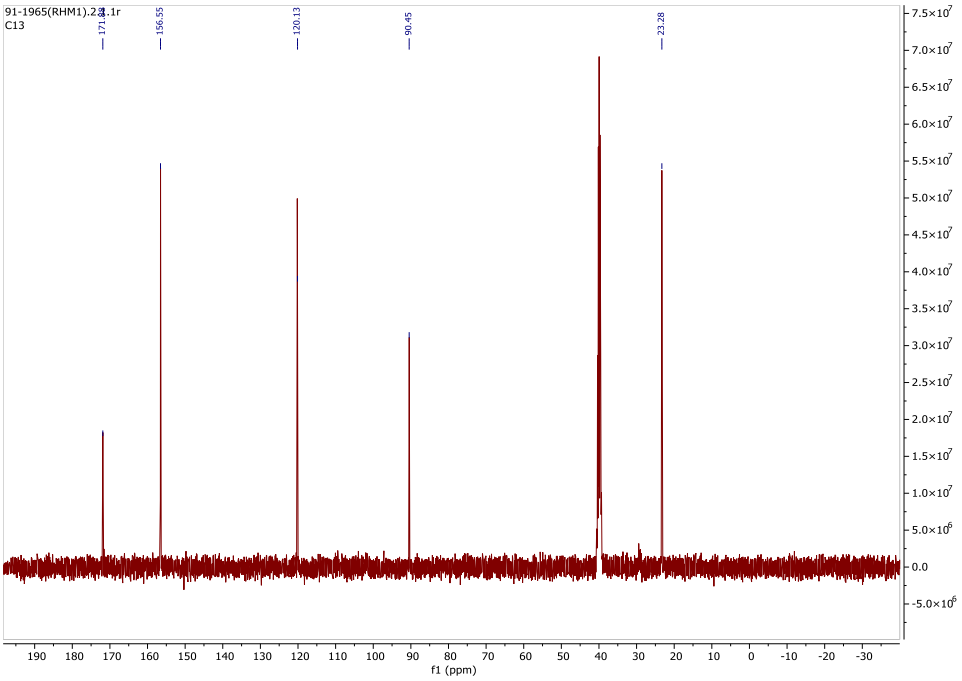


Figure 10. ^{13}C-NMR spectrum of RM-H1

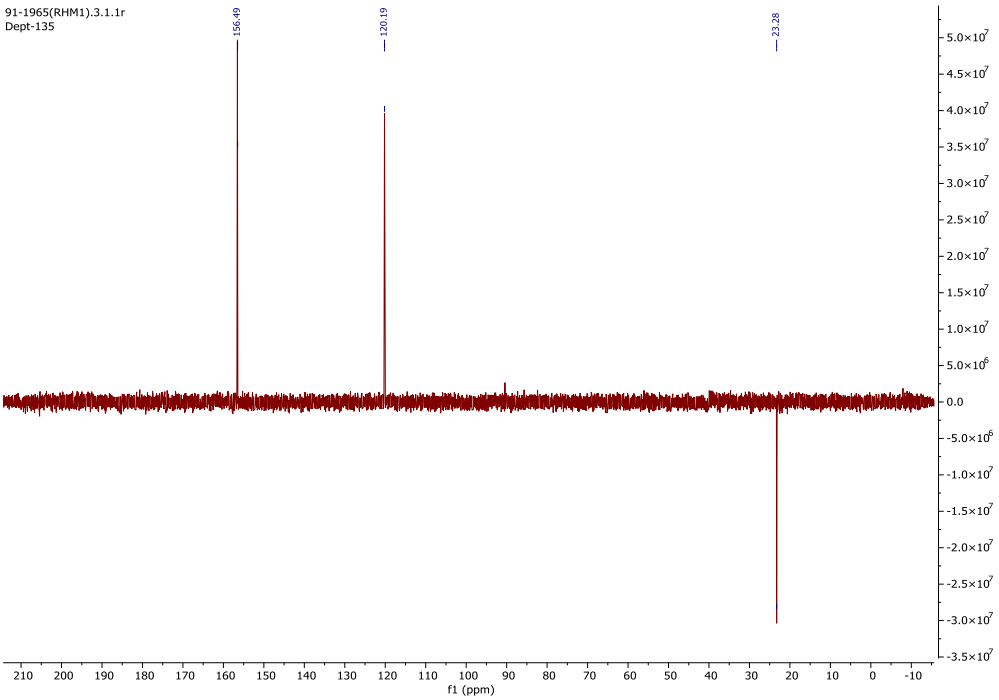


Figure 11. DEPT-135 spectrum of RM-H1

Table 7. Comparison of the ^{1}H NMR and ^{13}C NMR spectral data of the RM-H1 with ^{1}H NMR and ^{13}C NMR of anemonin reported by Ali *et al*. (2019)

No.	^{1}H NMR chemical shift (δ, ppm)		^{13}C NMR chemical shift (δ, ppm)	
	Anemonin	Ali *et al*. (2019)	Anemonin	Ali *et al*. (2019)
2 - 2'	-	-	171.8	170.8
3 - 3'	6.32, d (5.7Hz)	6.30, d (5.6Hz)	121.1	121.1
4 - 4'	8.28, d (5.7Hz)	8.26, d (5.6Hz)	156.5	153.2
5 - 5'	-	-	90.44	90.3
6 – 6'	6a – 6a'= 2.64	6a – 6a'= 2.61, m	23.27	23.8
	6b – 6b'= 2.25	6b – 6b' = 2.24, m		

d = doublet, *m* = multiplet

The complete assignments of ^{1}H and ^{13}C chemical shifts of RM-H1 are listed in Table 7. From the chemical shifts presented and by comparing the ^{1}H and ^{13}C-NMR spectral data of RM-H1 with the compound reported by Ali *et al*. (2019), Saidi *et al*. (2018), Nono *et al*. (2016) and Huang *et al*. (2008), RM-H1 was identified as anemonin (Figure 12).

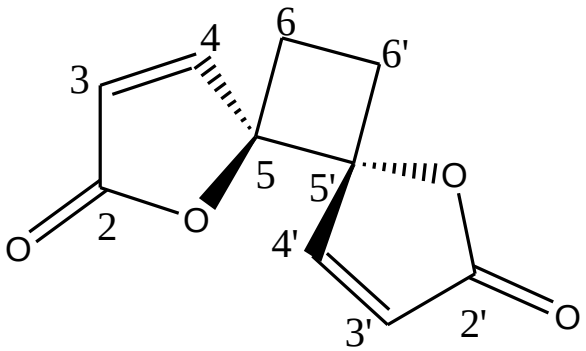


Figure 12. Structural formula of anemonin

4.8 Acute oral toxicity of anemonin

Results of the present study showed that anemonin and RM-H have similar oral acute toxicity profile. Thus, the LD_{50} of anemonin was determined to be above 175 mg/kg. Saidi *et al*. (2018) also reported that anemonin is toxic to primary keratinocytes above a concentration of 25 µg/ml in *in vitro* MTT assay.

