Evaluation of the Antiplasmodial and Antimicrobial Properties of the Medicinal Plants *Rumex nepalensis* Spreng and *Centella asiatica L*

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Evaluation of the Antiplasmodial and Antimicrobial Properties of the Medicinal Plants *Rumex nepalensis* Spreng and *Centella asiatica L*

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This is to certify that the Dissertation prepared by Asmamaw Habtamu Ayilet, entitled: Evaluation of the Antiplasmodial and Antimicrobial Properties of the Medicinal plants *Rumex nepalensis* Spreng and *Centella asiatica L* is submitted in fulfillment of the Requirements for the Degree of Doctor of Philosophy (Biology: Biomedical Sciences) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Infectious diseases are the major health problems in the world particularly in developing countries. To combat this problem various drugs, antibiotics and vaccines have been developed through time, but these successes have been challenged by the emergence of drug resistant pathogens. In Ethiopia, most people use traditional medicinal plants which are the natural source of medicine to treat different diseases. However, traditional healers in this country apply medicinal plants to heal different infections without scientific prescription and optimization of the right doses. This study was conducted to investigate antiplasmodial, antibacterial, antifungal properties, evaluate acute toxicity and possibly isolate the active compounds of two widely used medicinal plants: *Rumex nepalensis* Spreng and *Centella asiatica* L. The two plants were collected from Dejen District, Northen part of Ethiopia, and air dried in shade. The specific plant parts (leaves, roots. aerial parts) were separately ground into powder. Each plant powder was soaked in water, methanol and hexane in separate Erlenmeyer flasks and placed on shaker of 120 rpm for 72 hours. The extracts were concentrated in rotary evaporator and by lyophilizer (aqueous extract). The crude extracts of water, methanol and hexane of the two plants were tested for antiplasmodial, antimicrobial activities, acute toxicity and screened for phytochemicals. The higher antimicrobial performing extract, methanol extracts of the roots of *R. nepalensis* and further fractionnated by solvent-solvent extraction (hexane, chloroform, ethanol, water and methanol) and these fractions were evaluated on pathogenic microorganisms (*Plasmodium berghei, Esherchia coli, Pseudomonas aeruginosa, Shigella flexneri and Salmonella typhimurium, Klebsiella pneumonia, Candida albicans*). Similarly the best fraction, ethanol fraction, was further fractionnated by column chromatography (CC) and tested for antiplasmodial and antimicrobial activities. Furthermore, column Chromatographic profiles of

the roots of *R. nepalensis* were analyzed by thin layer chromatography (TLC). The crude methanol and hexane extracts of the root, the leaf extracts of *R. nepalensis* and the aerial parts of *C. asiatica* at the highest concentration of 5000 mg/kg BW was not toxic to the albino mice. Furthermore the phytochemical screening of the *R. nepalensis* revealed the presence of flavonoids, alkaloids, glycosides, triterpenoids, sterols, saponin and tannin. On the other hand, phytochemical screening of *C. asiatica* showed the presence of flavonoids, alkaloids, glycosides and saponin, triterpenoids and sterols but tannins were not clearly observed. CC and TLC profiles of ethanol fraction of the roots of *R. nepalensis* showed the presence of chrysophanol, emodin and 5 undefined compounds. The crude extracts of water, methanol and hexane of the two plants had shown suppression to *Plasmodium berghei in vivo* in mice. Particularly, the crude extracts of water, methanol and hexane of the roots of *R. nepalensis* had significantly higher day-4 suppressive effect 52.00 %, 59.90 % and 39.30 % respectively than the negative control (2% DMSO). But all extracts had significantly lower day-4 suppressive level than the positive control, chloroquine in *P. berghei* infected albino mice. The ethanol fraction of the roots of *R. nepalensis* showed higher plasmodium percent suppression *in vivo* in albino mice (70.08 %) compared to water (54.31 %), chloroform (19.61 %) and methanol (10.27 %) suppression at 500 mg/kg body weight (BW). Similarly the mean survival time value of the mice administered with ethanol fraction was significantly higher than all extracts (water, chloroform and methanol) at 500 mg/kg BW and negative control, 2% DMSO treated mice. More specificaly the ethanol fraction of the roots of *R. nepalensis* fractionnated by column chromatography in ethyl acetate: methanol (1:1) v/v showed 73.66% plasmodium suppression *in vivo* in albino mice at 300 mg/kg BW. The re-fractionnated ethyl acetate: methanol fraction by column chromatography in methanol increased its suppression to 79.66% at 150 mg/kg BW. In the antimicrobial test, the

ethanol fraction of the roots of *R. nepalensis* showed zone of inhibition for *P. aeruginosa* (19.33±0.33 mm), *S. aureus* (18.03±0.14 mm) and *S. flexneri* (12.77±0.15 mm) and *S. typhimurium* (8.33±0.33 mm) at 6 mg/well by using agar well diffusion method compared to the negative control (2% DMSO) which have no inhibition zone on the tested bacteria. However, the positive control (teteracyclin and chloramphenicol) significantly higher bacterial inhibition than ethanol fraction of the roots of *R. nepalensis.* From the result obtained it can be concluded that the five undefined compounds played a role in synergizing the atiplasmodial, antibacterial and antifungal activities of the roots of *R. nepalensis*. In conclusion this work has demonstrated the high potential of *R. nepalensis* and *C. asiatica* as sources for further exploitation of antiplasmodial, antibacterial and for use in herbal medicine.

Key words: antibacterial, antifungal, Plasmodium suppression, phytochemical screening, toxicity

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List of Abbreviations and Acronyms

AAU- Addis Ababa University

AIDS- acquired immune deficiency syndrome

ANKA - Angstromquelle Karlsruhe

ANOVA - Analysis of variance

BW- body weight

CC- column chromatography

CDC- Centers for Diseases Control and Prevention

CFU- colony forming unit

COX- cycloxygenase

DAEC- diffusely adherent *Escherichia coli*

DMSO- dimethylsulfoxide

DMU- Debre Markos University

MOH- Federal Democratic Republic of Ethiopia, Ministry of Health

EIEC- enteroinvasive *Escherichia coli*

EPEC- enteropathogenic *Escherichia coli*

EPHI - Ethiopian Public Health Institute

ESCMID - European Society of Clinical Microbiology and Infectious Diseases

EUCAST - European Committee for Antimicrobial Susceptibility Testing

HIV- human immune deficiency syndrome

MBC - Minimum Bactericidal Concentration

ME-TRAP - Thrombin Receptor-Activating Peptide

MHA- Mueller Hinton Agar

MIC - Minimum inhibitory Concentration

MSP- merozoite surface protein

MVA- modified vaccinia virus Ankara

OD- optical density

OECD - Organization for Economic Co-operation and Developmen

PCV- Packed Cell Volume

Rf- retention factor

RTS,S/AS01E - repeat region of circumsporozoite protein T-cell epitopes surface antigen of hepatitis B

SPSS - Statistical Package for Social Sciences

T-80 - tween 8

CHAPTER 1: GENERAL INTRODUCTION

1.1 Infectious diseases

Infectious diseases are caused by pathogenic microorganisms including bacteria, viruses, parasites and fungi. They affect 100 millions of people in the world. Among the 10 top infectious diseases, the lower respiratory infections, HIV/AIDS, diarrheal diseases, tuberculosis (TB) and malaria account for 22 percent of total deaths (Diseases Control Priorities Project, 2006). Infectious diseases are of the major health problems of the world in general and less developing nations in particular (CDC, 2011). Infectious diseases still remain as the major causes of morbidity, mortality, losses in productivity and economy in less developing nations as well as developed nations.

Malaria and food born diseases like typhoid fever, shigellosis and shiga diseases are among the infectious diseases that can cause public health problem in the world. The number of death reported in the world due to malaria alone is about 438 000 in 2015. More than 90% of the death is reportedly known in Africa (WHO, 2015).

Researchers have conducted to develop vaccine, chemotherapy and antibiotics to fight against pathogenic microorganisms. As a result many vaccines and chemotherapies have been applied to solve health the problems which are caused by pathogens. Although various antibiotics have been discovered to combat bacterial infections, the diseases remained as the major cause of mortality (Chanda and Rakholiya, 2011). In addition to this, antimicrobial resistance is challenging the efficacy of antibiotics and this resistance is spread in different parts of the world (Canton and Conque, 2006).

Researchers discover and develop vaccine, drugs and antibiotics to combat against pathogens. However, these discoveries are challenged by resistant microorganisms to antimicrobial agents (Peterson and Dalhoff, 2004).

Globally, the emergence of drug resistant bacterial strains has been reported and this challenge limits the effectiveness of current drugs and significantly causing failure in the treatment of infectious diseases (Hancock, 2005). The first agents of peniciline resistant was shown by the strains of *S. aureus* have resistance to penicillin (Rammelkamp and Maxon, 1941). Apart from antibiotics from microorganisms, medicinal plants offer treatment options with little or no transferred resistance. As consequence, evaluating the medicinal activities of plants is very important since it is the source of natural drugs against pathogens.

1.2 Medicinal plants

Medicinal plants have been used as sources of medicine for a long period of time in human history (Hoareau *et al*., 1999). Furthermore, medicinal plants are the natural source of secondary metabolites such as alkaloids, tannins, flavonoids, steroids, terpenoids and phenylpropa (Harvey, 2008). Some have nutritional value and others have antiplasmodial, antibacterial, and antifungal properties (Wang *et al*., 2009).

In Ethiopia, more than 80% of the population use traditional medicines to treat different types of infections (Bekele, 2007). Plant extracts from roots, barks, stems, leaves and seeds can be used to treat different types of infections (Ogbulie *et al*., 2007). However, herbalist traditionally uses the plant without evaluating and optimizing the doses of biologically active ingredients. Extract fractions from *Withania somnifera* shows significant suppressive effect of malaria at a dose of 200 mg/kg (Teklemariam *et al*., 2013). Similarly, the crude extracts of *C. myricoides*, *D.*

angustifolia and *A. debrana* also shows strong activities *in vivo* against *P. berghei* in mice (Deressa *et al*., 2010). *R. nepalensis* and *C. asiatica* are wildly distributed in all regions of Ethiopia and traditionally used to treat ascariasis, abdominal pain and gastric (Hedberg *et al*., 2003; Muthuswamy and Solomon, 2009).

1.3 Statement of the problems

Ethiopia is rich in plant biodiversity in addition to this it is found in the tropics where infectious diseases are more prevalent. It is also demonstrated that more than 80% of the people used traditional medicine to treat various system based on indigenous knowledge (Abebe, 2001). As a result of this, various medicinal plants which have therapeutic potential to treat infected persons are still given by traditional healers without scientific prescription and optimization of the right doses. Furthermore, the emergence of multi-drug resistant bacterial strains challenged the currently available antibiotics. Similarly available malarial therapies are challenged by resistant *Plasmodim* species. These led different researchers to conduct the research on the identification and documentation of traditional plants which are used by local people of Ethiopia. In this work, the antiplasmodial, antibacterial and antifungal properties of *R. nepalensis* and *C. asiatica* plant species were evaluated.

1.4 Hypothesis

It is hypothesized that the aqueous and solvent extract, fractions and sub-fractions of the roots and leaves of *Rumex nepalensis* and aireal parts of *Centella asiatica* have *in vivo* antiplasmodium *berghei*, *in vitro* antimicrobial and antifungal effects on some pathogenic organisms.

1.5 Objectives of the Study

1.5.1 General objective

To evaluate the antiplasmodial, antibacterial, antifungal properties and isoate the active fractions of the leaves and roots of *R. nepalensis* and the aerial parts of *C. asiatica*

1.5.2 Specific objectives

1) To determine the antiplasmodial effect of the crude extracts, fractions and sub-fractions of the leaves and roots of *R. nepalensis* and the aerial parts of *C. asiatica* in albino mice.

2) To determine the antibacterial and antifungal effects of the crude and fractions of leaves and roots of *R. nepalensis* and the aerial parts *C. asiatica* on some pathogenic bacteria and fungi.

3) To possibly isolate the active antiplasmodial, antibacterial and antifugal compounds from the plant extracts

CHAPTER 2: LITERATURE REVIEW

2.1 Malaria

Malaria is a life-threatening disease which is caused by *Plasmodium* species and transmitted to people through the bites of infected female anopheles mosquitoes. *P. falciparum* is the most severe and most prevalent in Africa. Nearly 3.2 billion people of the world's population are at risk of malaria. According to the WHO (2014), 207 million were cases of malaria and an estimated 627 000 deaths in 2012. About 198 million cases of malaria worldwide in 2013 and about 584 000 deaths were reported in the same year. 90% of all malaria deaths occur in Africa (WHO, 2014). Both malarial cases and the number of death declined from 262 million to 214 million and from 839 000 to 438 000, respectively in 2000 and 2015 (WHO, 2015).

Although the global burden of malaria declined in the above consecutive years, this progress is threatened by the development of insecticides resistance mosquitoes and drug resistance malarial parasites. Furthermore, a lot of researches have been conducted on malaria vaccine and tested in man since 1960, but successful and effective malaria vaccine has not yet developed. On the other hand chemotherapies to malaria have been practiced for the long period times. For instance, chloroquine has been discovered in 1960s and after decades of use it encounters chloroquine resistance plasmodium strains (Hay *et al*., 2004).

The chemotherapy of malaria can be broadly classified into the following four classes: the quinolines derivatives (chloroquine, quinine, mefloquine, amodiaquine, and primaquine), the antifolates (pyrimethamine, proguanil and sulfadoxine), the artmisinin derivatives (artemisinin, artesunate, artemether, arteether) and hydroxynaphthaquinones such as atovaquine (Saifi, 2013).

Although quinolines derivatives drugs had long history along malaria, their mode of actions on malaria parasites have not clearly described, they are thought to interfere with hemoglobin digestion in the blood stages of the malaria parasites (Ismail *et al*., 1996).

Quinoline containing drugs such as chloroquine are supposed to cause the swelling of food vacuole and increasing granular formation of malaria parasites and finally cause cell death (Singh and Singh, 2014). Recently, researches in Cambridg revealed the dual antimalarial and antiprion properties quinoline class compounds. Type-I antifolate drugs (sulfonamides and sulfones) acts on malaria parasites by inhibiting the synthesis of folate competing with p-aminobenzoic where as type II antifolate drugs prevent the utilisation of folate through conversion of dihydrofolate to tetrahydrofolate by inhibiting the enzyme dihydrofolate reductase (Jadhav and Shinde, 2015).

Artemisinin is sesquiterpene trioxane lactone associated with endoperoxide bridge which is essential for antimalarial activity. The mode of action of artemisinin derivatives on malaria parasites is not clearly elucidated, but it is suggested that the drug interfers with flavoenzyme function (Haynes *et al*, 2010). It is also suggested endoperoxide interact with the parasite electron transport chain to stimulate free radicals formation that inhibit mitochondrial functions of the parasite (Padmanaban *et al.*, 2012).

In spite of antimalaria drug discovery, the various malaria drugs are resisted by parasites. As a result artemisinin combination therapy is a currently used malaria drug. It has more than 98% efficacy in southwestern part of Nigeria (Ojurongbe *et al*., 2013). Although artemisin based combination therapies are currently used, there will be a need for new drug discovery since the

existing therapies are threatened by emergence of resistance (Jadhav and Shinde, 2015) and are challenged by poor bioavailability and rapid metabolism (Padmanaban *et al*., 2012)

RTS, S/AS01E is the only malaria vaccine which is able to enter 3rd phases and the most advanced candidate malaria vaccine after it has been evaluated for 5 to 10 years. Researches revealed that RTS,S/AS01E had reduced the rate clinical malaria in children of aged 5-17 months it was at second phases in Kenya and Tanzania (Lusingu *et al*. 2011). ChAd63/MVAME-TRAP is the second phase malaria vaccine which is given in prime-bost strategies. This can be boosted with modified vaccinia virus Ankara (MVA) which encodes the same ME-TRAP which is able to elicit strong and long-lasting CD8+ T cell responses in mice (Reyes-Sandoval, *et al*., 2010). Merozoite surface protein 3 (MSP3) is a blood malaria vaccine which enter to second phase b. Immuno-epidemiological studies reveal that MSP3 has associated with antibody responses and reduced risk of clinical malaria in multiple settings (Fowkes *et al*, 2010).

2.2 Pathogenic bacteria

Pathogenic bacteria are bacteria that causes or are capable of causing diseases to the host, however most bacteria are non pathogen or harmless. Pathogenic bacteria including pathogenic *Escherichia coli*, *Vibrio cholera*, Shigela species, Salmonella species, *Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are among the common disease caused bacteria (Ramita and Mukesh, 2012). According to World Health Organization (WHO) report on food borne diseases (2015), about 420,000 deaths were reported in 2015 due to food borne illness. Among this, foodborne diarrheal disease accounts about 230,000 deaths in 2010. *S. enterica* and *S. typhi* account 59,000 and 52,000 deaths respectively of all food borne death in the same year.

2.2.1 *Salmonella typhimurium*

Salmonella are Gram-negative rod-shaped bacteria which belong to genus *Salmonella* under family Enterobacteriaceae. They are able to grow and multiply outside living host having greater survival chances (Gray and Fedorka-Cray, 2002) and cause a wide range of human diseases such as enteric fever, gastroenteritis and bacteremia. Typhoid fever is caused by *Salmonella typhi* and transmitted by ingestion of contaminated food or water through faeces, urine. Salmonella species are leading causes of acute gastroenteritis in several countries and salmonellosis remains an important public health. It affects about 22 million new cases of enteric fever annually with 200,000 deaths (Crump *et al*, 2004). *S. typhi* has also developed resistance to most of the commonly prescribed antibiotics such as pefloxacin, ciprofloxacin, augmentin, gentamicin, co-trimoxazole and ampicillin (Sule *et al.*, 2012).