4.9 Antileishmanial activity of anemonin

The current study disclosed that anemonin possesses strong and comparable growth inhibitory effect against the promastigote and amastigote forms of *L. aethiopica* and *L. donovani.* The IC_{50} values of anemonin were 0.257 µg/ml (1.33 nM) and 0.303 µg/ml (1.58 nM) against *L. aethiopica* and *L. donovani* promastigotes, respectively. As shown in Table 8, anemonin also inhibited growth of the amastigote forms of the parasites in nanomolar concentrations. Amphotericin B was found to be much more ($p < 0.001$) active than anemonin against the promastigote and amastigote forms of both *Leishmania* spp. tested.

The *in vitro* macrophage cytotoxicity assay indicated that anemonin and amphotericin B have comparable CC_{50} values of 5.39 and 4.31 µg /ml, respectively. However, the selectivity indices of amphotericin B (453 and 684) were much higher than anemonin (22 and 14) against *L. aethiopica* and *L. donovani*, respectively (Table 8) indicating that amphotericin B exhibits more selective toxicity to the parasites than anemonin.

Table 8. Antileishmanial activity, macrophage cytotoxicity and haemolytic property of anemonin against promastigote and amastigote forms *Leishmania aethiopica* and *Leishmania donovani in vitro*

Test substance	Antileishmanial activity IC_{50} (µg/ml)				Cytotoxicity (µg/ml)		Selectivity index	
	Promastigotes		Amastigotes		Macrophage CC_{50}	Haemolysis LC_{50}		
	La	Ld	La	Ld			La	Ld
Anemonin	0.257±0.007[b3]	0.303±0.304[b3]	0.239±0.014[b3]	0.368±0.024[b3]	5.39±2.013	91.00±0.298	22	14
AMB	0.0157±0.08	0.0067±0.008	0.0095±0.004	0.0063±0.011	4.31±0.983	47.25±0.54	453	684

Data expressed as mean ± SEM; n = 3; a: compared to anemonin, b: compared to Amphotericin B; 1: $p < 0.05$, 2: $p < 0.01$, 3: $p < 0.001$; La: *Leishmania aethiopica*, Ld: *Leishmania donovani*; CC_{50}: concentration causing 50% cytotoxicity, LC_{50}: concentration causing 50% lysis; AMB: amphotericin B.

Leishmaniasis is still one of the endemics and highly prevalent diseases in Ethiopia (Assefa, 2018). Most people in Ethiopia use traditional medicine for the treatment CL and have no knowledge of modern medical treatment (Bsrat *et al*., 2015). Even community who has knowledge about modern medicine do not have access of healthcare service, the available diagnosis and treatment are expensive which further enhance the spread of the disease and reinforce poverty (Alvar *et al*., 2007). Most of the population of Ocholo (CL endemic area in Gamo Zone, South Ethiopia) has been exposed to *L. aethiopica* infection which leaves permeant scar causing disfigurement and resulting social stigmatization (van Henten *et al*., 2018; Bugssa *et al*., 2014; Negera *et al*., 2008). Due to high stigmatization and disfigurement local people around Ocholo village have a saying in Amharic "የኦቾሎ ልጆች በጣም ቆንጆ ናቸው፤ ጠንቀኛ በሽታ ቡልቦ ገደላቸው" which literally means the Ocholo people are beautiful but CL ("*Bulbo*" in Gamo) is disfiguring them. Most of the CL patients seek a folk medicine and majority had the attitude that treatment from traditional healers is effective (Kebede *et al*., 2016). In different parts of Ethiopia, fresh leaves of *R. multifidus* are used for the treatment of CL (Aschale *et al*., 2018; Birhan *et al*., 2017;

Wolde-Mariam *et al*., 2015), wound healing, and various types of skin diseases (Ayele, 2018; Abebe, 2016; Awas and Demissew, 2009; Giday *et al*., 2009; Teklehaymanot *et al*., 2007).

Results of the present study suggest that anemonin might be responsible for the antileishmanial activity of *R. multifidus* leaves. Several studies reported that anemonin possesses anti-inflammatory activity by different mechanisms of action (Hou *et al*., 2020; Wang *et al*., 2017; Xiao *et al*., 2016; Ning *et al*., 2016; Jia *et al*., 2014; Lee *et al*., 2008; Duan *et al*., 2006). There are also reports that anemonin exhibits antibacterial (Nazir *et al*., 2013; Baer *et al*., 1945), antipyretic (Martin *et al*., 1988), sedative (Martin *et al*., 1988), wound healing (Saidi *et al*., 2018), antioxidant (Qiao *et al*., 2019) and neuroprotective (Jia *et al*., 2014) activities. Saidi *et al*. (2018) reported that anemonin has potent wound healing activity making it a promising candidate as a therapeutic agent in tissue repairing processes. As *L. aethopica* is a cause of CL, which ranges between self-healing condition and a chronic and disfiguring disease, inhibition of proliferation of the parasite by anemonin justifies the traditional use of the plant for the treatment of CL.

4.10 Antimalarial activity of anemonin

The current study evaluated the antimalarial efficacy of anemonin using three models. The 4-day suppressive test was used to evaluate antimalarial activity in early infections, Rane's test was employed to study curative capability during established infections and the prophylactic test was carried out to determine efficacy as an antimalarial prophylactic agent.

4.10.1 Four-day suppressive test

Results of the 4-day suppressive test indicated that anemonin significantly reduced parasitaemia ($p < 0.001$) compared to negative control, with % suppression ranging from 78.3 to 83.04% (Table 9). However, all doses of anemonin exhibited significantly lower ($p < 0.001$) parasitaemia reduction compared to the positive control. Besides, all test substances prolonged MST significantly ($p < 0.001$) compared to negative control. However, MST was still significantly smaller ($p < 0.001$) than the standard drug chloroquine. Overall, significant statistical difference was not observed among doses of anemonin (Table 9).

Table 9. Antimalarial activity of anemonin in mice infected with *Plasmodium berghei* in 4-day suppressive test

Test substance (mg/kg/day)	% Parasitaemia	% Suppression	Mean survival time (in days)
2% TW80	26.61±0.82	-	9.2±0.37
Chloroquine	0.12 ± 0.32^{a3}	99.58	28.0 ± 0.0^{a3}
Anemonin 8.75	$5.78\pm0.13^{a3,b3}$	78.30	$14.0\pm0.3^{a3,b3}$
Anemonin 17.50	$5.28\pm0.18^{a3,b3}$	80.18	$14.4\pm0.24^{a3,b3}$
Anemonin 35.00	$4.52\pm0.09^{a3,b3}$	83.04	$14.8\pm0.4^{a3,b3}$

Data are expressed as mean ± SEM; n = 5; a: compared to 2% TW80, b: compared to CQ, c: compared to anemonin 8.75, d: compared to anemonin 17.50, e: compared to anemonin 35.00; 1: $p < 0.05$, 2: $p < 0.01$, 3: $p < 0.001$); 2% TW80: 2% tween 80 vehicle, CQ: chloroquine.

The 4-day suppressive test results showed that anemonin prevented reduction of body weight significantly ($p < 0.001$) in comparison with the negative control (Table 10). All doses of anemonin prevented significantly ($p < 0.001$) the reduction of rectal temperature due to infection with *P. berghei* as compared to negative control. Anemonin also displayed statistically comparable effect in temperature stabilization with the standard drug (Table 10). Anemonin

showed protection of RBCs from *P. berghei* infection associated anemia ($p < 0.001$) compared to negative control. However, its effect was lower ($p < 0.001$) than chloroquine (Table 10).