2.2.2 *Shigella flexneri*

Shigella species which belong to family Enterobacteriaceae are Gram-negative, non-spore forming rod-shaped bacteria. The genus can be classified into S. dysenteriae (13 serotypes), S. flexneri (15 serotypes), *S. boydii* (18 serotypes) and S. sonnei with 1 serotype. They cause shigellosis or bacillary dysentery. According to WHO report (2013), *Shigella* species are the major bacterial causes of diarrhea which account more 80 million cases of bloody diarrhea and about 700,000 deaths each year. The species primarily infect the lower intestinal tract high fever, abdominal cramps, bloody and mucoid diarrhea (Ashkenaz and Cleary, 2008).

Shigellosis is transmitted by the feco-oral route mainly with contaminated hands and less commonly through contaminated water or food. Then bacteria enter the colonic mucosa via microfold (M) cells by inducing micropinocytosis. The major problem in treating shigellosis is the development of antimicrobial resistannt Shigella species to drugs. The isolated shigella

plasmids are known to develop resistance against ampicillin, chloramphenicol, tetracycline, sulfonamides, streptomycin and trimethoprim (Rowe and Threlfall, 1984).

2. 2.3 *Escherichia coli*

Escherichia coli is a Gram-negative facultative anaerobic and rod-shaped bacterium commonly found in human and animal intestinal tracts. Most of the *E. coli* strains is harmless, but some serotypes can cause serious food poisoning and becomes intra and extraintestinal pathogen in humans and many other animals (Kaper *et al*., 2004). The most common intestinal pathogens are enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998).

2.2.4 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative, encapsulated rod shaped bacterium. It is non-motile lactose fermenting bacterium which is found in the normal flora of the mouth, skin, intestines and it belongs to *Enterobacteriaceae* family (Koneman *et al*., 2006). However, opportunistic *K. pneumoniae* cause community-acquired and nosocomial infections and typically infects patients in hospital under medical devices (Vuotto *et al*., 2014).

2.2.5 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa i*s a Gram-negative, aerobic and motile bacillus which is able to survive in a broad range of environmental conditions. *P. aeruginosa* an opportunistic pathogen causing infections in vulnerable patients with cystic fibrosis (Lucchetti-Miganeh *et al*., 2014). It is an important pathogen in hospitalized patients and cause morbidity and mortality due to its multiple resistance mechanisms (Porras-Gomez *et al*., 2012). This infection affects urinary tract,

surgical site, blood stream and wound. *P. aeruginosa* infection can be severe and life threatening and it is difficult to treat the bacteria since it develop resistance to antibiotics (Carmeli *et al*., 1999, Adedeji *et al*., 2007). The recent study investigated that *P. aeruginosa* showed a 79.23%, 95.32% and 95.68% resistance pattern against gentamycine, nalidixic acid and ceftazidim respectively (Rostamzadeh *et al*., 2016).

2.2. 6. *Staphylococcus aureus*

Staphylococcus aureus is a Gram positive bacterium which is capable of existing in both aerobic and anaerobic environments. Most of *S. aureus* are commensal organism which inhabits the skin, the nose throat and some cause staphylococcal infections (Lowy *et al*, 1998) which include impetigo, boils, carbuncles, abscesses and infected wounds. The anterior part of the nose is the most preferable site for *S. aureus* (Wertheim *et al*., 2005). The pathological effects of *S. aureus* are associated with the production of enzymes and toxin. For instance, coagulase converts fibrinogen to fibrin clot surround the bacterium in the host cells. This presence coagulase is an indiucation for a highly pathogenic strain (Wistreich, 2006).

S. aureus developed methicillin-resistance strain in hospitals and communities of USA (Boucher and Corey, 2008). The resistant strains of *S. aureus* produce a plasmid-encoded penicillinase to hydrolyze the β-lactam ring of penicillin deactivating the molecule's antibacterial properties (Chambers and Deleo, 2009). The emergency of methicillin-resistant bacteria led to use increase other alternative drugs such as in vancomycin (Jones, 2006). Vancomycin affects *S. aureus* bacterial cell wall precursors by binding irreversibly to the terminal D-alanyl-D-alanine of the cell wall component (Costa *et al*., 2013). However *S.areus* developed vancomycin resistance by

through the acquisition of resistance genes (*van*A) from vancomycin-resistant *enterococci* (Appelbaum, 2006, Chang, *et al*, 2003).

2.3 Pathogenic fungi

Most fungi are saprophytic which are involved in decomposing organic matters. However, some pathogenic fungi cause human diseases like candidiasis that can cause a serious health problem, especially in immuno-compromised individuals (Odds *et al*., 2006). The clinical use of antibacterial drugs and immunosuppressive agents including chemotherapy taken during organ transplantation, surgery and cancer are associated with increasing risk of human fungal infection (Karkowska-Kuleta *et al*., 2009). Candida species is leading human fungal pathogen to cause invasive fungal infections (Caston-Osorio *et al*., 2008).

2.3.1 *Candida albicans*

Candida albicans is a commensal fungus which inhabits oral cavity, the gastrointestinal tract, the vaginal and the urinary tracts. On the hand it can be opportunistic pathogens in the critical patients and of recipients of transplanted organs (Schmiedel and Zimmerli, 2015). *C. albicans* is the main Candida species that causes invasive candidiasis (Brissaud *et al*., 2012). *C. albicans* is an opportunistic fungus which causes infections such as fatigue, weight gain and joint pain (Pfaller and Diekema, 2007). The biofilm formation in *C. albicans* is one the factors for the pathogen to be drug resistant (Al-Fattani and Douglas, 2006).

2.4 Medicinal plants uses, toxicity and their antimicrobial activities

Crop plants manufacture the primary products such as carbohydrate, protein and lipid which are the sources of food for animals whereas medicinal plants produce secondary metabolites include alkaloids, tannins, flavonoids, steroids, terpenoids and phenylpropanoids which have medicinal

values in human health care system (Harvey, 2008). Essential oils and cosmetics are also derived from the secondary products of plant in the form of alkaloids, terpenoids and flavaonoids. These plants contain mixtures of different chemical compounds which can be used to treat diseases and improve health (Gurib-Fakim, 2011).

The use of natural products mainly from plants and animals in human health is as ancient as human civilization (Petrovska, 2012), but the discovery of modern drugs, vaccine and antibiotics decline the interest of traditional remedies and left them to be the only options for poor who could not afford the price the approved drugs in pharmaceutical market. However, today the demand for traditional medicines gets popularity and become mandatory to investigate the scientific basis of herbal remedies both in developing and industrialized countries (Efferth and Greten, 2012; Mishra and Tiwari, 2011).

Similarly in the last 20 to 30 years, the analyses of secondary metabolites of the plants have shown remarkable progresses (Okada *et al*., 2010). Thus medicinal plants are important in modern medicine in one of the following ways: directly as a drug in pure or extract form, they can be the bases for synthetic drug; the ingredients can be used as tool for the development of new drug (Lewington, 1991). Some others have antiplasmodial, antibacterial, antifungal properties and cytotoxicity (Wang *et al*., 2009). They have also a critical role in the development of human cultures and tradition in the world (Hassan, 2012).

Medicinal plants not only have medicinal and nutritional values but also they are toxic to the normal physiology of the animals. Plants therefore, produce secondary metabolites as defense against animals, parasites, bacteria, and viruses. These secondary metabolites are produced for

the purpose of the plants themselves (Robber and Huxtable, 1992). Before new drugs are approved, toxicological studies on the drugs are very essential experiments in animals such as mice and rat. As a result, toxicological studies help to make a decision whether the new drug is approved for clinical use or not (Alam *et al*. 2006). Therefore, it is important to have variety of toxicological investigations on drugs under the study before exposing the prospected drugs to humans (Rang *et al*. 2003).

The curative potential of medicinal plant can be due to the presence of complex composition of the secondary metabolites which can be derived from the bark, leaves, flowers, roots, fruits and seeds of the plants (Gordon and David, 2001). The ingredients of these medicinal plants are synergic which mean the secondary metabolites of the plants interact each other. As a result the secondary metabolites can complement or neutralize their negative effects in animals (Hassan, 2012).

Phytochemicals are a large group of plant-derived compounds which are responsible for the protection disease and obtained from fruits, vegetables, beans, cereals and plant-based beverages (Arts *et al*., 2005). Phytochemicals can be either primary or secondary compounds. Chlorophyll, proteins and common sugars are included in primary constituents whereas; terpenoid, alkaloids and phenolic compound are secondary compounds (Krishnaiah *et al*., 2007). Phytochemicals are not vitamins rather, they are physiologically active compounds which are produced in secondary metabolism and they in relatively small amounts, but they have significant health potentials.

Flavonoids are important group of polyphenols which are found in fruits, vegetables, coffee, tea and wine. Flavinoids include anthocyanins, flavanols, flavones, isoflavones, anthocyanins,

quercetin, kaempferol and myricetin and are believed to be used in antioxidant activity as hydrogen donating free radical scavengers, anti-inflammatory properties and maintain a healthy heart and urinary tract (Rice-Evans *et al*., 1996, Prior and Cao, 2000). Flavonoids deactivate carcinogens by inhibiting the expression of mutated genes and the activity of enzymes that promote carcinogenesis by promoting detoxification of xenobiotics (Kris-Etherton *et al*., 2002).

Alkaloids are largest group of secondary chemical constituents which are made up of mainly ammonia compounds and synthesized from amino acid. Alkaloids exist in solid such as atropine or stimulants caffeine, nicotine, codeine, atropine, morphine, ergotamine, cocaine, nicotine and ephedrine. Alkaloids are intensely bitter and function in the defense of plants against herbivores and pathogens. They are widely exploited as pharmaceuticals, stimulants, narcotics and poisons due to their potent biological activities. Alkaloids have pharmacological applied as anesthetics and centeral nervous system stimulants (Madziga *et al.,* 2010).

Terpenes are hydrocarbons of chemically diverse groups of natural products and are lipid-soluble compounds. Terpenoids include hydrocarbons of plant origin of general formula $(C_5H_8)_n$ and are classified as mono, di, tri and sesquiterpenoids depending on the number of carbon atoms. Terpenes include perillyl alcohol, limonene and carnosol. These help cells from becoming cancerous, slow cancer cell growth, strengthen immune function, limit production of cancer-related hormones, fight viruses and work as antioxidant. Among the pharmaceuticals, the anticancer drug taxol and the antimalarial drug artimesinin are two of the most renowned terpene-based drugs.

The application of traditional medicine against diseases has a very long history in Ethiopia. Traditional medicines mostly obtained from natural products especially from medicinal plants are not only concerned to curing diseases but also applied to physical, spiritual, social, mental and material wellbeing of human beings (Deriba *et al*., 2006).

2.5 *Rumex nepalensis*

Rumex nepalensis Spreng is an erect and perennial herb which measures about 2 m tall and it is called "yewsha tult" in the local language Amharic. It bears petioleted leaf with various shapes ranging from lanceolate to elliptic. The genus *Rumex* consisted of more than 250 species and is widely distributed in the worldwide including Europe, Asia, African and American countries (Vasas *et al*., 2017). *R. nepalensis* is distributed in the altitude range of 900–4000 m above sea level on moist and dry slopes in plains throughout India (Farooq *et al.,* 2013). It also grows in altitude range of 1200-3900 meter above sea level and distributed throughout Africa and in most parts of Ethiopia like Tigray, Amhara, Orromia and Southern parts of Ethopia (Hedberg, 2000).

Figure 1: *Rumex nepalensis Spreng* (Dejen district, Nothern Ethiopia October 2014)

Rumex species belong to family Polygonaceae with chemically complex and biologically active compounds. It has been used in traditional medicine, psychopharmacological, antioxidant, cytotoxic, anti-fertility, anti-inflammatory, purgative, antimicrobial, antidiarrhoeal and antiviral activities. Analysis of the five species of Rumex (*R. abyssinicus, R. usambarensis, R. baquaertii, R. ruwenzoriensis and R. crispus)* in Kenya showed that major compounds such as anthraquinones and naphthalenic derivative have been extracted from the above Rumex species (Midiwo *et al* 1985).

Fruit extraction obtained from six species of Rumex (*R. confertus*, *R. crispus*, *R. hydrolapathum* and *R. obtusifolius R. acetosa* and *R. acetosella*) in Poland reveal *in vitro* antimicrobial activities (Wegiera *et al.*, 2011). Methanolic extract of R. nepalensis showed antibacterial and antifungal activities at concentration of 400 μg/well. Another studies on crude extract of *R. nepalensis* root

exhibit highest activity against *A. niger* and showed moderate activity against *A. flavus* and *A. solani* (Hussain *et al*, 2010). Similarly, studies in India demonstrated that solvent extracts of *R. nepalensis* show significant antimicrobial activities (Yadev *et al*., 2011).

Different concentrations (ranging from 150 µg/mL to 500 µg/mL) of crude extracts of *R. dentatus* were determined to show antimicrobial activities of against different bacterial and fungal strains. In addition to this, the chloroform and ethyl acetate of the root extracts of *R. nepalensis* has anti-inflammatory, cycloxygenase (COX)-2, COX-1 inhibitory and free radical scavenging effects on mouse model and demonstrated a significant reduction in ear edema (Gautam *et al.*, 2010). The roots and leaves of *R. nepalenis* are used for dental care and the leaf juice in water is applied to stop bleeding during accidents (Joshi and Ananda, 2006).

On the other hand, a tea spoon of leaf powder of *R. nepalensis* boiled in 150 ml of water has been traditionally used to treat ascariasis, abdominal pain and gastric disorders in Gondar district, Ethiopia (Muthuswamy and Solomon, 2009). Rubbing the affected part of the body by ringworm has been traditionally treated with *R. nepalinsis* leaf extracts in Tgray Region, Ethiopia (Teklay *et al*., 2013).

2.6 *Centella asiatica*

Centella asiatica L is“yayit jero” in the local languate Amharic and it is a perennial, herbaceous creeper with kidney shaped leaves and grows about to 2 m high. It is characterized by reddish prostrate stolons and long rooting internodes (Jamil *et al*., 2007). It belongs to family Apiaceae which comprises 50 species. It is distributed in tropical and sub-tropical regions (James and

Dubery, 2009) and marsh and a swampy area with altitude range of 1000-3200 meter above sea level in different regions of of Ethiopia (Hedberg *et al.,* 2003).

Figure 2: Vegetative parts of *C. asiatica* L (Dejen District, Northern Ethiopia, October 2014)

Centella asiatica L. has been used as a medicinal herb for a long period in human history. It has been used for treating skin problems, healing wounds, revitalizing nerves and brain cells (Singh *et al*., 2014). *C. asiatica* is suggested to have a potential to treat systemic scleroderma, alcohol-induced liver cirrhosis, leg ulcers and as adjunctive treatment in leprosy (Chaudhury *et al*., 1987). Giving different doses of fresh leaf juice of *C. asiatica* to rat improved spatial learning performance and enhanced memory in neonatal rats treated with especially higher doses (Rao Mohandas *et al*., 2005). Similar studies also revealed that that this plant extracts enhances learning ability and memory retention power in adult rats (Rao Mohandas *et al*., 2007)

Chemical analysis of *C. asiatica* showed that the plant contains major bioactive constituents such alkaloids, terpenoids, carbohydrates and proteins compounds **(**Singh *et al*., 2012). It contains several active compounds such as triterpenoid saponins such as asiaticoside, centelloside, madecassoside, and asiatic acid. Different pharmacological studies revealed that *C. asiatica* are used to treat various skin conditions such asleprosy, lupus, varicose ulcers, eczema, psoriasis, diarrhoea, fever, amenorrhea, diseases of the female genitourinary tract and also for relieving anxiety and improving cognition (Gohil *et al*., 2010).

In addition, *C. asiatica* contains other components, including volatile oils, flavonoids, tannins, phytosterols, amino acids, and sugars. *C. asiatica* also contains triterpene, glycosides, asiatic acid (Zheng and Qin, 2007). Flavonoid derivatives such as quercetin, kaempferol, patuletin, rutin, apigenin, castilliferol, castillicetin, and myricetin have been reported in *C. asiatica* (Subban *et al*., 2008). Phenolic acids which include rosmarinic acid, 3,5-di-O-caffeoil quinic acid, 1 5-di-O-caffeoil quinic acid, 3,4-di-O-caffeoil quinic acid, 4, 5-di-O-caffeoil quinic acid, ettacrynic acid, chlorogenic acid and isochlorogenic acid are also the major components of *Centella asiatica.*

Flavonoids showed antioxidant activity as hydrogen donating free radical scavengers, anti-inflammatory properties and maintain a healthy heart and urinary tract (Rice-Evans *et al*., 1996, Prior and Cao, 2000). The other secondary matabolites alkaloids, has been pharmacological applied as anesthetics and CNS stimulants and it had antimalarial activities (Madziga *et al.,* 2010,). Saponin is known to have antiplasmodial activities (Traore *et al*., 2000). The hydrolysable tannin extracted from is known to show antibacterial and antiyeast activitieviteis and triterpnoids which are isolated from medicinal plant, *Momordica balsamina* is known to

inhibit both liver and blood stage plasmodium berghei (Ramalhete *et al*., 2014). Besides plant derivatived anthraquinone possess strong antiplasmodial activity in vitro on *P. falciparum* (Osman *et al*., 2010).