Table 10. Effect of anemonin on body weight, rectal temperature and packed cell volume of mice infected with *Plasmodium berghei* in four-day suppressive antimalarial test

Test substances (mg/kg/day)	Body weight (g)			Rectal temperature (°C)			Packed cell volume (%)		
	Day 0	Day 4	% Change	Day 0	Day 4	% Change	Day 0	Day 4	% Change
2% TW80	25.02 ±0.40	21.22±0.59	-15.18	36.68±0.03	33.02±0.23	-9.97	59.6±0.24	49.2±0.96	-17.44
CQ 25	26.26±0.57	30.58±0.85	16.45[a3]	36.42±0.32	36.46±0.16	0.10[a3]	58.4±0.38	58.8±0.51	0.68[a3]
Anemonin 8.75	24.88±0.05	26.84±0.96	7.87 [a3]	36.50±0.16	35.88±0.18	-1.69[a3]	57.2±0.86	51.2±0.73	-10.48[a3,b2]
Anemonin 17.50	26.10±0.63	28.72±1.05	10.03 [a3]	36.70±0.15	36.42±0.18	-0.76[a3]	56.8±0.86	52.4±0.67	-7.74[a3,b3]
Anemonin 35.00	26.80±0.99	30.30±0.63	13.05 [a3]	36.48 ±0.15	36.48±0.12	0.00[a3]	56.4±0.81	53.6±0.50	-4.96[a3,b1]

Data are expressed as mean ± SEM; n = 5; a: compared to 2% TW80, b: compared to CQ, c: compared to anemonin 8.75, d: compared to anemonin 17.50, e: compared to anemonin 35.00; 1: $p < 0.05$, 2: $p < 0.01$, 3: $p < 0.001$; 2% TW80: 2% tween 80 vehicle, CQ: chloroquine.

4.10.2 Rane's test

As shown in Figure 13, Rane's test resulted in gradual escalation of parasitaemia throughout the course of treatment in anemonin treated groups (Figure 13). Two-way repeated measures ANOVA analysis of parasitaemia showed significant ($p < 0.001$) difference in parasite development across the course of treatment. In all dose levels, anemonin treatment resulted in significant ($p < 0.001$) reduction of parasitaemia as compared to the negative control group, but the effect was less than the positive control group (Table 11). The efficacy of anemonin in mice at all doses was correlated significantly ($p < 0.001$) with increased MST compared to the untreated control animals. However, increase in MST of chloroquine treated group was significantly ($p < 0.001$) higher than anemonin treated group.

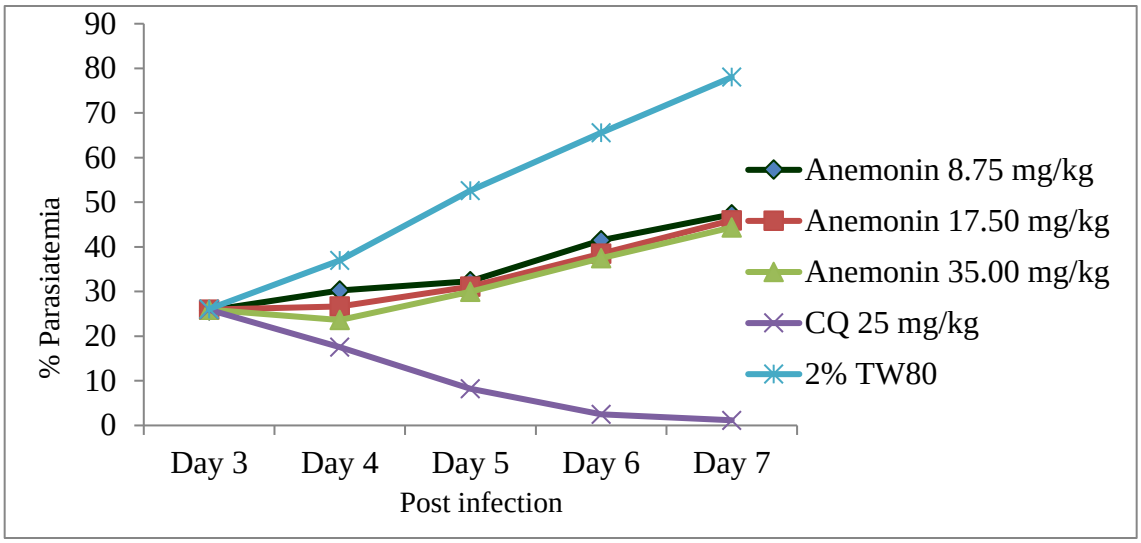


Figure 13. Parasitaemia development over the course of treatment with anemonin on *Plasmodium berghei*-infected mice in Rane's antimalarial test (CQ; chloroquine, 2% TW80: 2% tween 80)

Table 11. Antimalarial activity of anemonin in *Plasmodium berghei* infected mice in Rane's test

Test substances (mg/kg/day)	% Parasitemia	% Suppression	Mean survival time (in days)
2% TW80	78.02±0.25	-	8.60±0.10
CQ 25	1.16±0.24^{a3}	98.49	28.00±0.00^{a3}
Anemonin 8.75	47.28±0.44^{a3,b3}	38.84	11.60±0.24^{a3,b3}
Anemonin 17.50	45.96±0.85^{3,b3}	40.55	12.00±0.3^{a3,b3}
Anemonin 35.00	44.36±0.74^{a3,b3}	42.62	13.00±0.3^{a3,b3}

Data are expressed as mean ± SEM; n = 5; a: compared to 2% TW80, b: compared to CQ, c: compared to anemonin 8.75, d: compared to anemonin 17.50, e: compared to anemonin 35.00; 1: $p < 0.05$, 2: $p < 0.01$, 3: $p < 0.001$; 2% TW80: 2% tween 80 vehicle, CQ: chloroquine.

All the tested doses of anemonin protected body weight loss significantly ($p < 0.001$) compared to the negative control but, the effect of chloroquine was superior ($p < 0.001$) to that of anemonin. Similarly, anemonin protected body temperature reduction of mice and the effect was statistically significant ($p < 0.001$) compared to the negative control. There was no statistical

difference between anemonin in all dose levels and chloroquine in improving temperature drop. As shown in Table 12, when anemonin was administered to *R. berghei*-infected mice, a substantial improvement of PCV was observed when compared with the infected untreated group. Overall, in established infection with *P. berghei*, anemonin produced significant suppression of parasitaemia and maintained pathological parameters suggesting the curative potential of the compound.