In Ethiopia, ethnobotanical studies showed that the fresh leaves of *Centella asiatica* are applied in wound healing. The crushed leaves are tied to wound parts of the body to heal the wound infection in the local people of Western Shewa Zone, Oromia Regional, Ethiopia (Kassa *et al*., 2016). Ethno-medicinal studies in South Omo, Ethiopia revealed that traditional healers apply *C. asiatica* to treat abdominal/stomach disorders and internal parasites (Tolossa *et al.*, 2013). This plant is among the list of medicinal plants which is used by the Maale and Arsi ethnic communities in Ethiopia to treat black leg diseases (Kidane *et al*., 2013).

CHAPTER 3: PHYTOCHEMICAL ANALYSIS AND ACUTE TOXICITY OF *R. NEPALENSIS* AND *C. ASIATICA* IN SWISS ALBINO MICE

3.1 Introduction

Medicinal plants are type of plants which contain compounds that can be used for therapeutic purposes or those plants which synthesize metabolites to produce useful drugs. Plants synthesize compounds which can be used for the treatment or prevention of diseases and other health disorders of the body. The most important compounds extracted from medicinal plants are alkaloid, fixed oil, essential oil, tannins and resins. These chemicals can be used as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activity. Phytochemicals inhibit microorganisms by interfering with some metabolic processes, modulating gene expression and signal transduction pathways (Kris-Etherton *et al*., 2002).

Phytochemicals are a large group of plant-derived compounds which are responsible for the protection of diseases and obtained from fruits, vegetables, beans, cereals and plant-based beverages (Arts *et al*., 2005). Phytochemicals can be either primary or secondary compounds. Chlorophyll, proteins and common sugars are included as primary constituents whereas; terpenoid, alkaloids and phenolic compound are secondary compounds (Krishnaiah *et al*., 2007).

In medicinal plant research, extraction involves the separation of medicinally active portions of plant and animal tissues using selective solvents and standard procedures. The purpose of standardized extraction procedures for crude drugs (medicinal plant parts) is to attain the therapeutically desired portions and to eliminate unwanted material by treatment with a selective

solvent. In adition to extraction techniques, phytochemistry, the simplest and quickest methods of screeing plant extracts by using chemical regents.

Medicinal plants produce secondary metabolites as defense against animals, parasites, bacteria, and viruses (Robber and Huxtable, 1992). When new drugs are developed toxicological studies are employed on animal models such as mice and rat starting from crude extract stage upto adaption for clinical use (Alam *et al*., 2006). It is also important to follow variety of toxicological investigations that show the safety profile of the compound to which humans may be exposed (Rang *et al*., 2003). Since *R. nepalensis* and *C. asiatica* are medicinal plants usually practiced by traditional healers in Ethiopia, the determination of their acute toxicity is very important together with their antiplasmodial activities of the two medicinal plants are evaluated. This work was aimed at evaluating the acute toxicity of crude and fractions of the two medicinal plants, *R. nepalensis* and *C. asiatica*.

3.2 Materials and methods

3.2.1 Description of study area

The fresh leaves and roots of *R. nepalensis* Spreng which is locally named "yewha tult" in Amharic and *C. asiatica* which is locally named "Yayit jero" were collected from natural vegetation in Dejen District, Eastern Gojjam Zone, Amhara Regional State, Ethiopia. The area has an altitude of 1080 to 2576 meters above sea level with average minimum and maximum annual temperatures of 18°C and 30°C respectively. The annual mean rainfall is 954.6 mm. Like other people in Ethiopia, people of Dejen practiced traditional plants treat various infectious diseases.

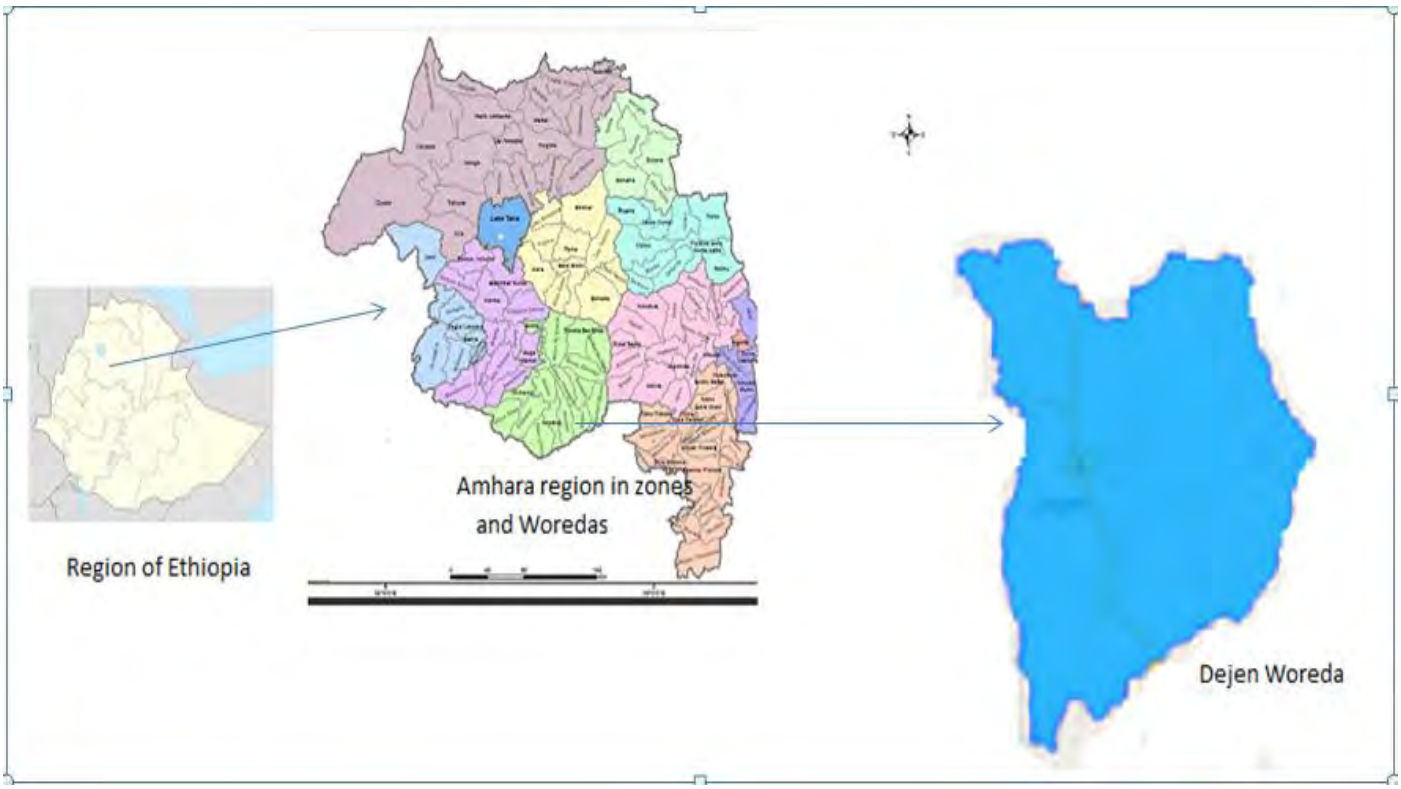


Figure 3: Collection area *of R. nepalensis* and *C. asiatica* (Dejen Woreda Government Communication Affairs office, 2016)

The plants were collected during their flowering time in October 2014 and the specimen were identified and authenticated by Mr. Melaku Wondafrash at College of Natural Sciences of Addis Ababa University. Then the specimens of the plants (*R. nepalensis C.* asiatica) were deposited with voucher number (ah001) and (ah002), respectively at the National Herbarium of Collage of Natural Science at Addis Ababa University. The plant samples were washed 3 times with tap water once with sterile distilled water and air dried in shade place at room temperature (25-30 ◦C) for a week as described in (Thippeswamy *et al*., 2011). About 1.5kg of each plant part was powdered by milling and sieved through a fine mesh (Canadian Series sieves with 500 µm opening), and the powders were packed in glass bottles and stored at room temperature for further use.

3.2.2 Aqueous extract preparation

The powdered leaves and roots of *R. nepalensis* (100 gm) and the aerial parts of *C. asiatica* (100 gm) were soaked with 1000 ml sterile distilled water in erlenmeyer flasks and placed on shaker (GFL, model 3020, Germany) for 72 hours according to (Subbarayan *et al.,* 2010). The macerates were first filtered through double-layered muslin cloth. Then, supernatant was filtered through filter paper and the extracts were concentrated by using lyophilizer at -40^{o}C with vacuum pressure. The dry and concentrate extract were placed in a brown battle at -20°C for further use.

3.2.3 Preparation of solvent extracts

About 1kg extract powders of of *R. nepalensis* and *C. asiatica* were soaked in separate 500 ml Erlemeyer flascks and placed on a platform shaker at 120 rpm at room temperature for 72 hours and the prescipitates were separatly re-soacked with the same solvents and and in similar conditions according to Mohana *et al* (2009). The subsquents filtrates were then concentrated in a rotary evaporator (Buchi type TRE121; Switzerland) at a temperature of 45°C and 60 rpm. Finally, the percentage yield of each extract was preserved I screw caped at -20ºC for futre tests

3.2.4 Phytochemical screening

The crude methanol, water and hexane extracts of the root, the leaf extracts of *R. nepalensis* and the aerial parts of *C. asiatica* were screened for the presence of alkaloids, saponins, tannins, anthraquinones, glycosides and flavonoids in accordance to standard phytochemical methods described in Harborne (1998).

Test for Alkaloids

To identify presence of alkaloids in the give active fractions, 4mL of 1% HCl were added to the 250mg of plant extract and then it was warmed and filtered. 6 drops of Mayor's reagents were added to 1ml filtrate. Creamish precipitate/orange precipitate indicated the presence of respective alkaloids.

Test for Flavonoids

Alkaline reagent test was used to test the presence and absence of flavonoids. For this test few drops of sodium hydroxide was added to the extract then an intense yellow color formed. Few drops of dilute acetic acid were again added to yellow color mixture and allowed for a colorless change that indicated the presence of flavanoids.

Test for glycosides

About 0.5 g of the plant was hydrolyzed with HCl and neutralized with NaOH solution and treated with a few drops of Fehling's solution. The formation of red color indicates the presence of glycosides

Test for Sterols and Triterpenoids

Salkolwski's test was used for triterpenoids test. For this, 200 mg extract was treated with chloroform and a few drops of concentrated H_2SO_4 was added, and the test tube was shaken well and allowed to stand for some time. The formation of red color in upper layer indicated the presence of sterol and formation of yellow color at the lower layer indicated the presence of triterpenoids.

Test for anthraquinones

About 0.5g of the plants powder of was boiled with 10 % HCl in water for few minutes. Then 5 ml of chloroform was added and shaken for 5 min. The mixture was filtered and shaken with equal volume of 10% ammonia solution. A pink, red or violet color in the aqueous layer after shaken indicated the presence of free anthraquinone (Evans, 2002)

Test for saponin

About 200 mg of powdered sample was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent broth. Formation of foam indicated the presence of saponins.

Test for tannin

About 200 mg of plant extract was treated with few drops of 0.1% ferric chloride and observed for blue or black coloration. Formation of blue black color confirmed the presence of tannins.

3.2.5 Fractionation of solvent extracts

The better bioassay performing the methanol extracts of the roots *R. nepalensis* were further fractionated using hexane, chloroform, ethanol, water and finally fractionated by methanol in 1 mg extracts to 10 ml solvent ratio by using separatory funnel. The ethanol fraction of the roots of *R. nepalensis* which showed higher aniplasmodial and antibacterial activities was further fractionated by column chromatography.

The final extracts, after having been tested, for preminarly antagonistic tests *in vitro* against test bacteria fungal and Plasmodial (*P. berghei*) test pathogens were selected for fractionation experiment. Accordingly, the best performing extract from the roots of *R. nepalensis,* was further fractioned with different solvents (hexane, chloroform, ethanol, water and methanol) using

solvent solvent fractionation techniques according to Akinpelu *et al*., (2008). The procedure is shown in Figure 4.

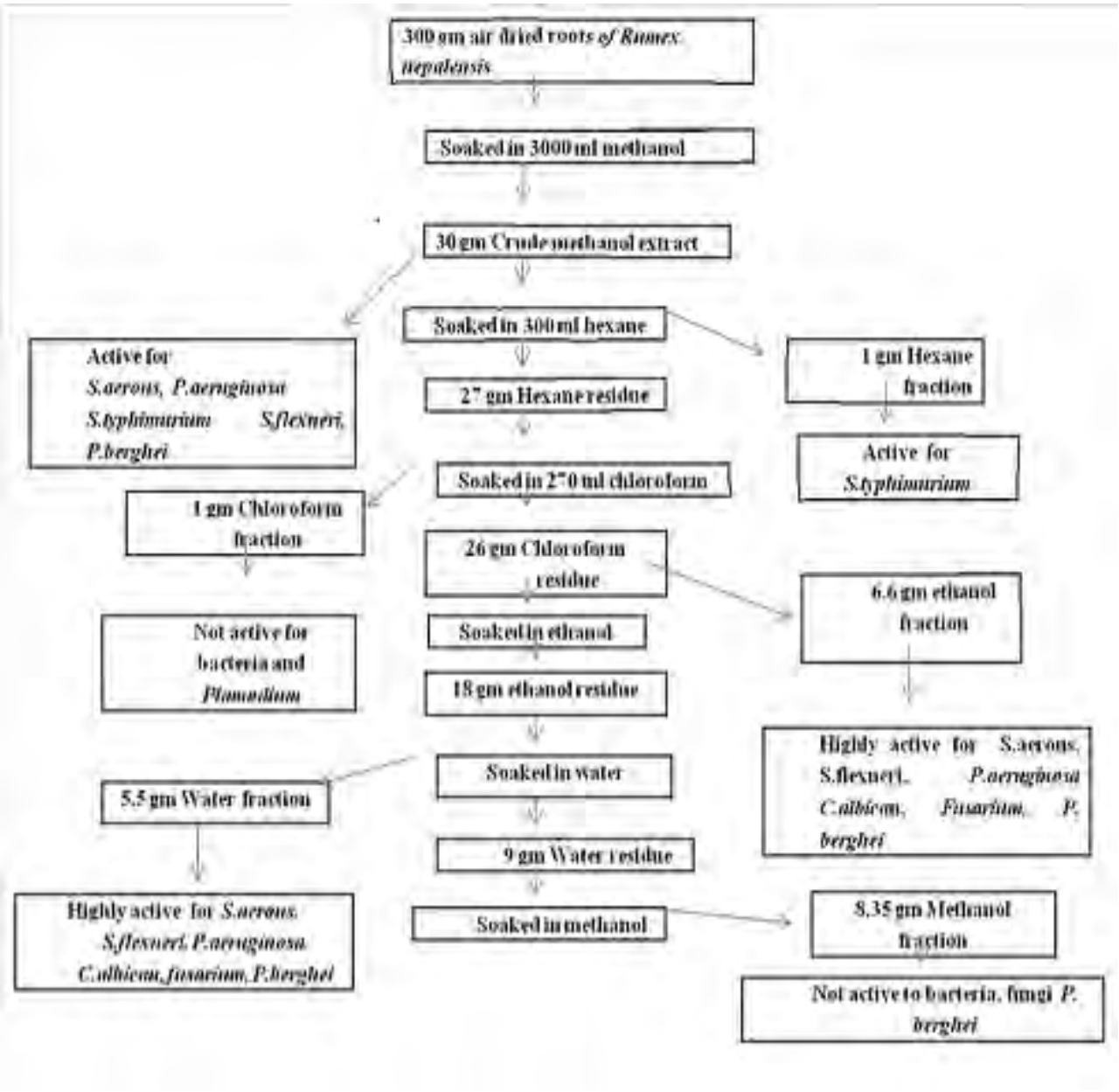


Figure 4: Flow shart showing fractionation of the roots of *R. nepalensis* (Akinpelu *et al*., 2008)

3.2.6 CC fractionation and TLC profiles

The ethanol fraction of the roots of *R. nepalensis* which had been relatively effective in inhibition growth of the test bacteria fungi and *Plasmodium* was further fractionnated by Column chromatography with three solvent systems (Chloroform, ethyl acetate and ethyl acetate: methanol) as described in Figure 5.