Table 12. Body weight, rectal temperature and packed cell volume of *Plasmodium berghei*-infected mice treated with anemonin in Rane's antimalarial test

Test substances (mg/kg/day)	Body weight (g)			Rectal temperature (°C)			Packed cell volume (%)		
	Day 3	Day7	% Change	Day 3	Day7	% Change	Day 3	Day7	% Change
2% TW80	26.63±0.41	20.54±0.81	-22.86	36.61± 0.20	32.29 ± 0.36	-11.8	58.2±0.36	46.2±0.42	-20.61
CQ 25	26.30±0.76	27.30±0.72	3.8[a3]	36.88±0.09	36.38 ± 0.16	-1.35[a3]	58.0±0.70	53.0±0.70	-8.62[a3]
Anemonin 8.75	26.48±0.82	23.64±0.75	-10.72 [b3]	36.70±0.16	35.68 ± 0.20	-2.77[a3]	57.8±0.58	51.4±0.74	-10.67[a3]
Anemonin 17.50	28.50±0.38	27.29±1.14	-3.91[a3,b3]	36.70±0.15	36.22 ± 0.07	-1.3[a3]	56.0±0.70	50.2±0.80	-10.35[a3]
Anemonin 35.00	26.30±0.76	25.30±0.72	-3.80[a3 b3]	36.88±0.09	36.38 ± 0.34	-1.35[a3]	58.1±0.74	53.0±0.70	-8.77[a3]

Data are expressed as mean ± SEM; n = 5; a: compared to 2% TW80, b: compared to CQ, c: compared to anemonin 8.75, d: compared to anemonin 17.50, e: compared to anemonin 35.00; 1: $p < 0.05$, 2: $p < 0.01$, 3: $p < 0.001$; 2% TW80: 2% tween 80 vehicle, CQ: chloroquine.

4.10.3 Prophylactic test

In the prophylactic test, all the tested doses of anemonin significantly ($p < 0.001$) suppressed parasitaemia compared to the negative control group (Table 13). However, the effect of anemonin was significantly ($p < 0.001$) lower than the chemosuppression displayed by the positive control. Similarly, anemonin caused significant ($p < 0.001$) prolongation of MST compared to the negative controls, although chloroquine was far more effective ($p < 0.001$) than anemonin.

Table 13. Prophylactic antimalarial activity of anemonin in *Plasmodium berghei*-infected mice

Test substances (mg/kg/day)	% Parasitemia	% Suppression	Mean survival time (in days)
2% TW80	33.80±0.91	-	8.4±0.56
CQ 25	3.80±0.73^{a3}	88.62	27.4±0.40^{a3}
Anemonin 8.75	13.12±0.43^{a3,b3}	60.71	14.0±0.3^{a3,b3}
Anemonin 17.50	12.12±0.55^{a3,b3}	63.71	14.0±0.3^{a3,b3}
Anemonin 35.00	11.12±0.30^{a3,b3}	66.70	14.4±0.24^{a3,b3}

Data are expressed as mean ± SEM; n = 5; a: compared to 2% TW80, b: compared to CQ, c: compared to anemonin 8.75, d: compared to anemonin 17.50, e: compared to anemonin 35.00; 1: $p < 0.05$, 2: $p < 0.01$, 3: $p < 0.001$; 2% TW80: 2% tween 80 vehicle, CQ: chloroquine.

Treatment with anemonin significantly prevented loss of body weight associated with parasitaemia on day 7 compared to the body weight on day 3 at all dose levels. The results also revealed that anemonin-treated groups at all dose levels significantly ($p < 0.001$) prevented the loss of body weight compared to that of the negative control. Similarly, anemonin attenuated significantly ($p < 0.001$) the rapid decline in rectal temperature of infected mice compared to negative control, and there was no statistical difference between the effect of anemonin and chloroquine. Also, anemonin prevented significantly ($p < 0.001$) reduction of PCV due to parasite infection in comparison with the negative control group. Effect of the higher dose of anemonin (35.00 mg/kg/day) was comparable to that of chloroquine (Table 14).

Table 14. Body weight, rectal temperature and packed cell volume of *Plasmodium berghei*-infected mice treated with anemonin in prophylactic antimalarial test

Test substances (mg/kg/day)	Body weight (g)			Rectal temperature (°C)			Packed cell volume (%)		
	Day 3	Day 7	% Change	Day 3	Day 7	% Change	Day 3	Day 7	% Change
2% TW80	26.79±0.51	21.69±0.02	-19.03	36.66±0.06	32.52±0.22	-11.29	58.9±0.42	47.8± 0.65	-18.84
CQ 25	26.10±0.63	27.90±0.58	6.89[a3]	36.70±0.17	36.58±0.10	-0.32[a3]	57.8±0.58	53.4±0.81	-7.61[a3]
Anemonin 8.75	26.48±0.82	23.64±0.75	-10.72[a3,b3]	36.70±0.16	35.68±0.20	-2.77[a3]	58.4±0.60	51.0±0.44	-12.67[a3,b1]
Anemonin 17.50	27.40±0.51	27.50±0.48	0.36[a3,b3]	36.58±0.13	36.18±0.25	-1.09[a3]	58.0±0.54	50.8±0.37	-12.41[a3,b1]
Anemonin 35.00	26.90±0.31	27.26±0.52	1.33[a3,b3]	36.50±0.25	36.22±0.07	-0.76[a3]	58.60±0.5	53.4±0.81	-8.87[a3]

Data are expressed as mean ± SEM; n = 5; a: compared to 2% TW80, b: compared to CQ, c: compared to anemonin 8.75, d: compared to anemonin 17.50, e: compared to anemonin 35.00; 1: $p < 0.05$, 2: $p < 0.01$, 3: $p < 0.001$; 2% TW80: 2% tween 80 vehicle, CQ: chloroquine.

Increase in parasitaemia levels in rodents usually results in decreased metabolic rates and develop severe hypothermia (Dascombe and Sidara, 1994), which might result in death. The decreasing body weight in malaria has been associated with decreased food intake, disturbed metabolic function and hypoglycemia (Basir *et al*., 2012). An ideal antimalarial agent would, therefore, prevent this occurrence. In the current study, anemonin showed temperature stabilizing and weight maintenance effect in all models. In addition to parasite suppression this might indicate that anemonin controlled the immune system of infected mice along with controlling some pathological processes and balance the reduction in metabolic rate that produced drop in rectal temperature.

As shown in Figure 14, the effective median dose (ED_{50}) of anemonin was determined by non-linear regression analysis from sigmoidal dose–response curves. It was found that the ED_{50}s of anemonin are 0.4172, 0.5356 and 0.5196 mg/kg (2.17, 2.78 and 2.70 μM) in 4-day suppressive, Rane's tests and prophylactic, respectively.

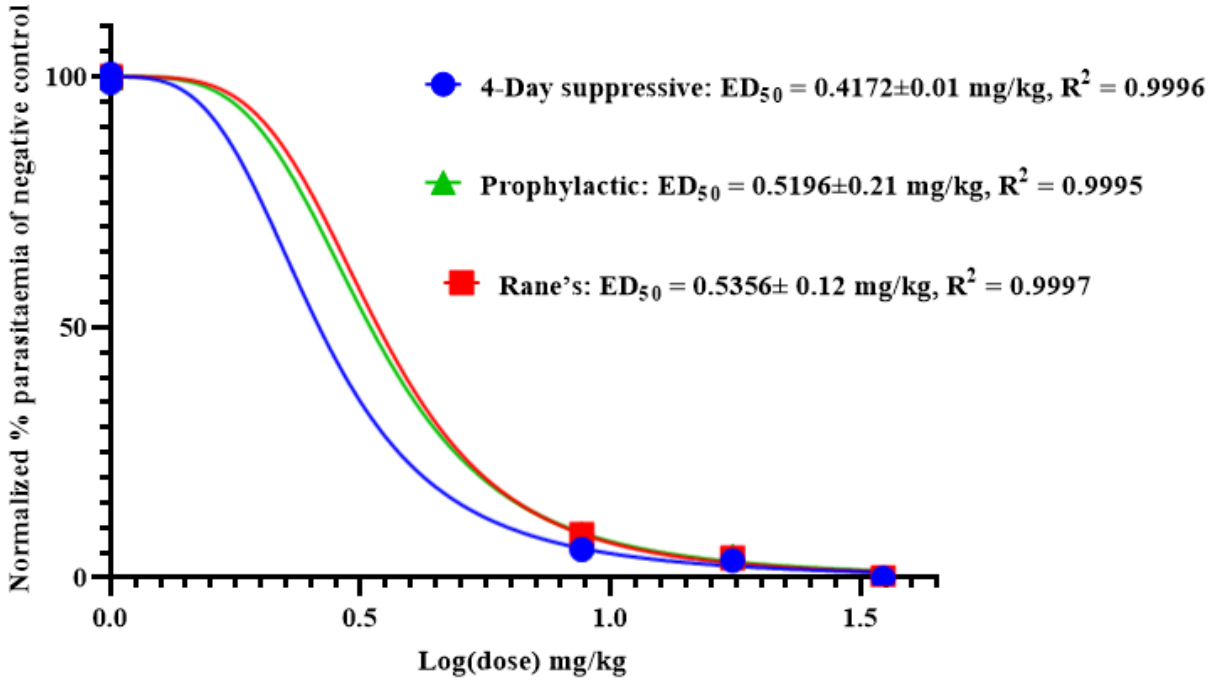


Figure 14. ED_{50} of anemonin in mice infected with *Plasmodium berghei* in 4-day suppressive, Rane's and prophylactic antimalarial tests; ED_{50} was estimated from a plot of Log dose against % parasitaemia of negative control (normalized); values are presented as mean ± SEM; n = 5.

4.11 Antitrypanosomal activity of anemonin

4.11.1 *In vitro* activity

In *in vitro* antitrypanosomal activity test, anemonin ceased motility of trypanosomes within 5 min at 4 mg/ml test concentration and drastically reduce motility of trypanosomes within 15, 20 and 35 min at 2, 1 and 0.1 mg/ml test concentrations, respectively (Table 15). In the *in vivo* infectivity test, all infected mice which received 4 mg/ml of anemonin and diminazine aceturate were found to be free from parasite (lost infectivity) during the study period (30 days). All the animals in the negative control group developed infection at the 12th day (Table 15). At a concentration of 4 mg/ml anemonin immobilized motility of trypanosomes within 5 min, while the standard dug diminazene diaceurate immobilized the parasites within 10 min. Therefore,

anemonin can be considered a promising anti-trypanosomal agent, since its activity was stronger than that of the positive control diminazene aceturate.

Table 15. *In vitro* antitrypanosomal effect of anemonin against *Trypanosoma congolense* motility and *in vivo* infectivity test

Dose (mg/ml)	Effect	1% DMSO	DA	Anemonin
4 .0	Change in motility (in min)	NE	10*	5*
	No of infectious mice/Total no of mice	5/5	0/5	0/5
	Infection interval (in days) ± SEM	12±0.00	Ni	Ni
2.0	Change in motility (in min)	NE	15**	15**
	No of infectious mice/Total no of mice	5/5	1/5	1/5
	Infection interval (in days) ± SEM	12±0.00	22±0.58	22±0.58
0.4	Change in motility (in min)	NE	30**	20**
	No of infectious mice / Total no of mice	5/5	3/5	2/5
	Infection interval (in days) ± SEM	12±0.00	16±0.56	18±0.56
0.1	Change in motility (in min)	NE	45**	35**
	No of infectious mice / Total no of mice	5/5	4/5	3/5
	Infection interval (in days) ± SEM	12±0.00	14±0.48	16±0.37

Data are expressed as mean ± SEM; n = 5; Ni: no infection; DA: diminazine aceturate; 1% DMSO (dimethyl sulfoxide): vehicle; *: motility ceased; **: motility drastically reduced; NE: no effect on motility.

4.11.2 *In vivo* activity

In the *in vivo* antitrypanosomal assay, mice treated with all dose levels of anemonin had significantly ($p < 0.001$) low parasitaemia throughout the observation period compared to the negative control group. There was no statistical difference between the activity of diminazine aceturate and anemonin (Table 16). At all dose levels, anemonin eliminated trypanosomes from infected mice. Furthermore, mice treated with anemonin did not show relapse, while in diminazine aceturate-treated group the parasites reappeared.

Table 16. *In vivo* antitrypanosomal effect of anemonin on parastaemia (Log number trypanosomes/ml) on *Trypanosoma congolense* infected mice

Test substance (mg/kg/day)	Log number trypanosomes/ml							
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
1% DMSO	7.26±0.06	7.74±0.14	8.28±0.07	8.58±0.07	8.64±0.06	8.70±0.00	8.82±0.07	8.94±0.06
DA 28	7.32±0.07	2.16±1.32[a3]	0.00±0.00[a3]	0.00±0.00[a3]	0.00±0.00[a3]	0.00±0.00[a3]	1.08±1.08[a3]	2.16±1.32[a3]
Anemonin 8.75	7.32±0.07	2.16±1.32[a3]	0.00±0.00[a3]	0.00±0.00[a3]	0.00±0.00[a3]	0.00±0.00[a3]	0.00±0.00[a3]	0.00±0.00[a3]
Anemonin 17.50	7.38±0.07	2.16±1.32[a3]	0.00±0.00[a3]	0.00±0.00[a3]	0.00±0.00[a3]	0.00±0.00[a3]	0.00±0.0 [a3]	0.00±0.00[a3]
Anemonin 35.00	7.38±0.07	1.08±1.08[a3]	0.00±0.00[a3]	0.00±0.00[a3]	0.00±0.00[a3]	0.00±0.00[a3]	0.00±0.00[a3]	0.00±0.00[a3]

Data expressed as mean ± SEM; n = 5; a: compared to 1% DMSO, b: compared to DA, c: compared to anemonin 8.75 d: compared to anemonin 17.50, e: compared to anemonin 35.00; 1% DMSO: vehicle; DA: diminazine aceturate.

All tested doses of anemonin prevented body weight loss ($p < 0.001$) compared to the negative control. Positive control group and mice treated with anemonin exhibited similar body weight gain at day 14 compared to the uninfected mice (Figure 15). Mice treated with anemonin showed reduced rectal temperature when compared with the negative control ($p < 0.001$). Prevention of rectal temperature rise by anemonin was even superior ($p < 0.001$) to that of the positive control, diminazine aceturate (Figure 16). As shown in Figure 17, *T. congolense* caused a significant PCV reduction. However, seven days post-treatment the PCV level significantly ($p < 0.001$) enhanced in those groups which are treated anemonin compared to the negative control.