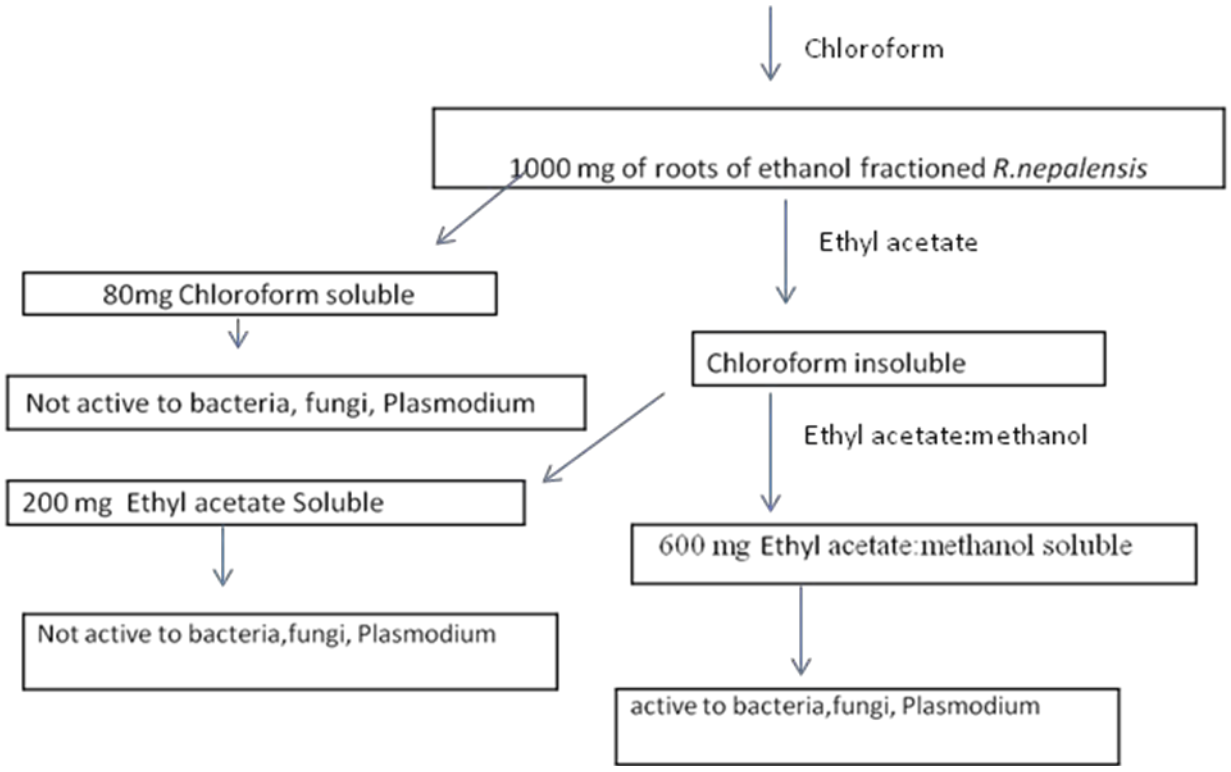


Figure 5: Column Chromatographic isolations of ethanol fractions of roots of R. nepalensis

The constituent of profile of each each Chromatographic fraction was monitored by TLC in hexane, chloroform and ethyl acetate visualized under UV-Vis light (λmax 254 and 366nm). The better antimicrobial performing fraction, the ethyl acetate: methanol fraction was profiled in TLC.

Furthermore, the Thin Layer Chromatography (TLC) profiles were compared with reference materials (emodin and chrysophanols) from the Department of Chemistry, Addis Ababa University (AAU). Then, the antiplasmodial, antibacterial and antifungal activities of the three Chromatographic fractions were evaluated against *P.bergei in vivo* and against bacteria and fungi *in vitro* in Biomedical laboratory AAU. The TLC profile was compared with reference materials such as chrysophenol and emodin. Fractions with similar Rf value on TLC were combined together and kept for bioautography screening and were tested for microbial activities as it is described by Tomer *et al*., (2009)

3.2.8 CC re-fractionation of ethyl acetate: methanol CC fractions

The ethyl acetate: methanol in 1:1 (v/v) Column Chromatographic fraction which has 5 spots on TLC was further fractionnated by Column chromatography with 4 solvent systems (hexane, chloroform, acetone and methanol) as shown in Figure 6. The Chromatographic fractions which were eluted in hexane: chloroform methanol and chloroform: methanol was loaded on TLC.

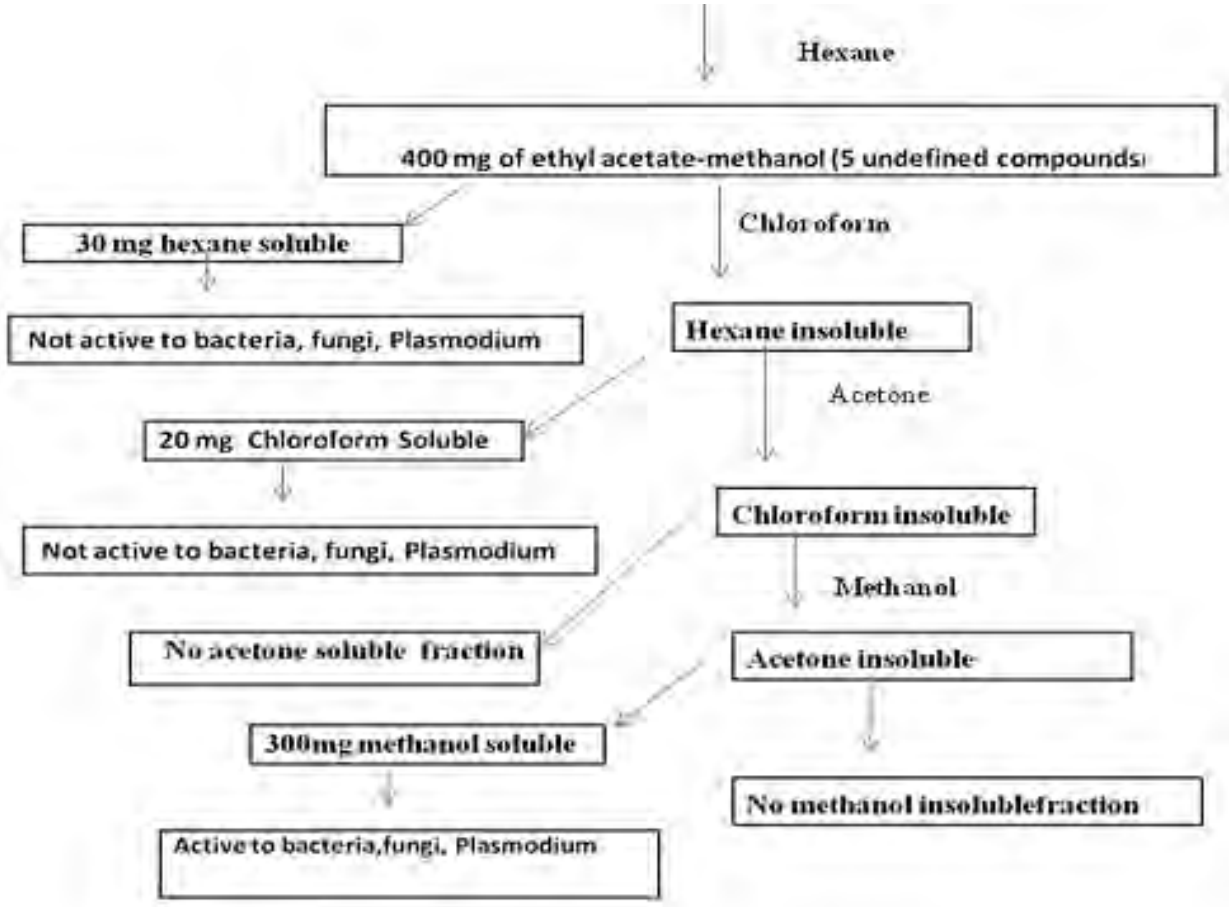


Figure 6**:** CC isolations of ethyl acetate: methanol fractions of root of *R. nepalensis*

3.2.9 Oral acute toxicity determination

OECD guideline (2001) was used as a reference for oral acute toxicity in mice. Female Swiss albino mice, 5 in each group were administered with the highest oral dose of 5000 mg/kg body weight from each crude extract which was dissolved in 2% DMSO since the acute toxicity of the two plants were not toxic at oral dose of 4000 mg/ BW in the previous studies (Chuhan *et al*., 2012, Kumar *et al*., 2011). 0.2 ml 2% DMSO were administered as a test and 0.2 ml of water was administered to negative control mice. For acute toxicity studies, gross physical changes and body weight were assessed for 14 days.

3.2.10 Body weight (BW) determination for oral acute oral toxicity

The mice were randomly grouped and each group had five mice. All mice were housed in standard cages in the animal House and fed a standard pellet diet and tap water *ad libitum.* Then each mouse was measured by using electronics beam balance before the administration of extracts before treatment. 5000 mg/kg BW of the crude methanol and hexane crude extracts aerialplant parts of C. *asiatica,* leaves and roots *of R. nepalensis* was dissolved in 2 % DMSO.

Then 0.2ml from each extracts of *R. nepalensis* and C. *asiatica* were administered orally by using oral gavage (an oral needle). At the same time 0.2ml of 2 % DMSO and 0.2ml of distilled water were given to mice as negative controls. The physical signs of acute toxicity such as depression, decrease in feeding activities and hair erection after extract administration were observed within 24 hours. The body weights of the mice in each group were measured at the first day when the experimental mice were administered with extracts, day 7 and day 14. The body weight of the extract administered mice was measured and their body weight changes were compared with mice which were treated with water and 2 % DMSO. The physical activities of the mice were also assessed in 14 days follow up.

3.2.11 Statistical analysis

Data were analyzed by using window software; SPSS, version 20. The results were presented as the Mean ± standard error of the mean (Mean±SEM) and statistical significance was considered at 95% confidence interval ($P<0.05$). The toxicity, antiplasmodial supressioin tests antimicrobial suscepitibllity were compared by using one way ANOVA followed by Tukey's test.

3.2.12 Ethical considerations

This study was conducted after having obtained ethical clearance from the institute review board of the College of Natural Sciences, Addis Ababa University, Research Ethics Review Committee (Appendix 1).

3.3 Results and discussion

3.3.1 Percentage yield of the crude extracts of *R. nepalensis* and *C. asiatica*

The highest (17.26 %) and minimum (0.63%) yield of crude extracts were obtained from methanol extracts of the roots of *R. nepalensis* and hexane extracts of the roots of *R. nepalensis,* respectively (Table 1). From this data, it is possible to conclude that the roots of *R. nepalensis* contained more polar compounds compared to its leaf parts. Similarly, the highest (13.34%) and the lowest (1.5%) yield of the aerial parts of *C. asiatica* were obtained from methanol and hexane extracts, respectively indicating that methanol dissolves dissolves more active components of the plant parts other solvents such as water and hexane.

Table 1: The percentage yield of crude extracts the roots and leaves *of R. nepalensis* and *C. asiatica* at concentration of 80 mg powder dissolved in 800 ml of solvents

Plant part	Solvent	Yield in (g)	Yield in (%)
C. asiatica/	MeOH	10.59	13.34
R. nepalensis root	MeOH	13.81	17.26
R. nepalensis leaf	MeOH	7.65	9.56
C. asiatica	H_2O	9	11.25
R. nepalensis root	H_2O	3.06	3.82
R. nepalensis leaf	H_2O	4	5
C. asiatica	n-Hex	1.2	1.5
R. nepalensis root	n-Hex	0.5	0.63
R. nepalensis leaf	n-Hex	1.1	1.48

Note: %= percent; g = gram; H_2O = water; MeOH = methanol; n-Hex. = normal Hexane

3.3:2 Phytochemical screening test *R. nepalensis* and *C. asiatica*

The present phytochemical screening test on the roots and leaves of *R. nepalensis* and aerial parts of *C. asiatica* revealed the presence of different secondary metabolites as it is shown in Table 2. Acoordingly, the phytochemicals including alkaloids, anthraquinone, flavonoids, glycosides, triterpnoids and saponins were present in both *R. nepalensis* and *C. asiatica* plants, but tannin and steroids were absent in methanol, water and hexane extracts of *C. asiatica*. In agreement with previous studies the two plants were known to have biologically active metabolites (Yadev *et al*., 2011, Muthuswamy and Solomon, 2009, Kassa *et al*., 2016, Kidane *et al*., 2013).

3.3.3 Phytochemical screening test of the roots and leaves of *R. nepalensis*

The color of roots *of R. nepalensis* was yellow, whereas the methanol extracts of the roots of *R. nepalensis* were brown in color and were found to have anthraquinone, flavonoids, glycosides, triterpnoids, tannin, saponin and steroids, but alkaloids were not detectable in the present phytochemical screening tests of the roots *of R. nepalensis*. This study was combarable with the previous work showing that *Rumex* plants contain anthraquinones, naphthalenes, flavonoids and other phenolic compounds (Mei *et al*., 2009; Zhang *et al*., 2009). Similarly, phytochemical screening tests in other studies reveals the presence of anthraquinone, steroids, saponins and tannins in the methanol extracts of the same plant (Ghosh *et al*., 2002).

In comparable with this study, phytochemical screening test on the related spices, *Rumex hastatus,* showed the presence of alkaloids, anthraquinones, flavonoids and saponins (Sahreen *et al*., 2015). Similarly phytochemical analysis of the water extracts of the roots of *R. nepalensis* showed the presence of anthraquinone, flavonoids, glycosides, triterpnoids, tannin and steroids, there were detectable saponin in this test. On the other hand the hexane extracts roots *of R. nepalensis* was yellow in color and the phytochemical analysis showed the presence of anthraquinone, alkaloides, glycosides, tannin, saponin and steroids, but there were no flavonoids and triterpnoids in hexane extracts of the roots *of R. nepalensis*.

The color of leaves of *R. nepalensis* was green, whereas the methanol and water extracts of the leaves *of R. nepalensis* were brown. In this work phytochemical screening test revealed the presence of anthraquinone, flavonoids, glycosides, tannin, saponin and steroids in both methanol and water extracts of the leves of *R. nepalensis* as it is shown in Table 2, but saponin and resin was not present in the water extracts of leaves of *R. nepalensis.*

3.3.4 Phytochemical screening test of the of the aerial part of *C. asiatica*

In this work, phytichemical studies on methanol and water extracts of the aerial parts of *C. asiatica* revealed the presence of anthraquinone, flavonoids, glycosides and triterpnoids. In agreement with previous studies, qualitative phytochemical analysis indicates the presence phytochemicals including alkaloids, flavonoids, tannins, terpenoids, saponin, steroids, carbohydrates and cardiac glycosides in aqueous extracts of *C. asiatica* (Madhusudhan *et al*., 2014). But in this work, tannin was not detected in methanol, water and hexane extracts of *C. asiatica*.

The phytochemical screening test on *R. nepalensis* in this work revealed the presence of major biologically active compounds including anthraquinone, alkaloids, flavonoids, glycosides, triterpnoids, tannin, saponin and steroids. Similaaarly *C. asiatica* contains all secondary metabolites except tannin. The above mentioned compounds have antibacterial, antimalarial, antiinflamatory and antioxidant activities.

Table 2: Phytochemical analysis of the crude extracts of the roots and leaves of *R. nepalensis* and the aerial plants of *C. asiatica*

Phytochemical/ Test	*R. nepalensis* roots			*R. nepalensis* leaves			*C. asiatica*		
	MeOH	H_2O	n-Hex	MeOH	H_2O	n-Hex	MeOH	H_2O	n-Hex
Alkaloids/ Mayer's Test	-	-	+	-	_	+	-	-	+
Flavonoids/ Ferric Chloride test	+	+	-	+	+	+	+	+	-
Glycosides/ Fehling's solution	+	+	+	+	+	-	+	+	-
Triterpnoids/ Liebermann-Burchardt test	+	+	-	+	+	-	+	+	-
Resins/turbidity test	+	-	+	+	-	+	_	-	-
Anthraquinone/ Borntrager's test	+	+	+	+	+	+	+	+	+
Saponins/ Foam Test	+	-	+	+	-	+	-	-	+
Steroids/ Liebermann-Burchardt test	+	+	+	+	+	-	+	_	-
Tannins/ Braemer's test	+	+	+	+	+	+	-	-	-

Note: H_2O = water; MeOH = methanol; n-Hex = normal hexane, + = presence of phytochemicals; - = absence of phytochemicals

3.3.5 CC profile of ethanol fraction of R. nepalensis

The CC technique was applied to isolate the better boiologically active solvent fractions, ethanol fractions of the roots of *R. nepalensis.* This brown color fraction was further fractionnated by using chromatography in different solvent systems as is shown in Table 3. In this work, 80 mg (8%), 200 mg (20 %) and 600 mg (60 %) of 1000 mg of ethanol fractions of the roots of *R.*

nepalensis was obtained by using CC technique in chloroform, ethyl acetate and ethyl acetate: methanol solvent system respectively as shown in Table 3.

From this it can be drawn that the ethanol fraction of the roots of *R. nepalensis* pereferably dissolved in ethyl acetate: methanol (50:50) v/v. The CC fraction obtained in chloroform solvent system was yellow and sticky solid. On the other hand, the chromatographic fraction obtained from ethyl acetate: methanol brown and sticky solid. Whereas the chromatographic yields in ethyl acetate: methanol solvent system was characterized by dark brown and non sticky solid powder.