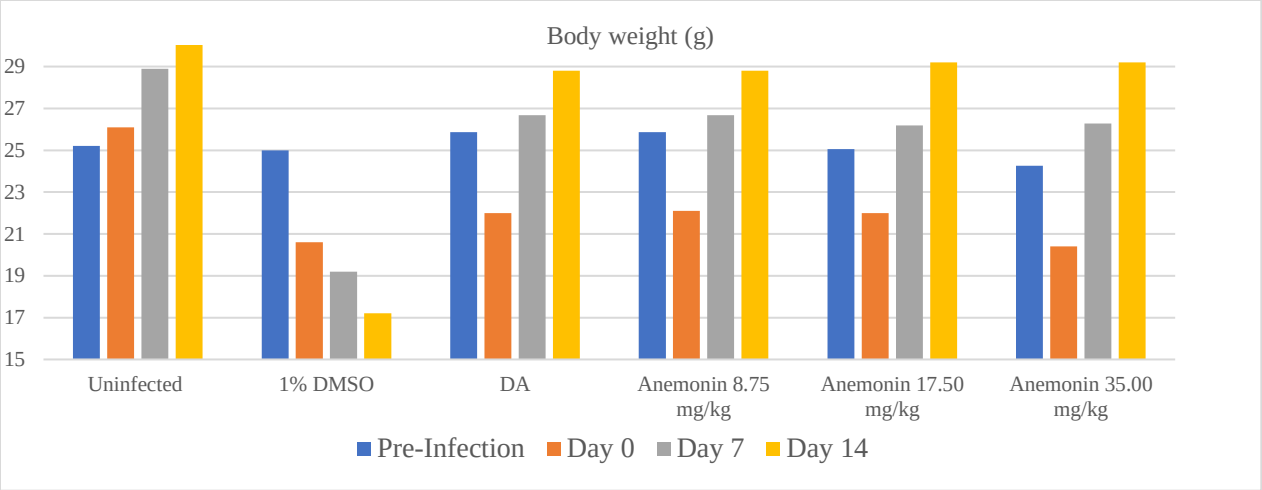


Figure 15. Body weight (g) of healthy uninfected and *Trypanosoma congolense*-infected mice treated with anemonin in *in vivo* antitrypanosomal assay

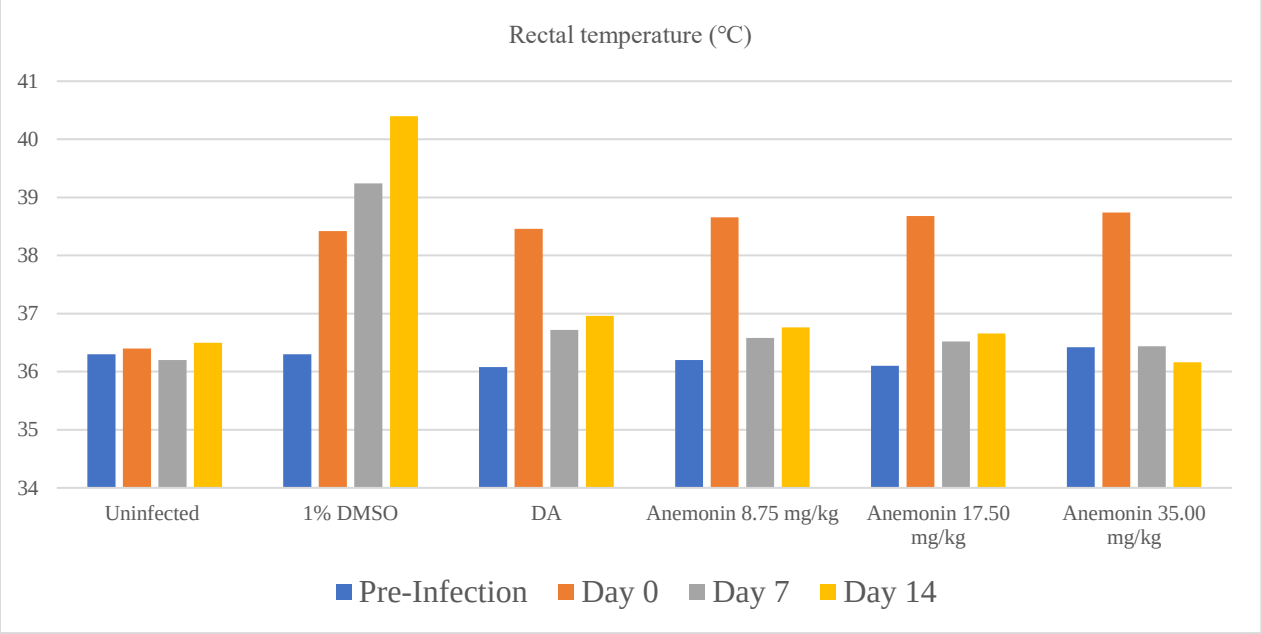


Figure 16. Rectal temperature (°C) of healthy uninfected and *Trypanosoma congolense*- infected mice treated with anemonin in *in vivo* antitrypanosomal assay

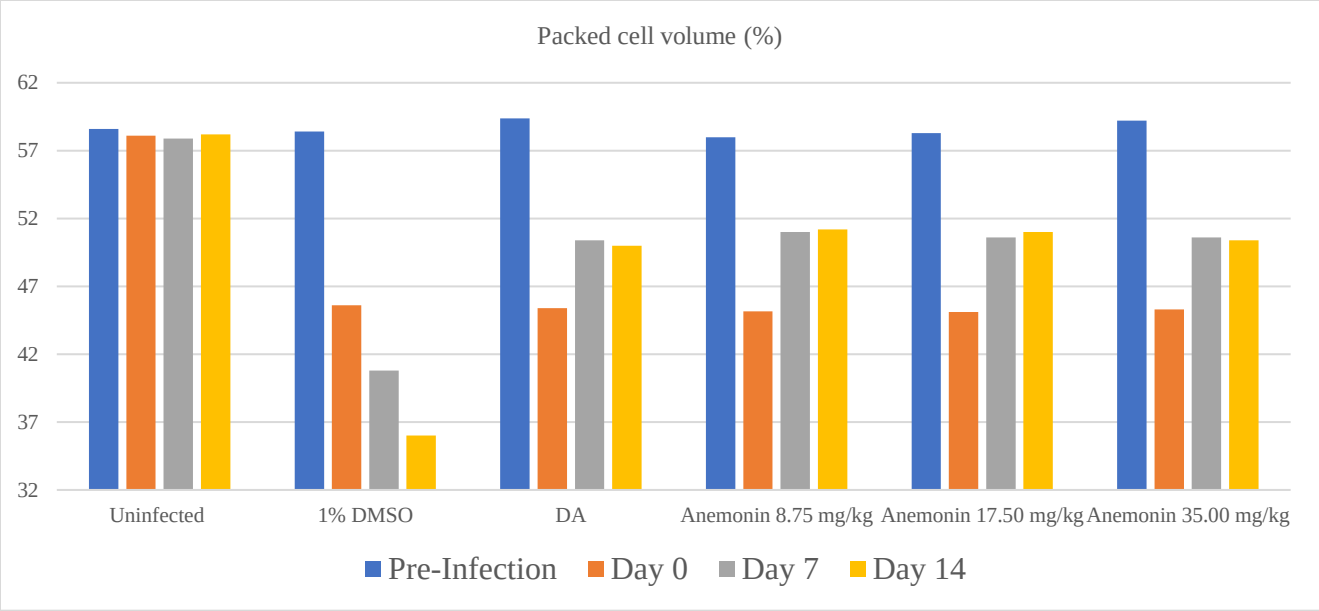


Figure 17. Packed cell volume (%) of healthy uninfected and *Trypanosoma congolense*-infected mice treated with anemonin in *in vivo* antitrypanosomal assay

Perusal of literature unveiled that only limited scientific investigations have been carried out on *R. multifidus*. These include the work done by Naidoo *et al*. (2013) which reported *in vitro* antimicrobial activity of the aqueous extract of the leaves of *R. multifidus* against sexually transmitted disease associated pathogen *Ureaplasma urealyticum*, which confirmed its inhibitory activity with minimum inhibitory concentration (MIC) of 0.02 mg/ml. In addition, Begashawu *et al*. (2016) demonstrated that the chloroform extract of the leaves of *R. multifidus* possesses antibacterial activity against *Staphylococcus aureus* at a concentration of 200 mg/ml with inhibition zone of 26.67 ± 0.83 mm. Similarly, the 95% methanol leaf extract of the plant has also been shown to have activity against the gram-positive bacterium *Clavibacter michiganense* (inhibition zone of 9.0 ± 0.8 mm), an organism that causes canker on tomatoes and ring rot of potato (Pretorius *et al*., 2003). It was also reported that the 80% methanol extract of the aerial part of *R. multifidus* inhibits carrageenan-induced mice paw oedema at a dose of 300 mg/kg

confirming that it is more effective in reducing oedema than the positive control indomethacin at a dose of 10 mg/kg (Dilebo *et al*., 2010).

To the best of our knowledge, this is the first report on the antileishmanial, antimalarial and antitrypanosomal activities of *R. multifidus*. The current investigation on the antiprotozoal potential of *R. multifidus* extract is of special relevance for the country as it may help to obtain indigenous sources of drugs to combat these diseases. From the results of the study, it appears that anemonin is, in full or in part, responsible for the antiprotozoal activity of the plant.

It has been reported that protozoal parasites such as *Leishmania*, *Plasmodium* and *Trypanosoma* utilize glutathione (GSH) and trypanothione as primary antioxidant to minimize oxidative damage when they are exposed to endogenous and exogenous oxidative stress during life cycle (Meister and Anderson, 1983). Apart from its use as antioxidative defense in human erythrocytes, GSH maintains the reducing environment of the cytosol, supports rapid cell growth and many of the known GSH dependent processes are directly related to the specific lifestyle of plasmodium parasites (Becker *et al*., 2003; Zhao *et al*., 1997; Munday and Winterbourn, 1989; Zhang *et al*., 1988). Similarly, trypanosomatids including *Leishmania* and *Trypanosoma* utilize GSH in the trypanothione biosynthesis (König *et al*., 2011). Trypanothione plays a crucial role in regulation of intracellular thiol redox balance and in defense against chemical and oxidative stress (Flohe *et al*., 1999). Since trypanothione is absent in humans, trypanothione biosynthesis pathway is a potential drug target (Agnihotri *et al*., 2016; König *et al*., 2011; Meister, 1974). Low GSH and trypanothione levels affect parasite development through the impairment of oxidative stress reduction systems and damage to the DNA (Padín-Irizarry *et al*., 2016; Davioud-Charvet *et al*., 1999). GSH is a water-soluble tripeptide composed of the amino acid: glutamine, cysteine, and glycine (Griffith Mulcahy, 1999; Meister and Anderson, 1983). The cysteine thiol acts as a

nucleophile in reactions with both exogenous and endogenous electrophilic species (Townsend *et al*., 2003; Meister and Anderson, 1983). As a consequence, reactive oxygen species are frequently targeted by GSH in both spontaneous and catalytic reactions (Becker *et al*., 2003).

Compounds containing an α,β-unsaturated lactone such as anemonin **(7)** undergo specific alkylation between unsaturated lactone with the thiol group (sulphydryl (-SH)) of L-cysteine **(10)** by a Michael-type addition (Mares, 1987; Hall *et al*., 1977; Cavallito and Haskell, 1945) (Figure 18).

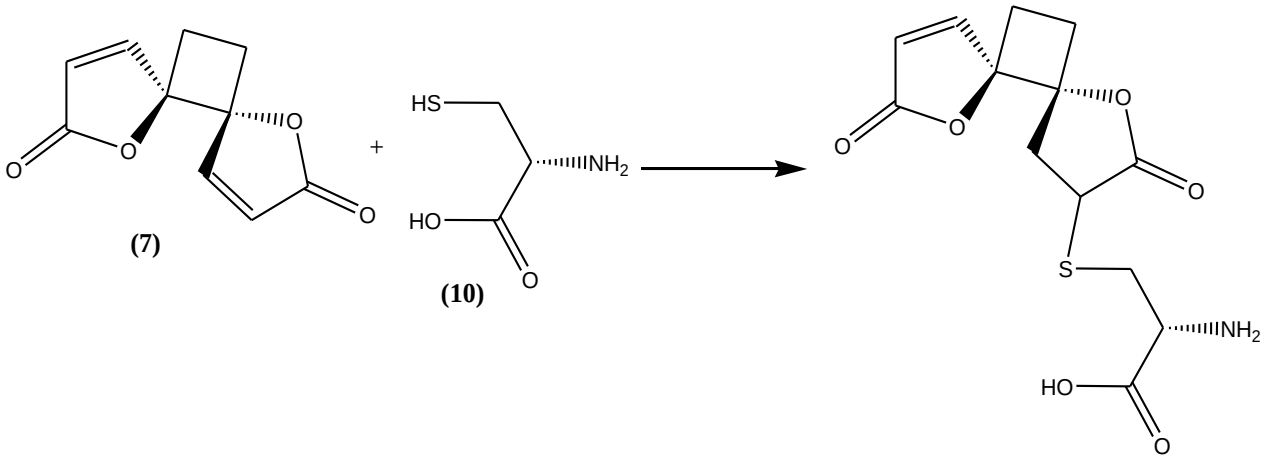


Figure 18. Michael-type addition reaction between anemonin **(7)** and L-cystine **(10)**

In the present study, the observed antiprotozoal activity of anemonin might be due increasing oxidative stress in the parasite, since it interacts with the thiol group of the precursor amino acid L-cystine by Michael-type addition (Figure 18). Meanwhile, L-cysteine is relevant as a substrate in the synthesis of GHS and trypanothione (Canepa *et al*., 2009). The capability of anemonin to interact with this important amino acid might offer it a potential to increase oxidative stress and growth inhibition in the parasites.