Table 3: The chemical profile of ethanol fraction of the roots of R. nepalensis by using CC

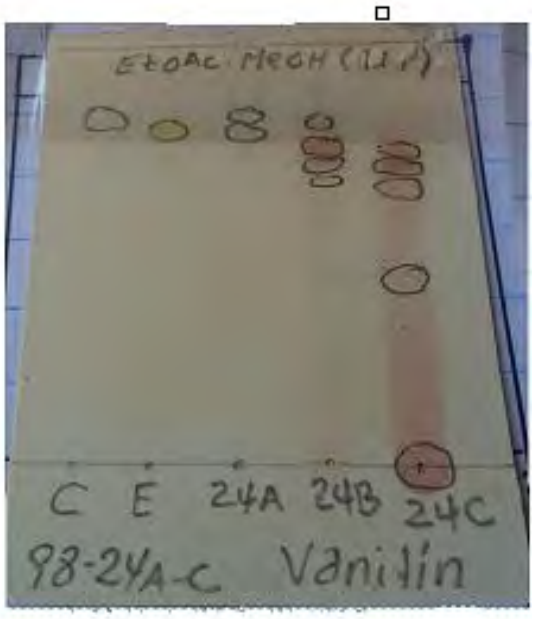
Solvent	Amount (mg)	% yield	Code	Major spot	Rf. value	Known compound
CHCl3	80	8	98-24A	2 (1:2)	0.78:0.83	Chrysophanol emodin
EtOAc	200	20	98-24B	4 (1:2:3:4)	0.67:0.71:0.76:0.78	Undefined
EtOAc:MeOH	600	60	98-24C	5 (1:2:3:4:5)	0:0.44:0.67:0.71:0.76	undefined

Note: CHCl3 = chloroform; EtOAc = ethyl acetate; Rf = retention factor; MeOH = methanol

The Thin Layer Chromatography (TLC) analysis on column Chromatographic fractions was shown in Figure 7. In this work, the two known compounds (chrysophanol and emodin) were used as a reference. Based on these references, Chromatographic fraction of the roots of *R. nepalensis* in chloroform solvent system had had 2 spots (compounds) with similar retardation factor (Rf) values (0.78 and 0.83) as a referenced chrysophanol and emodin, respectively. From these data, it can be concluded that the present Chromatographic fraction of the roots of *R. nepalensis* obtained in chloroform solvent system was related to be chrysophanol and emodin.

In comparable to this study, the previous researches isolated emodin and chrysophanol from the ethanol extracts of the roots of *R. nepalensis* (Liang *et al*., 2010).

The TLC profile of second chromatography fraction in ethyl acetate consisted of chrysophanol and 3 undefined compounds. These undefined compounds 1, 2 and 4 had the Rf values of 0.68, 0.71 and 0.76, respectively on TLC in ethyl acetate: methanol solvent system. The undefined compound in acetyl acetate isolate (1, 2 and 4) had the same Rf values as compound 2, 3 and 5 obtained in the chromatographic ethyl acetate: methanol fraction. Therefore it is possible to conclude that the second column Chromatographic fraction in ethyl acetate system and the third column Chromatographic fraction in ethyl acetate: methanol had 3 similar isolates having the same Rf values.



Note: C = Chrysophanols; E= Emodin; EtoAc = Ethyl acetate

Figure 7: TLC profile of chloroform, ethyl acetate and ethyl acetate: methanol column chromatographic fractions of roots of *R. nepalensis*

Since the five undefined compounds were the highest biologically active isolates, these fractions were further re-fractionnated by column chromatography in different solvents as it is presented in Table 4. In this CC refraction technique, about 30 mg (7.5 %), 20 mg (5 %) and 300 mg (75 %) out of 400 mg chromatographic ethyl acetate: methanol fractions was obtained in hexane, chloroform and methanol solvent syststem, respectively. The first two, hexane and chloroform Chromatographicre-fraction were yellow and greasy solid, but the methanol Chromatographi cre-fraction was brown and solid powder. The first hexane re-fraction had 0.79 Rf value on TLC with chloroform as elutant solvent.

On the other hand the second chloroform fraction consisted of compound 1 and 2 with the Rf values of 0.68 and 0.79, respectively as shown in Figure 8. In the third methanol fraction, compound 1, 2, 3 and 4 had Rf values of 0, 0.13, 0.21 and 0.68, respectively in TLC within chloroform solvent as elutant. In this work, compound 1 in hexane chromatogram, compound 2 in chloroform Chromatographic showed similar Rf values. This leads to the conclusion that the two are related comounds. In addition to this, compound 1 in chloroform fraction and compound 4 in methanol chromatographic fraction had similar Rf values.

The brown solid Chromatographic re-fraction was the most biologically active isolates. This activity may due to the presence of active seconday metabolites whcich were confirmed by phytochemical screening technique from methanol extracts of the roots of *R. nepalensis*. The groups of compounds identified are anthraquinone, flavonoids, glycosides, triterpnoids, tannin and steroids. The CC and TLC techniques alone did not help to identify and isolate the 5 unkown compounds. More material was required to go on further isolation technique.

Table 4: The chemical profile of ethyl acetate-methanol Chromatographic of the roots of *R. nepalensis* by column chromatogram

Solvent	Amount (mg)	% yield	Code	Major spot	Rf values	Known compound
n-Hex	30	7.5	98-24C1	1	0.79	Undefined
CHCl3	20	2	98-24C2	2 (1:2)	0.68:0.79	Undefined
EtOAc	-	-	98-24C3	-	-	-
MeOH	300	75	98-24C4	4(1:2:3:4)	0:0.13:0.21:0.68	Undefined

Note: CHCl3 = chloroformm; EtOAc = ethyl acetate; Rf = retention factor; MeOH = methanol

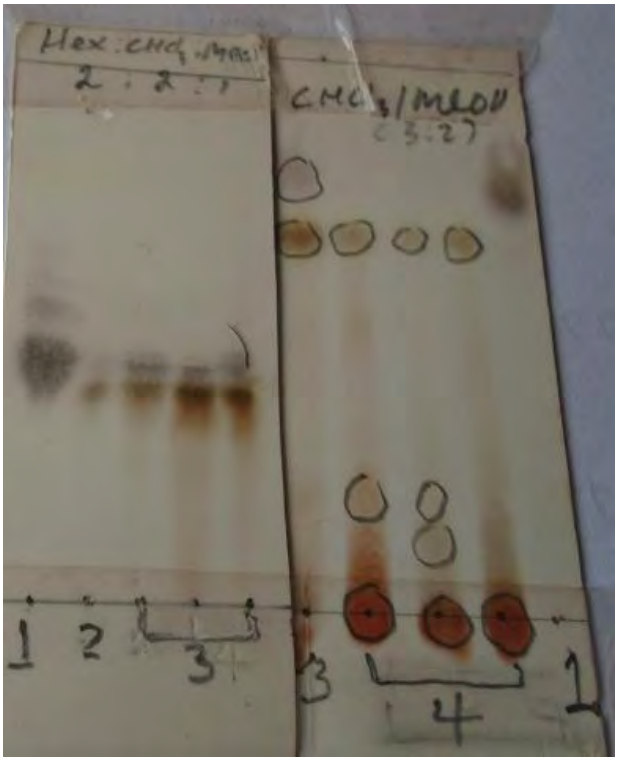


Figure 8: TLC profiles of hexane, chloroform, acetone and methanol CC fractions of roots of *R. nepalensis*

3.3.6 Acute toxicity of methanol and hexane crude extracts *C. asiatica* against body weight

In this study, the mean BW of the mice treated with 5000 mg/kg BW of crude methanol extracts of the aerial parts of C. asiatica at the first day (D0) was 24.88±0.75 gm. The mean BW at D0 significantly increased their BW to 31.76±0.74 gm at D14 as shown in Table 5. The BW of the mice administered with crude methanol extracts of the aerial parts of *C. asiatica* increased by 27.65 % at D14. This increment in the BW of the mice indicates that the methanol extracts of *C. asiatica* were not toxic to albino mice at 5000 mg/kg BW. The effect of 2% DMSO which was used as a solvent to the plant extract was tested for oral acute toxicity. The mean BW of mice treated with 2% DMSO significantly increased their body weight at D14 with 26.48 %. The negative control mice which was administered with water had a significant BW change with 25.8 % at D14 compared to the mean BW of mice at D0

It is known that body weight can be one of the parameter which indicates the extent of oral acute toxicity. Based on the data obtained from the body weight changes in mice from D0 to D14, the BW increment among the methanol extract of *C. asiatica,* 2 % DMSO and water administered groups were not significantly different from each other. From this it is possible to say the methanol extracts of *C. asiatica* and 2 % DMSO had no significant effect on the body weight of mice at D14.

This data revealed that this extract and 2 % DMSO are no toxic at the hifhest oral dose of 5000 mg/kg BW. Similarly the mice treated with the hexane extracts of *C.asaiatica* at 5000 mg/kg BW significantly increased their body weight at D14. As a result the hexane extracts of *C. asiatica* at 5000 mg/kg BW had no effect on the BW of the mice. It is possible to say that the hexane extracts of this plant had toxicity effect on the albino mice at D 14. In agreement with

this study, the previous work revealed that the the acetone leaf extract of *C. asiatica* did not show any change in body weight of extract treated mice at 400 mg/kg BW (Chuhan *et al*., 2012).

Table 5: Acute toxicity of methanol and hexane crude extracts aerialplant parts of *C. asiatica* against body weight at 5000 mg/kg body

Treatment	Solvent	Meam BW (gm) ± SEM			
		D0	D7	D14	% change b/n D0 and D14
C. asiatica	MeOH	24.88±0.75	27.68±0.87$^{\#a}$	30.76±0.74^{*a}	27.65
	n-Hex	26.32±0.49	28.4±0.91$^{\#b}$	32.55±0.89^{*b}	23.67
Negative control	0.2ml H_2O	26.8±0.63	28.02±0.98$^{\#c}$	33.76±0.95^{*c}	25.8
Positive control	0.2 ml 2% DMSO	24.54±1.2	26.84±1$^{\#d}$	31.04±0.99^{*d}	26.48

Note: BW= Body weight; D0= Day 0; D7= day 7; D14= day 14; H_2O = water; MeOH = methanol; n-Hex = normal hexane; SEM= Standard error of the mean; % = percent ^{a} = weight of mice at D0 for MeOH, ^{b} =weight of mice at D0 for n-hex *C. asiatica*, ^{c} = for waterat D0, ^{d} = for DMSO at D0; *=is significantly higher than; $^{\#}$= not is significantly higher than

3.3.7 Acute toxicity of methanol and hexane crude extracts the leaves of *R. nepalensis* against body weight (BW)

The oral acute toxicity of the leaves of *R. nepalensis* was determined in the albino mice before the antimicrobial and antiplasmodial activities of the extracts are evaluated. As a result of this 5000 mg/kg BW of the leaves of *R. nepalensis* which dissolved in 2 % DMSO was orally administered to mice. The 5000 mg/kg body weight of the extracts of the leaves of *R. nepalensis* which is the highest dose used OECD guideline (2001). 0.2 ml of water and 2 % DMSO was given to negative and positive control mice, respectively.

The body weight of the mice which was treated with crude methanol extracts of leaves of *R. nepalensis* significantly increased at D14 with the BW values of 32.14±0.38 mg compared to their BW at D0 (26.17±0.87) as shown in Table 6. The mice which were administered with 0.2 ml of water also significantly increased their BW from D0 (26.8±0.63 mg) to D7 (28.02±0.98 mg) and then to D14 (32.76±0.95 mm).

The percent body weight changes of the mice treated with 5000 mg/kg BW of crude methanol extracts of leaves of *R. nepalensis* was 22.81 % at D14 which is almost similar to the percent body eight changes (22.23 %) of the mice treated with 0.2ml of water. Based the body weight of the mice as a parameter it is possible to say crude methanol extracts of leaves of *R. nepalensis* at 5000 mg/ kg had no effect on the extract treated mice indicating that the extract was not toxic to the mice.

In similar fashion the mice treated with the crude hexane extracts of the leaves of *R. nepalensis* significantly increased their BW at D14 (29.24±1.1) from their initial BW at D0. As it is described in crude methanol extracts of leaves of *R. nepalensis*, the hexane extracts had significant effects on the body weight of the mice as it is compared to the body weight of the mice administered with 0.2ml of water. As a result of this it can be interpreted that the both methanol and hexane extracts of the leaves of *R. nepalensis* were not toxic to the albino mice at the concentration of 5000 mg/ kg BW.

Table 6: Acute toxicity of methanol and hexane crude extracts of the leaves of *R. nepalensis* against body weight at 5000 mg/kg body

Treatment	Solvent	Meam body weight (gm) ± SEM			
		D0	D7	D14	% change b/n D0 and D14
leaves of R.	MeOH	26.17±0.87	28.58±0.58$^{\#a}$	32.14±0.38^{*a}	22.81
nepalensis	n-Hex	22.62±.092	26.5±1$^{\#b}$	29.24±1.1^{*b}	21.62
Negative control	0.2ml H_2O	26.8±0.63	28.02±0.98$^{\#c}$	32.76±0.95^{*c}	22.23
Positive control	2% DMSO	24.54±1.2	26.84±1$^{\#d}$	30.04±0.99^{*d}	22.41

Note: D0= Day before extract administration; D4= fourth day after extract administration; D14= fourteenth day after extract administration ^{a} = weight of mice at D0 for MeOH, ^{b} =weight of mice at D0 for n-hex, ^{c} for water at D0, ^{d} for DMSO at D0; *=is significantly higher than; $^{\#}$= not is significantly higher than

3.3.8 Acute toxicity of methanol and hexane crude extracts the roots of *R. nepalensis* against body weight (BW)

The mice which were treated with both extracts of the roots of *R. nepalensis* had no any clinical sighs of toxicity such hair erection, loss of appetite, restlessness and there were no mortality within 24 hours. In this work the BW of the mice treated with 5000 mg/kg BW of the roots of *R. nepalensis* significantly increased their BW at D14 (30.78±0.5 mg) from their original mean BW of mice at D0 (25.14±0.32 mg). Similarly the BW of mice which were treated with 0.2 ml of water and 2% DMSO significantly increased their BW at D14 compared to their corresponding original BW at D0 as shown in Table7.

Form this it can be concluded that the methanol extracts of the roots of *R. nepalensis* did not affect the BW of the mice at 5000 mg/kg BW within 14 days follow up. Hence this extract was no toxic to albino mice at the concentration of 5000 mg/kg. This was in agreement with Kumar *et al*., (2011) in that this plant had no acute toxixcity at the concentration of 4000 mg/kg on the rat model.

Table 7: Acute toxicity of methanol and hexane crude extracts of the roots of *R. nepalensis* against body weight at 5000 mg/kg body

Treatment	Solvent	Meam body weight (gm) ± SEM		
		D0	D7	D14
Root of *R. nepalensis*	MeOH	25.14±0.32	$28.64\pm0.73^{\#a}$	$30.78\pm0.5^{*a}$
	n-Hex	21.32±0.59	$26.14\pm0.64^{\#b}$	$28.16\pm0.61^{*b}$
Negative control	0.2ml H_2O	26.8±0.63	$28.02\pm0.98^{\#c}$	$32.76\pm0.95^{*c}$
Positive control	0.2 ml 2 % DMSO	24.54±1.2	$26.84\pm1^{\#d}$	$31.04\pm0.99^{*d}$

Note: BW= Body weight; D0= Day 0; D7= day 7; D14= day 14; H_2O = water; MeOH = methanol; n-Hex = normal hexane; SEM= Standard error of the mean; % = percentNote: BW= body weight of mice

3.4 Conclusion

The phytovhemical screening method revealed the presence of the major secondary metabolites in the two medicinal plants (*R. nepalensia* and *C. asiatica*) including alkaloids, anthraquinone, flavonoids, glycosides, triterpnoids and saponins. In addition to this, *R. nepalensis* contained tannin. The above plant derived natural products could be the major sources of modern medicine. Since medicinal plants are not only medically useful, their harmful effects should also be considered.

In this work there were no observable clinical signs in crude methanol and hexane extracts of the root, the leaf extracts of *R. nepalensis* and the aerial parts of *C. asiatica* treated mice compared to the negative control, water and 2% DMSO. The result sowed that the crude methanol and hexane extracts of the root, the leaf extracts of *R. nepalensis* and the aerial parts of *C. asiatica* did not affect body weight the mice at the highest concentration of the extracts (5000 mg/kg BW).

CHAPTER 4: THE ANTIPLAMODIAL ACTIVITIES OF THE EXTRACTS OF *R. NEPALENSIS* AND *C. ASIATICA* IN SWISS ALBINO MICE

4.1 Introduction

Malaria is caused by *Plasmodium* species (*P. falciparum, P. vivax, P. ovale* and *P. malariae)* and transmitted to people through the bites of infected female anopheles mosquitoes. Malaria remains the world public health problem and 90% of malaria deaths were reported in Africa (WHO, 2014). *P. falciparum* alone causes more than 90% of the world's malaria mortality, particularly Sub-Saharan Africa countries are severely affected (Snow, 2015).

In Ethiopia, malaria transmission mainly occur bellow 2000 meter and malaria is among the ten causes of death which accounts 2% of the total deaths in Ethiopia (MOH, 2016). *Falciparum* malaria and *P.vivax* account about 67% and 32 % of malarial cases bellow 2000 meter above sea level respectively in Ethiopia (CDC, 2016). Although both malarial cases and the number of death declined in the last consecutive years (WHO, 2015), the progress is threatened by the development of insecticides resistannt mosquitoes, drug resistannt malarial parasites and lack of successful and effective approved malaria vaccine. As a result the use of medicinal plants in treating various infectious diseases like malaria can be one of the alternatives means in drug discovery.

Folk medicine (traditional medicine) is traditional based custom and belief which include knowledge acquired through indigenous methods for treatment of various diseases (Yadav, 2013). Medicinal plants are the major sources of traditional medicine and applied to treat diseases and prevent human well beings (WHO, 2008).

In Ethiopia, about 90% of the population uses traditional medicine to treat various diseases (WHO, 2005). However traditional medicine practitioners use them without scientific dose prescription and without considering toxological studies of medicinal plants. As a result the herbal medicine can have negative side effects in body organs of the traditional medicine users. Furthermore traditional knowledge is transferred from generation to generation orally.

Therefore different ethnobotanical and pharmacological studies have been conducted on medicinal plants. Ethnobotanical studies showed that the apex part of *Croton macrostachyus* and the root and leaves of *Vernonia amygdalina* mixed with water are applied to treat malaria in Dega Damot District, Amhara Region, Ethiopia (Limenih *et al*., 2015). Similarly the methanol and chloroform of crude extracts of *B. antidesenterica* showed 47.70 % and 46.44 % day four antiplasmodial suppression respectively, at 600 mg/kg BW *in vivo* in Swiss Albino mice (Kefe *et al*., 2015).

In other related studies, the crude methanol extract of *Clerodendrum myricoides, Dodonea angustifolia* and *Aloe debrana* showed 82.5, 84.52 and 73.95 percent suppression respectively in mice showed 82.5, 84.52 and 73.95 percent suppression respectively in mice (Deressa *et al*., 2010). The 80 % crude methanolic extract of the seeds of *Brassica nigra* had 53.13 % chemosuppressive of malaria parasite *in vivo* in mice at 400 mg/kg BW (Muluye *et al*., 2015). The above studies mostly evaluate the antiplasmodial activities of the crude extracts of the plant parts *in vivo* in Swiss Albino mice.

R. nepalensis and *C. asiatica* are among medicinal plants used by herbalist to treat various diseases in Ethiopia (chapter 3). Phytochemical screening tests revealed the presence of anthraquinone, steroids, saponins and tannins in the methanol extracts of the *R. nepalensis* (Ghosh *et al*., 2002, Sahreen *et al*., 2015). Similarly qualitative phytochemical analysis indicated the presence phytochemicals including alkaloids, flavonoids, tannins, terpenoids, saponin, steroids, carbohydrates and cardiac glycosides in aqueous extracts of *C. asiatica* (Madhusudhan *et al*., 2014).

Triterpnoids, anthraquinone, tannin and saponin are kown to have antiplasmodial activities (Ramalhete *et al.,* 2014, Osman *et al.,* 2010, Traore *et al.,* 2000). Thus, this study aimed at evaluating the *in vivo* antiplamodial activities of the crude extracts of leaves and the solvent fractionation and CC isolatess of roots of *R. nepalensis* and the crude extracts of aerial part of *C. asiatica.*

4.2 Materials and methods

4.2.1 Animal model

In this study, Swiss albino mice that had been obtained from Ethiopian Public Health Institute (EPHI) were reared in the Animal House of the Department of Biology for *in vivo* anti plasmodial test.

4.2.2 Plasmodium pathogen

Chloroquine sensitive *Plasmodium berghei* strain which was obtained from EPHI was used to infect the mice. This parasite was subsequently maintained in the laboratory by serial blood passage from mouse to mouse.

4.2.3 Infection of experimental mice with *P. berghei*

For antimalarial test, female mice with age of 6-8 weeks and weight (22-29g) were selected and grouped into 5 (each group has 5 mice). The test mice were acclimatized for two weeks and randomly placed into five groups (OECD, 2001). The mice were infected intraperitoneally with 0.2 ml diluted blood containing 1×10^7 infected erythrocytes from a donor mouse with the parasitemia of 30% to 40% using standared methods (Builders *et al*., 2011).

4.2.4 Extract and drug administration to mice

Three hours after parasite inoculation, the crude extracts orall were administered with 200, 400 and 500 mg/kg body weight (one tenth of its toxicity dose) crude extracts by dissolving the extracts in 0.2ml of 2% DMSO for each mouse orally by using gavage (an oral needle) for four consecutive days starting from day 0 to day 4 in a 24 hr interval (Deressa *et al*., 2010**)**. The fractionated and sub-fractionnated extracts were administered with appropriate doses according to their percent yield obtained from their corresponding crude extracts. The positive controls were treated daily with 5 mg/kg body weight chloroquine while the negative control groups were treated with 0.2ml 2% DMSO.

4.2.5 Determination of parasitemia and percent suppression

Antiplasmodial test was undertaken using a 4- day suppressive standard test as described in David *et al*., (2004). Accordingly, the fresh blood samples were collected from the tail snip of each mouse and thin smears were prepared at day 4 (D4) as described Krettli *et al*., (2007). Blood films were first fixed with methanol for 30 seconds and stained with 10% Giemsa for 10 minutes to exmine parasitaemia under light microscopy fitted with counting chamber under oil immersion objective of 100x magnification power (WHO, 1991). Then, percent parasitaemia and

suppression were calculated for each dose level of treatments by comparing the parasitaemia against infected negative control (no extract treatment) according to the following formula (Samanta *et al*., 2011)

$$\% \text{ Parasetemia} = \frac{\text{Number of infected RBCs}}{\text{Number of infected RBCs + Number ofun infected RBCs}} \times 100$$

$$\% \text{ Suppression} = \frac{\text{No of parasites in negative control- no of parasites in treated groups}}{\text{No of parasite in negative control}} \times 100$$

4.2.6 BW and PCV determination

Each test and control mouse was measured with digital weighing balance (AND: FX-320, Japan) at D0 and D4. Packed cell volume was determined by using Wintrobe's method (Gilmour and Sykes, 1951). Accordingly half filled fresh blood was taken from the tail snip of each mouse in heparinized microhaematocrit tube and sealed with sealing clay. Then the tubes were centrifuged by microhaematocrit centrifuge (HAWKSLEY1500, England) at 12,000 rpm for 4 minutes. Finally, the result was compared with control groups. The mean Body weight and packed cell volume were calculated as described in Bull *et al.,* (2000).

$$\text{Mean body weight} = \frac{\text{Total weight of mice in a group}}{\text{Total number of mice in that group}} \times 100$$

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

4. 3 Results and discussion

4.3.1 Determination of parasitemia and percent suppression of methanol, water and hexane extract of the roots, leves of *R. nepalensis* and aerial part of *C. asiatica*

The data on mean parasitemia, percent suppression and survival time of the crude hexane extract treated mice are shown in Table 8. Accordingly, in the 4-day suppressive test, the mean parasitaemia level which is less than or equal to 90 % of the negative control animals mostly indicated that the test extract is active to Plasmodium in standard screening studies (Peter and Anatoli., 1998). Based on this, the hexane extracts of *C. asiatica* at 200 mg/kg body weight which had the highest mean parasetemia level (34.86±0.56) in mice is 80.86 % of the negative control, 2% DMSO which is less than 90 % of the negative control. Accordingly all extracts treated against malarial activities in this work are active against Plasmodium *in vivo* in mice.

The crude hexane extracts of *R. nepalensis* root supressed *P. berghei* by (39.3%) at 500 mg/kg body weight and followed by leaves of *R. nepalensis* by 35.4 % inhibition. The lowest suppression recorded for hexane crude extract of *C. asiatica* with (19.05%) percent suppression of *P. berghei* at 200 mg/kg body weight compared to 100 percent suppression of chloroquine indicating that the 2.5-5 times more potent in parasite suppression than the crude extracts as it is shown in Table 8. Furthermore the mean survival time (14.2±0.73) of chloroquine treated groups (Table 8) were significantly higher than of all the groups whereas, the DMSO treated had significantly the lower the mean survival time than hexan extracts treated mice.

Table 8: Parasitemia, percent suppression and mean survival time of mice treated with crude hexane extract of the root, leaf *R. nepalensis* and the aerial parts of *C. asiatica* under 4 day ssupression

Extract	Dose (mg/kg)	Mean parasitemia ±SEM	Percent suppression	Mean Survival time (day)±SEM
	200	^{a*}25.5±1.74 $^{\#b}$	27.97	^{b*}8.8±0.34$^{\#a}$
R. nepalensis root	400	^{a*}24. ±1.49$^{\#b}$	32.2	^{b*}9.4±0.4$^{\#a}$
	500	^{a*}21.5±1.42$^{\#b}$	39.3	^{b*}10±0.31$^{\#a}$
R. nepalensis leaf	200	^{a*}24.56±1.24$^{\#b}$	29.67	^{b*}8.6±0.4$^{\#a}$
	400	^{a*}24.91±2.02$^{\#b}$	30.61	^{b*}9±0,44$^{\#a}$
	500	^{a*}23.2±0.92$^{\#b}$	35.4	^{b*}9.4±0.51$^{\#a}$
C. asiatica aerial	200	34.86±0.56	19.04	7.55±0.47$^{\#a}$
part	400	32.43±0.47	24.68	^{b*}8.8±0.59$^{\#a}$
	500	30.38±0.64	29.5	^{b*}9.4±0.6$^{\#a}$
2% DMSO	0.02 ml	43.06±0.89	00	6.2±0.37$^{\#a}$
Chloroquine	5 mg/kg	00	100	14.2±0.73

Note: = ^{a} Chloroquine; ^{b}=2%DMSO; H_2O = water; MeOH = methanol; n-Hex. = normal hexane
*= significantly higher than; $^{\#}$= significantly lower than

Similarly the crude methanol plant extracts of *R. nepalensis* and *C. asiatica* showed varation in the parasite suppressions of the mice in (Table 9). Accordingly, the highest and lowest percent parasite suppressions were recoreded from the extract figures 59.90 % and 29.89 % from root extracts of *R. nepalensis* and *C. asiatica* at the concentration of 500 mg/kg body weight and 200 mg/kg body weight, respectively. The previous studies in Ethipia revealed that the crude

methanol extract of leaves of *Withania somnifera* showed percent parasite suppressions (53.34 %) at the concentration of 900 mg/kg BW (Teklemariam *et al*., 2013). In the other related studies the methanol extract of the leaves of *Buddleja polystachya* displayed 66.67 % parasite suppression at 400 mg/kg and this percent suppression increase as concentration increaesaed (Mohammed *et al*., 2016).

On the contrary Umar *et al* (2012) reported 88.5 % suppressions of the parasite in mice from crude of methanol of root extract of *Morinda lucida* at 400 mg/kg BW. The mean survival time (10±0.31 days) of mice treated with crude of methanol of root of *R. nepalensis* extract this study was was lower than the mean survival time (29.25 days) of mice treated with crude of methanol of root extract of *Morinda lucida* at 400 mg/kg BW (Umar *et al*; 2012).

The crude methanol extract of the root of *Clerodendrum myricoides, Dodonia angustifolia and Aloe debrana in* Ethiopia showed higher parasite suppression (82.50%, 84.52% and 73.95%) suppression, respectively at 600 mg/kg (Deressa *et al.,* 2010*)* compared to the present sutudy. Similarly the data in this study showed that the crude methanol extracts of aerial part of *C. asiatica* displayed parasite suppression (40.8 %) at 500 mg/kg (Table 9), however the ethanol extracts of this plant displayed comparatively higher parasite suppression (44.80 %) at 500mg/kg (Banyal *et al*., 2014).

Table 9: Parasitemia, percent suppression and mean survival time of mice treated with crude methanol extract of the root, leaf of *R. nepalensis* and the aerial parts of *C. asiatica*

Extract	Dose (mg/kg)	Mean parasitemia ±SEM	Percent suppression	Mean Survival time (day)±SEM
R. nepalensis root	200	$^{b\#}$20.82±0.46 * ^{a}	49.09	^{b*} 9.4±0.6$^{\#a}$
	400	$^{b\#}$19.4±0.50* ^{a}	52.54	^{b*}10.2±0.37$^{\#a}$
	500	$^{b\#}$16.38±0.67* ^{a}	59.90	^{b*}10.8±0.37^{a}
	200	$^{b\#}$24.56±1.2* ^{a}	39.92	^{b*}8.40±0.25$^{\#a}$
R. nepalensis leaf	400	$^{b\#}$24.2±0.91* ^{a}	40.8	^{b*}9.60±0.81$^{\#a}$
	500	$^{b\#}$23.19±0.48* ^{a}	43.27	^{b*}10.4±0.60$^{\#a}$
C. asiatica aerial	200	$^{b\#}$28.66±0.53* ^{a}	29.89	7.6±0.0.55$^{\#a}$
part	400	$^{b\#}$26.66±0.47* ^{a}	34.78	^{b*}8.48.9±0.24$^{\#a}$
	500	$^{b\#}$24.2±0.77* ^{a}	40.8	^{b*}8.88.9±0.20$^{\#a}$
2% DMSO	0.2ml	40.88±0.86* ^{a}	0	6.60±0.24$^{\#a}$
Chloroquine	5 mg/kg	0	100	15.88.9±0.20

Note: ^{a} = for Chloroquine administered mice; ^{b} = for 2%DMSO administered mice; *= significantly higher than; $^{\#}$= significantly lower than

From the above data (Table 9), the percent suppression of *P. berghei* in mice increases as the concentration of methanol extracts of both plants increases (from 200 body weight to 500 500 mg/kg body weight). Thus the crude methanol extracts of the roots *R. nepalensis* performed the best percent suppression of *P. berghei* 59.9 (%) *in vivo* in mice at 500 mg/kg body weight compared to the water suppressions (52.00 %) and hexane suppressions (40.8 %) at the same concentration.

Furthermore, the mice which were treated with 200 mg/kg body weight of *C. asiatica* had significantly lower mean survival time than those groups which were treated with 500 mg/kg extracts of roots, leaves of *R. nepalensis* and aerial parts of *C. asiatica*. In agreement with Adinew (2014), the mean survival time of increaseed as the concentration increases from 200 mg/kg body weight to 400 mg/kg body weight and then to 500 mg/kg body weight.

The mean parasitemia and mean survival time of the crude water extracts of of the two plants is shown in Table 10. Accordingly the data showed that the water extracts of the roots of *R. nepalensis* imposed the highest percent suppression (52.00 %) at 500 mg/kg body weight whereas the lowest was recorded from *C. asiatica* (23.08 %) at 200 mg/ml body weight. This showed that the roots extracts of *R. nepalensis* was significantly higher parasite suppression than the extracts from *C.asiatica,* but it showed significantly lower parasite suppression than the standared drug.

Table 10: Parasitemia, percent suppression and mean survival time of crude water extract of the root, leaf of *R. nepalensis* and the aerial parts of *C. asiatica*

Extract	Dose (mg/kg)	Mean parasitemia ±SEM	Percent suppression	Mean Survival time (day) ±SEM
R. nepalensis root	200	$^{b\,\#}22.5\pm0.72^{*\,a}$	46.49	$^{a\#}11.00\pm0.31^{*b}$
	400	$^{b\,\#}20.52\pm0.28^{*\,a}$	51.2	$^{a\#}11.00\pm0.31^{*b}$
	500	$^{b\,\#}20.18\pm0.48^{*\,a}$	52.00	$^{a\#}11.20\pm0.31^{*b}$
R. nepalensis leaf	200	$^{b\,\#}32.02\pm0.33^{*\,a}$	23.87	$^{a\#}9.80\pm0.58^{*b}$
	400	$^{b\,\#}29.74\pm0.37^{*\,a}$	28.74	$^{a\#}10.40\pm0.68^{*b}$
	500	$^{b\,\#}28.14\pm0.27^{*\,a}$	31.8	$^{a\#}10.40\pm0.60^{*b}$
C. asiatica	200	$^{b\,\#}32.8\pm0.34^{*\,a}$	23.08	$^{a\#}7.2\pm0.37^{*b}$
	400	$^{b\,\#}30.28\pm0.31^{*\,a}$	29.28	$^{a\#}8.40\pm0.51$
	500	$^{b\,\#}27.94\pm0.44^{*\,a}$	33.56	$^{a\#}9.00\pm0.45^{*b}$
2% DMSO	0.2ml	$42.05\pm0.89^{*\,a}$	0	$^{a\#}6.00\pm0.37$
Chloroquine	5 mg/kg	0	100	14.80±0.20

Note: ^{a} = for Chloroquine administered mice; ^{b} = 2%DMSO administered mice; * = significantly higher than; $^{\#}$= significantly lower than

4.3.2 BW and PCV determination of methanol, water and hexane extract *R. nepalensis* and *C. asiatica*

The body weight (BW) and packed cell volume (PCV) of etracts of the roots of *R. nepalensis* and control mice were measured at day 0 and day 4 Table (Table 11). The BW and PCV of *P. bergei* infected mice with crude water, methanol and hexane extracts of the roots of *R. nepalensis* reduced at D4, but the methanol extracts of the roots of *R. nepalensis* prevents the loss of BW with reduction changes (-0.59 %) compared to methanol extracts of leaves of *R. nepalensis* with

reduction changes (-2.95 %) and *C. asiatica* with reduction changes (-3.82 %) at the concentration of 500mg/ kg BW.

Similarly the methanol extracts of roots of *R. nepalensis* prevented the loss of the PCV with reduction changes (0.47%) of the infected mice better than the PCV of mice treated with the methanol extracts of the aerial parts of *C. asiatica* with reduction changes (-2.68 %) and the leaves of *R. nepalensis* with reduction changes (-2.22 %) at the concentration of 500mg/ kg BW at D4. However the previous studies showed that *P. bergei* infected rodents lost their PCV from 43 % to 44 % within 48 hours post-infection (Taylor and Hurd, 2001). But in this study, the mice treated with negative control, 2 % DMSO, reduced their PCV significantly by 10.59 % at D4.