5. Conclusion

In the present study, initial bioassay tests conducted on the 80% methanol and hydrodistilled leaf extracts of *R. multifidus* indicated the potency of the plant as antileishmanial, antimalarial, and antitrypanosomal agent. The hydrodistilled extract was proven to express the highest potency in inhibiting growth of these pathogenic protozoan parasites, by lowering the rate of parasite multiplication. Further phytochemical analysis of the hydrodistilled extract resulted in the isolation of an α,β-unsaturated dilactone characterized as anemonin. Investigation for antiprotozoal activity has demonstrated that anemonin exerts strong potency in inhibiting growth of the parasites suggesting that it is responsible, in full or in part, for the activity of the plant. In summary, this study has provided scientific evidence for the genuine activity of *R. multifidus* against the protozoan parasites *Plasmodium*, *Leishmania* and *Trypanosoma*. To our knowledge, this study is the first to demonstrate the antiprotozoal activity of anemonin, isolated from the leaves of *R. multifidus*. Finally, the results produced in this study might be additional reference in natural product research and will contribute to the further study of antiprotozoal drug discovery. The findings also support the use of the plant for the treatment of leishmaniasis, malaria and animal trypanosomiasis in traditional medicine.

Recommendations

Based on the findings of the present study, the following recommendations are forwarded.

- Isolation of compounds from the hydroalcoholic extract and investigate their antiprotozoal activities;
- To carry out further toxicological studies (subchronic, chronic and dermatological) on both the extracts and anemonin; and
- To use anemonin as lead compound for the development of safe and cost-effective antiprotozoal drugs.

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Appendices

Appendix 1

Appendix 1a: Preparation of NNN medium

Novy-MacNeal-Nicolle medium (NNN medium) is prepared by 9.2g Nutrient agar, 0.6g D-(+) Glucose (anhydrous) and 2.4 g sodium chloride were weighed, mixed and dissolved in 400 ml of distilled water by boiling in hot plate with repeated shaking until clear solution is obtained. Then it was autoclaved at 121℃ for 30 min. Sheep blood priorly collected with a sterile bottle containing glass beads was defibrinated by shaking and heat inactivated by keeping it in 37 ℃ water bath for 50 min and then transformed into 56 ℃ water bath for 20 min. The autoclaved ingredients (400 ml) and of heat inactivated blood (100 ml) were mixed at 50 ℃. 2 ml of the mix is dispensed among culture vials, allowed to settle slant and stored at 4 ℃ until use.

Appendix 1b: Preparation of Locke's solution

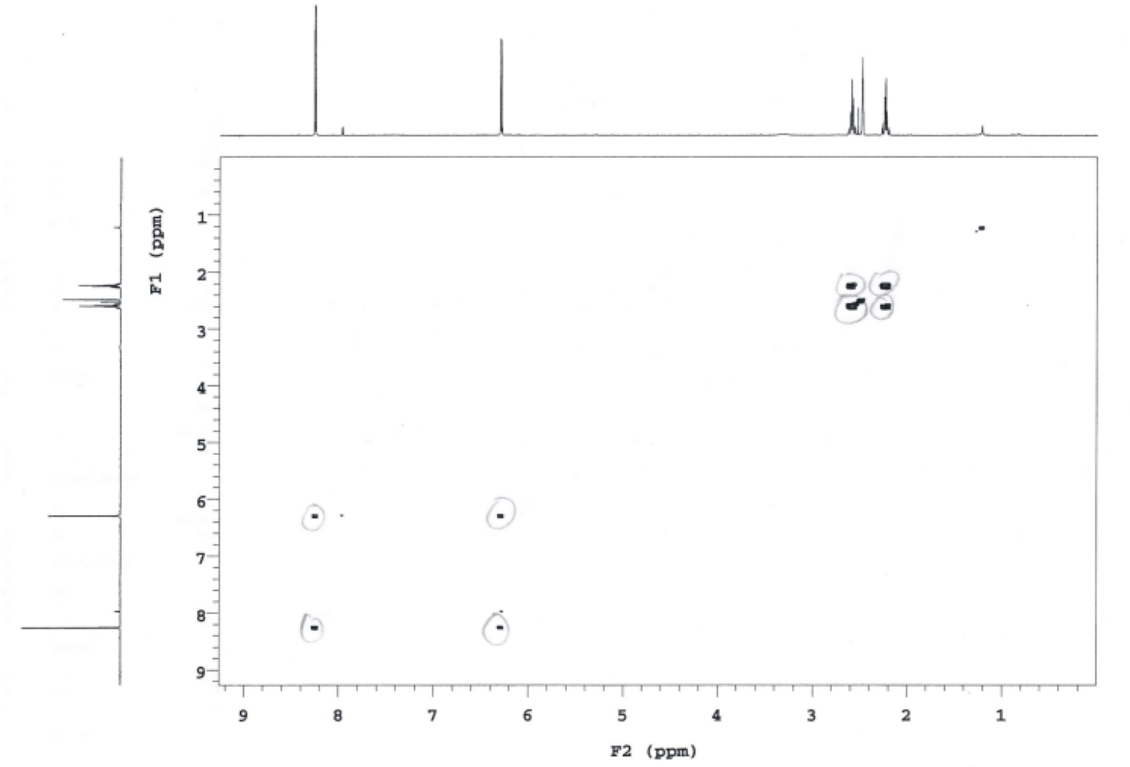
Locke's solution is preprepared by 4.5 g sodium chloride ,0.2 g potassium chloride, 0.1 g calcium chloride, 0.1 g sodium bicarbonate and 1.25 g D-(+)-glucose(anhydrous) mixed and dissolved in 500 ml distilled water. The mixture was autoclaved at 121 ℃ for 30 min and stored at 4 ℃ until use.

Appendix 1c: Preparation of Phosphate-Buffered Saline (PBS)

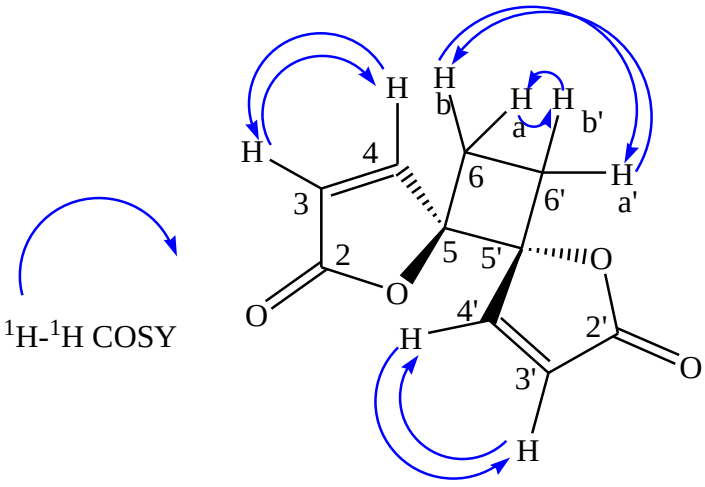
Phosphate-buffered saline (PBS) was prepared by dissolving 80 g sodium chloride, 2 g potassium chloride, 14.4 g disodium hydrogen phosphate (Na_2HPO_4) and 2.4g potassium dihydrogen phosphate (KH_2PO_4) in one liter of distilled water.

Appendix 2

Appendix 2a: 2D NMR ^{1}H-^{1}H COSY spectrum of RM-H1



Appendix 2b: ^{1}H-^{1}H COSY correlation of RM-H1



Appendix 3: Some photographs during laboratory work

Plant collection

Extraction (Maceration)

Extraction (Hydrodistillation)

Compound isolation (PTLC)

T. congolense collection from infected cattle

In vitro antitrypanosomal study