Table 11: Packed cell volume and the body weight of infected mice treated with crude extracts of the roots of *R. nepalensis*

Treatment	Parameter	Solvent	Dose mg/kg	D0	D4	% change
			200	50.8±0.37	49.67±0.34	-2.22
		H_2O	400	50.8±0.40	50.25±0.25	-1.08
	PCV		500	49.3±0.96	48.83±1.6	-0.95
			200	50.1±0.49	48.65±0.69	-2.89
		MeOH	400	50.16±0.60	49.23±0.5	-1.85
			500	48.66±0.48	48.38±0.66	-0.47
			200	50.94±0.22	49.58±0.36	-2.67
***R. nepalensis* roots**		n-Hex	400	50.80±0.37	49.6±0.53	-2.36
			500	50.56±0.42	49.41±1.2	-2.27
			200	27.78±048	26.56±0.34	-4.39
		H_2O	400	27.16±0.64	26.56±0.73	-2.19
			500	26.80±0.67	26.4±0.67	-1.4
	BW		200	27.96±0.41	27.26±0.6	-2.5
		MeOH	400	27.74±0.26	27.48±0.41	-0.934
			500	26.68±0.72	26.52±0.72	-0.59
			200	26.4±0.45	25.43±0.64	-3.37
		n-Hex	400	25.8±0.83	24.96±0.78	-3.25
			500	25.2±0.5	24.86±0.64	-1.35
2%DMSO	PCV		0.2ml	51.16±0.22	$45.42\pm0.5^{\#a}$	-11.22
	BW		0.2ml	26.08±0.32	$22.6\pm0.25^{\#b}$	-13.34
Chloroqune	PCV		5 mg/kg	50.08±0.35	49.8±0.48	-0.5
	BW		5 mg/kg	27.88±0.86	28.1±0.75	0.79

Note: D0 = day 0; D4 = day 4; P H_2O = water; MeOH = methanol; n-Hex. = normal Hexane CV= Packed cell volume; [a]= PCV at day 0; [b] = body weight at day 0; [#]= significantly higher lower; - = reduction; + = increment

The BW and PCV of *P. bergei* infected mice treated with water, methanol and hexane extracts of the leaves of *R. nepalensis* was shown in Table12. As it is described above, the BW and PCV of infected mice treated with extracts of the leaves of *R. nepalensis* decreased at D4 according to their concentrations, but change of in BW and PCV of the infected mice at D0 and D4 were not significant. In the contrary, the BW and PCV of infected mice treated with distilled water significantly reduced by 10.35 % and 12.34 %, respectively. The data in this study showed that

all extracts of leaves of *R. nepalensis* prevent the loss of BW and PCV of *P. bergei* infected mice which was comparable to the former studies conducted on methanol extract of the leaves *Strychnos mitis* in *P. berghei* infected mice (Fentahun *et al*., 2017).

Table 12: Packed cell volume and the body weight of infected mice treated with crude extracts of the leaves of *R. nepalensis*

Treatment	Parameter	Solvent	Dose mg/kg	D0	D4	% change
			200	50.5±0.8	48.65±0.47	-3.66
		H_2O	400	50.5±0.44	48.75±0.48	-3.46
	PCV		500	50.92±0.49	48.25±0.24	-2.07
			200	50.14±0.61	48.65±0.7	-2.97
R. nepalensis		MeOH	400	50.23±0.29	48.98±0.5	-2.48
Leaves			500	49.56±0.34	48.47±0.67	-2.22
			200	50.5±0.81	48.18±0.59	-4.59
		n-Hex	400	50.66±0.43	48.56±0.42	-4.14
			500	50.86±0.49	48.62±0.54	4.40
			200	27.04±0.62	25.26±0.6	-6.58
		H_2O	400	25.62±0.47	24.84±0.41	-3.04
			500	28.28±0.66	27.62±0.72	-2.33
	BW		200	25.86±0.77	25.04±0.77	-3.17
		MeOH	400	25.68±0.53	25.24±0.57	1.71
			500	26.8±0.66	26.54±0.57	-0.97
			200	26.45±0.33	25.44±0.23	-3.82
		n-Hex	400	25.86±0.89	24.93±0.66	-3.59
			500	26.02±0.45	25.25±0.37	-2.95
	PCV		0.2ml	50.42±0.31	$45.20.37^{\#a}$	-10.35
2%DMSO	BW		0.2ml	25.28±0.36	$22.16\pm0.51^{\#b}$	-12.34
	PCV		5 mg/kg	49.92±0.55	49.86±0.53	-0.12
Chloroqune	BW		5 mg/kg	2726.41±0.82	26.76±0.	0.35

Note: D0 = day 0; D4 = day 4; P H_2O = water; MeOH = methanol; n-Hex. = normal Hexane CV= Packed cell volume; [a]= PCV at day 0; [b] = body weight at day 0; [#]= significantly higher lower; - = reduction; + = increment

The PCV of infected mice treated with extracts of *C. asiatica* reduced from day 0 to day 4 in a dose-dependent manner for all of the three extracts at the three doses as shown in Table 13. The crude water, methanol and hexane extracts of *C. asiatica* significantly reduced the loss of BW and the PCV of mice at extract concentration of 200, 400 and 500 mg/kg body weight at day 4 compared to negative control (2% DMSO), but the changes were not significant. In contrast to this the PCV of 2 % DMSO treated groups significantly declined from D0 to D4. In this work, both BW and PCV of mice were reduced after the administration of extracts in the fifth day (D4), but slight body weight increment in chloroquine treated mice.

Table 13: Packed cell volume and the body weight of infected mice treated with crude extracts of *C. asiatica*

Treatment	Parameter	Solvent	Dose mg/kg	D0	D4	% change
			200	50.4±0.89	48.2±0.58	-4.37
		H_2O	400	50.43±0.39	48.7±0.47	-3.43
	PCV		500	50.13±1.4	48.76±0.22	-2.73
			200	50.00±1.1	48.12±0.43	-3.76
		MeOH	400	50.15±0.33	49.42±0.28	-1.56
			500	50.56±0.63	49.71±0.11	-2.68
		n-Hex	200	49.56±0.33	48.2±0.27	-2.74
C. asiatica			400	50.45±0.27	48.8±0.15	-3.27
			500	49.68±0.87	48.01±0.34	-3.36
			200	26.3±0.58	25.98±0.49	-1.22
		H_2O	400	28.84±0.46	27.8±0.46	-3.61
			500	27.26±0.37	26.5±0.41	-2.79
	BW		200	27.5±0.71	26.42±0.66	-2.93
		MeOH	400	28.22±0.61	27.16±0.65	-3.76
			500	25.68±0.48	24.7±0.35	-3.82
			200	26.4±0.98	25.17±1.3	-4.65
		n-Hex	400	25.43±1.2	24.2±1	-4.84
			500	25.8±0.99	24.4±0.89	-5.43
2%DMSO	PCV		0.2ml	50.8±0.37	$45.42\pm0.50^{\#a}$	-10.59
	BW		0.2ml	27.36±0.79	$23.8\pm0.78^{\#b}$	-13.36
Chloroqune	PCV		5 mg/kg	50.92±0.41	50.7±0.8	-0.62
	BW		5 mg/kg	27.88±0.86	28.29±0.75	1.47

Note: D0 = day 0; D4 = day 4; H_2O = water; MeOH = methanol; n-Hex. = normal Hexane PCV= Packed cell volume; [a] = PCV at day 0; [b] = body weight at day 0; [#]= significantly higher lower; - = reduction; + = increment

4.3.3 Determination of parasitemia and percent suppression of ethanol, water, chloroform and methanol fractions of the roots of *R. nepalensis*

In this study, the crude methanol extracts of the roots of *R. nepalensis* performed the best antimalarial activity at 500 mg/kg BW of all crude extracts based on a 4- day malaria suppression test. As a result the crude methanol extracts of the roots of *R. nepalensis* were further fractionnated by with different solvents.

The data showed that the mean parasitemia (12.2±1.5) of mice treated with ethanol fraction was significantly lower than the mean parasitemia of of mice treated with water extracts (19.96±2.3), chloroform (35.12±0.43) and methanol (39.2±0.58) fractions at 500 mg/kg BW (Table 14). But it is significantly lower than chloroquine treated mice.

The ethanol fraction of the roots of *R. nepalensis* also showed better plasmodium percent suppression *in vivo* in albino mice (70.08 %) compared to water (54.31 %), chloroform (19.61 %) and methanol (10.27 %) suppression at 500 mg/kg BW as shown in Table 14. In agreement to to thiswork, the previous studies showed comparatively higher parasite suppression (77.24 %) from the methanol fractions of leaves of *Clerodendrum myricoides* and followed by hexane fractions with parasite suppression of (17.45 %) in vivo in mice (Gebretsadik and Mekonnen, 2016).

The mean survival time of the ethanol, water, chloroform, methanol fraction at 500 mg/kg BW, 2 % DMSO and chloroquine at 5 mg/kg BW were 11.4±0.25, 8.6±0.25, 6.4±0.25, 6.4±0.39, 6.6±0.24 and 15.2±0.38, respectively as it is presented in Table 14. The mean survival time value of the mice administered with ethanol fraction (11.40±0.25) was significantly higher than water,

chloroform and methanol at 500 mg/kg BW at 500 mg/kg BW, but this value was significantly lower than chloroquine treated mice at 5 mg/kg BW. In agreement with Asnake *et al*., (2015) the mean survival time of the ethanol and water fractions treated mice in this work increased with concentration as shwn in Table 14.

Table 14: The parasitemia, percent suppression and mean survival time of ethanol, water, chloroform and methanol fraction of the root of *R. nepalensis*

Solvent	Dose (mg/kg)	Mean parasitemia ±SEM	Percent suppression	Mean Survival time (day)±SEM
EtOH	400	$18.37\pm0.72^{*\,d}$	57.95	$^{b*}9.2\pm0.58^{\#a}$
	500	12.2±1.5	70.08	$^{b*}11.4\pm0.25^{\#a}$
H_2O	400	$20.83\pm0.89^{*\,d}$	52.32	$^{b*}8.2\pm0.37^{\#a}$
	500	$19.96\pm2.3^{*\,d}$	54.31	$^{b*}8.6\pm0.25^{\#a}$
CHCl3	400	$35.00\pm1.61^{*\,d}$	19.89	$6.4\pm0.25^{\#a}$
	500	$35.12\pm0.43^{*\,d}$	19.61	$6.4\pm0.25^{\#a}$
MeOH	400	$40.70\pm0.70^{*\,d}$	6.84	$6.6\pm0.24^{\#a}$
	500	$39.2\pm0.58^{*\,d}$	10.27	$6.4\pm0.39^{\#a}$
DMSO	0.2ml	$43.69\pm2.42^{*\,d}$	0	$6.6\pm0.24^{\#a}$
	5 mg/kg	0	100	$^{b*}15.2\pm0.38$

Note: a = mean survival value for chloroquine; b = mean survival value for 2% DMSO; ^{c} = parasitemia for DMSO; ^{d} = parasitemia for EtOH fraction at 500 mg/kg; $CHCl_3$ = chloroform; DMSO = dimethylsulfoxide H_2O = water; EtOH = ethanol MeOH = methanol; SEM = Standard error of the mean; * = significantly higher than $^{\#}$ = significantly lower than

4.3.4 The body weight and packed cell volume of infected mice treated ethanol, water, chloroform and methanol fraction of the root *R. nepalensis*

The body weight (BW) and packed cell volume (PCV) of the mice treated with different crude fraction were evaluated *in vivo* in mice as shown in Table 15. The crude fractions reduced the BW and PCV of the treated mice at fifth day post infection except the BW of mice that were treated with chloroquine which had a 2.11% BW increment at day 4 (fifth day). The infected mice mice treated with ethanol fractions at 500 mg/kg BW with BW change (-0.14 %) significantly prevented the reduction of BW compared to the BW of mice treated with 2% DMSO (-16.71), methanol (-8.56) and chloroform (-8.67) extract fractions.

In agreement with previous studies (Mekonnen *et al*., 2015) this study showed that the ethanol fraction at 400 and water fraction at 400 and 500 mg/kg BW significantly prevented the loss of BW at day 4 and showed increment dose dependently. The ethanol fractions treatments at 500 mg/kg BW also significantly prevented the reduction of PCV with PCV changes (0.12%). In general, the ethanol fraction at 500 mg/kg BW showed better BW and PCV reduction performance *in vivo* in *P. berghei* infected mice at day 4 post infections. As a result this fraction may contain potentially active antimalaria compounds. Thus this fraction was further re-fractionnated by column chromatography.

Table 15: Packed cell volume and body weight determination of ethanol, water, and chloroform and methanol fraction of the roots of *R. nepalensis*

Parameter	Solvent	Dose mg/kg	Mean value±SEM		
			D0	D4	% change
PCV	EtOH	400	49.64±0.68	49.44±0.41	-0.40
		500	49.71±0.34	49.77±0.23	0.12
	H_2O	400	48.22±0.77	48.62±0.88	-0.82
		500	49.63±0.28	49.62±0.26	-0.0002
	CHCl3	400	49.76±0.37	46.6±0.25$^{\#b}$	-6.35
		500	50.1±1.5	47.00±0.44$^{\#b}$	-6.18
	MeOH	400	50.12±0.39	47.2±0.37$^{\#b}$	-5.83
		500	50.32±0.67	47.4±0.51$^{\#b}$	-5.80
	2 % DMSO	0.2ml	50.24±0.45	45.7±0.49$^{\#b}$	-9.03
	Chloroquine	25 mg/kg	49.99±0.45	49.92±0.41	-0.014
	EtOH	400	27.0±1.3	26.14±1.12	-0.86
		500	28.5±1.2	28.46±1.1	-0.14
	H_2O	400	23.08±0.33	21.66±0.5	-6.15
		500	24.18±0.89	24.08±1.01	-0.41
	CHCl3	400	25.58±0.4.9	22.9±0.67$^{\#a}$	-10.47
		500	25.62±0.43	23.4±0.68$^{\#a}$	-8.67
	MeOH	400	25.46±0.5	23.28±0.42$^{\#a}$	-8.56
		500	26.12±0.63	24.12±0.58$^{\#a}$	-7.66
	2 % DMSO	0.2 ml	25.37±0.74	21.13±0.59$^{\#a}$	-16.71
	Chloroquine	5 mg/kg	23.56±0.63	24.06±0.66	2.11

Note: CHCl3= chloroform; D0 = day 0; D4 = day 4; EtOH = ethanol, H_2O = water; MeOH = methanol; PCV= Packed cell volume; SEM = Standared error of the mean; ^{a}= PCV at day 0; ^{b} = body weight at day 0; $^{\#}$= significantly lower than; - = reduction; + = increment

4.3.5 Determination of parasitemia and percent suppression of column Chromatographic fractions

The ethanol fraction of the roots of *R. nepalensis* which had higher parasite suppression, BW and PCV loss reduction was further isolated by colomun chromatography with three solvent systems (Chloroform, ethyl acetate and ethyl acetate: methanol). The five undefined compounds which were fractionnated by column chromatography in ethyl acetate: methanol (50 to 50 v/v) showed 73.66% *Plasmodium* suppression (Table 16).

This suppression was higher than of the parasite suppression (28.03 %) of CC buthanol fraction of the leaves *Clerodendrum myricoides* treated *mice* at 100 mg/kg BW (Gebretsadik and Mekonnen, 2016). Whereas chrysophanol and emodin displayed 21.42 % suppression and chrysophenol and 3 undefined compounds with percent suppression of (45.75 %) at 100 and 40 mg/kg BW respectively as it is shown in Table 15.

Similarly the mean survival time (11.5±0.35) of infected mice treated with 300 mg/kg BW ethyl acetate: methanol Chromatographic fraction was significantly higher than the mean survival time of infected mice treated with 40 mg/kg BW chloroform fractions (7.2±0.62), 100 mg/kg BW ethyl acetate fractions (8.6±0.88) and 2% DMSO (6.5±0.75). But the mean survival time (11.5±0.35) of 300 mg/kg BW ethyl acetate: methanol Chromatographic fraction treated mice was significantly lower than 5 mg/kg BW chloroquine (15.68±0.45) treated mice.

Table 16: Parasitemia, percent suppression, mean survival time PCV and BW of Column Chromatographic fractions of the roots of *R. nepalensis*

Solvent fraction/ dose mg/kg	Mean parasitemia ±SEM	Percent suppression	Mean Survival time (day)±SEM	PCV			BW		
				D0	D4	% change	D0	D4	% change
CHCl3/40	38.75±0.9 *c	21.42	7.2±0.62	50.4±1.02	47.06±2.2	-6.62	27.04±1.56	25.9.04±1.5	-4.21
EtOAc/100	26.25±2.3 *c	45.75	8.6±0.88	48.78±0.57	47.62±0.59	-4.42	29.54±1.01	29.16±1.6	-1.29
EtOAc-MeOH/300	12.99±2.5	73.66	a# 11.5 *b	50.06±0.88	49.86±1.2	-0.39	27.44±1.3	27.36±0.59	-0.91
2%DMSO	49.31±0.85 *c	00	6.5±0.75	49.72±0.74	44.46±1.6 # d	-10.56	31.48±1.2	27.7±2.8 # e	-11.79
Chloroquine/5	00	100	15.68±0.45 *b	48.68±0.58	48.58±0.58	-0.21	27.18±0.82	27.42±0.96	+0.88

Note: CHCl3 = chloroform; DMSO = Dimethyl sulphoxide; EtOAc = ethyl acetate; MeOH = methanol; SEM = Standard error of the mean [a] = mean survival value for chloroquine; [b] = mean survival value for 2% DMSO; [c] = mean parasitemia for EtOAc- MeOH, [d] = PCV at D0; [e] = BW at D0; [*] = significantly higher than [#] = significantly lower than

4.3.6 The BW and PCVof infected mice treated with CC fractions of the roots of *R. nepalensis*

The BW and PCV of the *P. berghei* infected mice treated with different Chromatographic fractions showed among extract fractions. In all treatments there was andecrease in BW and PCV except the BW of mice administered with chloroquine. However, the infected mice treated with 300 mg/ kg BW ethyl acetate: methanol, 40 mg/ kg BW chloroform and 100 mg/ kg BW

ethylacetate chromatographic fractions significantly prevented the loss of BW at day 4 post infections compared to the negative control mice treated with 2% DMSO.

The negative control mice that were treated with 2 % DMSO significantly lost their PCV with PCV percent reduction (-10.56 %) at day 4 on the other hand the chloroform with PCV change (-6.62 %), ethyl acetate with PCV change (-4.42 %) and ethyl acetate: methanol with PCV change (-0.39 %) chromatographic fractions of the roots of *R. nepalensis* significantly prevented PCV at day 4 post infection.

4.3.7 Determination of parasitemia and percent suppression of fractions

The effect of Chromatographicactive fractions of *R. nepalensis* is shown in Table 17. The methanol column Chromatographicre-fractions (3 undefined compounds) showed the highest percent Plasmodium suppression (79.66 %) at 150 mg/kg BW, but the lowest percent suppression (25 %) was recorded from hexane re-fractions at 20 mg/kg BW and chloroform re-fraction.

Table 17: Parasite densities, percent parasite and survival time of Column Chromatographicre-fraction in hexane, chloroform and methanol of the roots of *R. nepalensis*

Solvent fraction/ dose mg/kg	Mean parasitemia ±SEM	Percent suppression	Mean Survival time (day)±SEM	PCV			BW		
				D0	D4	% change	D0	D4	% change
n-Hex/ 20	36.66±0.86^{*c}	25	$^{a\#}$7.2±0.37	49.74±0.45	44.83±0.46	-9.88	25.52±0.29	22.96±0.31	-10.03
CHCl3/15	33.6±0.93^{*c}	31.3	$^{a\#}$7.4±0.25	49.66±0.41	44.80±0.97	-9.7	26.1±0.56	23.8±0.49	-8.81
MeOH/150	9.94±0.55	79.66	$^{a\#}$11.8±0.37^{*b}	50.1±0.96	49.9±0.72	-0.39	25.1±0.67	25.15±0.67	+0.2
2 % DMSO	48.88±0.99^{*c}	00	$^{a\#}$6.66±0.5	50.5±0.39	43.47±0.32	-15.05	24.44±0.45	21.96±0.79	-10.14
Chloroquine/5	00	100	14.66±0.4^{*b}	50.26±0.85	49.94±0.53	-0.63	26.14±0.55	26.32±0.48	+0.69

Note: CHCl3 = chloroform; DMSO = Dimethyl sulphoxide; EtOAc = ethyl acetate; MeOH = methanol; n-Hex = normal hexane; SEM = Standard error of the mean ^{a} = mean survival value for chloroquine; ^{b} = mean survival value for 2% DMSO; ^{c} = mean parasitemia of MeOH, ^{d} = PCV at D0; ^{e} = BW at D0; * = significantly higher than $^{\#}$ = significantly lower than

The percent plasmodium suppression (79.66) of the methanol column chromatographicre-fractions was higher than the percent suppression (73.66) of ethyl acetate: methanol column chromatographic fractions shown in Table 17. In agreement with Teklemariam *et al*., (2013), the percent parasite suppression in this study increased as the crude methanol extracts further fractionated by using column chromatography. Similarly the present phytochemical study revealed the presence of alkaloids, terpenes and flavonoids in the extracts of the roots of *R. nepalensis* indicating that these secondary metabolites of the plant may suppress *P. berghe in vivo* in mice model.

According to Deharo *et al.*, (2001) *in vivo* antiplasmodial activity of plant extracts are moderate, good, and very good if an extract displayed percent parasite suppression ≥50% at extract concentration of 500, 250 and 100 mg/kg BW, respectively. Based on this, the methanol and ethyl acetate: methanol CC fractions at 150 and 300 mg/kg BW, respectively showed a good and moderate *in vivo* antiplasmodial activity.

The mean survival time value (11.2±0.37) of infected mice treated with the methanol fractions was significantly higher than once treated with 2 % DMSO (6.66±0.5), hexane re-fractions (7.2±0.37) and chloroform re-fraction (7.4±0.25) of column chromatogram. In agreement with Fentahun *et al*., (2017), the mean survival time of the mice increased from 10.8±0.37 at 500 mg/kg BW crude extracts to survival values (11.4±0.25) at 500 mg/kg BW of ethanol fraction of the roots of *R. nepalensis.* The mean survival time also increased to (11.8±0.37 day) at 150 mg/kg BW methanol chromatographicre-fractions.

4. 3. 8 BW and PCV of infected mice treated with CC fractions of the roots of *R. nepalensis*

The BW and PCV of the mice treated with different re-fractions were shown in Table 17. Accordingly, the methanol chromatographic re-fraction significantly prevented the BW at 150 mg/kg BW methanol chromatographic re-fraction, but the ones treated with other refractions significantly lost their BW at day 4 post infections.

The BW of treated with 150 mg/kg BW of methanol Chromatographic re-fraction and 5 mg/kg BW chloroquine slightly increased with 0.2 % and 0.69 %, respectively. In consistant with this data, the previous study showed that the butanol fractions of methanolic root extract of *Dodonaea angustifolia* significantly reduced BW of the mice at 600 mg/kg, but chloroform

fractions showed significantly lost its BW in comparison with both control and standard drug treated group (Amelo *et al*., 2014).

Similarly the PCV of mice treated with 20 mg/kg BW of hexane, 15 mg/kg BW chloroform re-fractions and 2 % DMSO significantly lost their PCV at day 4 whereas the ones treated with 150 mg/kg BW methanol Chromatographic re-fraction and 5 mg/kg BW chloroquine significantly prevented the loss of PCV at day 4 post infections. This PCV loss prevention in this study is in consisitant with previous studies that showed the PCV of *P. berghei* infected mice treated with the 80 % crude methanol extract of aerial part of *Artemisia abyssinica* reduce the loss of PCV with BW changes (-4.88 %, -0.96 % and 2.52 %) at 100, 200 and 400 mg/kg, respectively in dose dependent manner (Adugna *et al.,* 2014).

4. 4 Conclusion

The two plants were found to have suppression on plasmoidal parasistes in the tested mice. In this work 500 mg/kg BW methanol crude extract of the roots of *R. nepalensis* showed highest plasmodial suppression (59.9 %) than the ones treated with crude methanol extracts from the leaf plants (43.27 %) and the aerial parts of *C. asiatica* (40.8 %) at 500 mg/kg BW. The ethanol fraction of the roots of *R. nepalensis* at 500 mg/kg BW again showed the highest plasmodial suppression (70.08 %).

The ethyl acetate: methanol Chromatographic fraction at 300 mg/kg BW displayed significantly higher *parasite* (73.66 %) suppression in mice and reduced the loss of BW and PCV values of infected mice. Among these Chromatographic refractions, 150 mg/kg BW methanol re-fraction showed the highest percent suppression (79.66 %) of the parasite. Similarly treatment values 150 mg/kg BW Chromatographicmethanol re-fraction significantly prevented the loss of the BW and

PCV of the infected mice the compared to negative control mice which is treated. On the other hand, the chloroquine treated ones displayed 100 % parasite suppression.

From the above data it can be concluded that the roots and leaves of *R. nepalensis* and the aerial parts of *C. asiatica* showed antimalarial activity in reducing plasmodium parasite load. The roots of *R. nepalensis* dissolved in slightly polar compounds (ethanol) and polar (water) showed better antiplasmodial activities than ones dissolved in non polar compounds (hexane, chloroform) and are good for herbal medication.

CHAPTER 5: THE ANTIMICROBIALACTIVITIES ACTIVITIES OF *R. NEPALENSIS* AND *C. ASIATICA* ON SOME PATHOGENIC BACTERIA AND FUNGUS

5.1 Introduction

Infectious diseases are caused by pathogenic microorganisms including bacteria, viruses, parasites and fungi. Infectious diseases remain the major cause of death of the world especially among poor people and it causes nine million deaths each year in the world (WHO, 2012). They are the major health problem especially in tropical and subtropical developing countries of the world (Girish and Satish, 2008, WHO, 2012).

Infectious diseases are mostly food and water borne (Craun *et al*., 2006, Scallan *et al*., 2011). Some of them are bacterial diseases caused by *Salmonella* species, Shigella species, *Escherichia coli* (Lynch *et al*., 2009). *P. aeruginosa* is an opportunistic pathogen which mainly causes nosocomial infections in immunocompromised patients and it naturally exists in the environment (Streeter and Katouli, 2016). *S. aureus* is a commensal bacterium which is associated with diseases of skin, skin glands, mucous membranes and mostly in the nose of healthy individuals (Verbrugh, 1997). These and other bacterial pathogenic bacteria are important pathogens which cause the community and hospital acquired infections throughout the world (Ravichitra *et al*, 2014). Some fungi such as *Candida albicans* also cause cutaneous, mucocutaneous and opportunistic infections (Ezzat, 2001).

Different drugs and antibiotics have been discovered to treat and prevent the human bacterial and fungal pathogens. However, these discoveries are challenged by emergency resistant

microorganisms to antimicrobial agents (Peterson and Dalhoff, 2004). Thus medicinal plants which are the natural source of medicine have been used as source of alternative medicine (Rates, 2001). In Ethiopia the use and practice of medicfinal plants had long history (Negussie, 1988). *R. nepalensis* and *C. asiatica* are among medicinal plants used by herbalist to treat various diseases in Ethiopia. In these work antibacterial and antifungal properties of the leaves and roots of *R. nepalensis* and the aerial parts of *C. asiatica* are evaluated.

5.2 Materials and Methods

The description of study areas, extract, fraction and re-fraction preparation of the leaves and roots of *R. nepalensis* and the aerial parts of *C. asiatica* were done as described in material and methods part (Chapter 3).

5.2.1 Test microorganisms

For *in vitro* experiment, the standard Gram negative bacteria (*Escherichia coli* ATTC 25922), *Pseudomonas aeruginosa* ATTC 27853, *Shigella flexneri* ATTC 12022, *Salmonella typhimurium* 13311 and *Klebsiella pneumonia* ATTC 700603) and the Gram positive bacteria (*Staphylococcus aureus*, ATTC 25923) were taken from Ethiopian Public Health Institute (EPHI), Microbiology Department, Addis Ababa. The test bacteria were allowed to grow on their corresponding selective media (*S. aureus* on Mannitol salt agar, *S. flexneri* and *S. typhimurium* on Salmonella Shigela Agar, *E. coli*, *P. aeruginosa* and *K. pneumoniae* on MacConkey agar) for 24 hr at 37^0 C.

Then test bacteria were transferred through sterilized loop into test tube having nutrient broth to grow over night in the incubator at 37^0 C. After 24 hours, the test bacteria grown in nutrient broth were streaked on Petri dish having nutrient agar which were prepared according to the manufactures protocol. Bacterial culture grown in nutrient agar for 24 hours was prepared to a

density of 10^8 cells ml^{-1} which approximately equal to 0.5 McFarland standards (Wanger, 2007). The 10^8 cells ml^{-1} bacteria culture were swabbed on the Petri dish having Muller Hinton Agar for bacterial susceptibility test. For fungal *in vitro* test, standard *Candida albicans* ATCC 10231 was taken from Ethiopian Public Health Institute (EPHI), Microbiology Department, Addis Ababa.

5.2.2 Standard drugs

30µg tetracycline and chloramphenicol were used as positive control for the antibacterial susceptibility test by disc diffusion and 30 µg Ketoconazole for antifungal test; whereas 2%Tween 80 and 2% DMSO served as a negative control. 30µg Ketoconazole and 2 % Tween 80 were used as positive and negative controls for the antifungal test respectively.

5.2.3 Antibacterial susceptibility test to crude extracts *R. nepalensis* and *C. asiatica* by disc diffusion methods

The paper disc diffusion technique was applied to determine the antimicrobial activities of the tested plant extracts as described by Bauer (1966). *E. coli, P. aeruginosa*, *S. flexneri, S. typhimurium* and *K. pneumoniae* were screened for bacterial susceptibility test. Each test was repeated 3 times. The 24 hours growth nutrients bacterial broth cultures were adjusted to a density of 10^8 cells ml^{-1} which approximately equal to 0.5 McFarland standards were uniformly spread on Mueller Hinton Agar (MHA) using sterile cell spreader. The discs were carefully placed on inoculated MHA plates.

Similarly the standard antibiotics teteracycline (30 µg/disc), and chloramphenicol (30 µg/disc) as a positive control and discs soaked with 2% DMSO as a negative control were carefully placed on bacteria the same agar plates swabbed MHA. All plates were incubated at $37C^o$ for 24 hours.

The inhibition zone of bacterial was measured from the edge of each disc after the incubation period by using sliding calipers and ruler.

5.2.4 Antibacterial activities of solvent fractions roots of *R. nepalensis* by disc diffusion method

The methanol extracts which showed better antibacterial activities on with paper disc diffusion method were assayed for their antimicrobial activities as described in (5.2.3).

5.2.5 Antibacterial susceptibility determination of ethanol and water fraction of roots of *R. nepalensis* by using agar well diffusion method

The ethanol and water fractions that further showed higher antibacterial activities on some pathogenic bacteria were further evaluated antibacterial activities by using agar well diffusion method as described above in (section 5.2.6).

5.2.6 MIC of crude extracts of methanol, water and hexane of the leaves and roots of R. *nepalensis* and *C. asiatica* by agar dilution method

The Minimum inhibitory concentration (MIC) of the solvent extracts on the test microorganisms was determined by agar dilution methods as described by European society of clinical microbial and infectious diseases (ESCMID, 2000). Nineteen ml of molten MHA and one ml of the solvents of extracts were prepared at different concentration (50, 25, 12.5, 6.75, 3.125 mg/ml) and mixed thoroughly and poured on Petri dish. One ml of culture in 0.85 % saline adjusted to $1.5x10^8$ cells ml^{-1} colony forming unit (CFU) was incupated into MHA at 37^0C for 24 hours. The MIC was determined by observing the growth of the test pathogens. The MIC of the ethanol and water fraction was also determined in similar procedure.

5.2.7 MBC determination of crude extracts

The loop full tests from last MIC were subcultured on solid nutrient agar and incubated at 37°C for 24 hours to determine MBC. The least concentration which inhibited growth of the test bacteria was considered as the MBC (Joshua and Takudzwa, 2013).

5.2.8 Antibacterial susceptibility test of CC fractions in solvents of the roots of *R. nepalensis* by agar well diffusion method

About 0.24 mg/well chloroform CC fraction, 1.2 mg/well ethyl acetate CC fraction and 3.6 mg/well ethyl acetate: methanol CC fraction were prepared based on their yield obtained from column chromatography fractions. The procedures for antimicrobial activities assay were as above (5.2.8) except the concentration differences.

5.2.9 Antibacterial susceptibility CC re-fractions in hexane, chloroform and methanol of the roots of *R. nepalensis*

The better antibacterial chromatographic fractions (ethyl acetate: methanol was further re-fractionnated by CC in hexane, chloroform and methanol. The re-fraction components were prepared for bacterial assay at 0.045 mg/well chloroform CC re-fraction, 0.09 mg/well hexane CC re-fraction and 0.75 mg/well methanol CC re-fractions. The procedures for antiplasmodial activities were the same as before.

5.2.10 Antifungal sensitivity test determination by Agar well diffusion

Agar well diffusion method was used to determine the activities of methanol, water and hexane extracts of the test plants on *C. albicans. C. albicans* were adjusted to a density of 10^8 cells ml^{-1} which approximately equal to 0.5 McFarland standards. Sabouraud dextrose plates were streaked evenly with a swab dipped into the standardized inoculums suspension. The plates were allowed

to dry and a sterile cork borer of diameter 6 mm were used to bore wells in the agar plates. The 6 mg/disc, 3mg/disc and 1.5 mg/disc methanol, water and hexane extracts extracts were prepared be separately introduced into the wells and allowed to stand for 1 hours for diffusion to take place at room temperature and then the plated was incubated for 48 hours at 30^{o} C (Olowosulu *et al*., 2005).

Similarly the different solvent fractions (hexane, chloroform, ethanol water and methanol) obtained from methanol crude extracts of roots of *R. nepalensis* were assayed against *C. albicans* using agar well diffusion method as described above. Chloroform, ethyl acetate and ethyl acetate: methanol CC fractions were were assayed against *C. albica* at the concentration of 0.24, 1.2 and 3.6 mg/well. The hexane, chloroform and methanol re-fraction obtained from ethyl acetate: methanols in (1:1 v/v) ratio were tested against *C. albica* at 0.18, 0.27 and 2.7 mg/well, respectively in the same procedure as described above.

5.2.11 MIC and MFC of root fractions of *R.nepalensis* against *C. albica*

The minimum inhibitory concentration and minimum fungicidal concentration (MFC) were done in the same way as it is described in (section 5.2. 6 and 5.2.7).

5. 3 Results and Discussion

5.3.1 Antibacterial susceptibility test determination of crude extracts of the root, leaf of *R. nepalensis* and the aerial parts of *C. asiatica* by disc diffusion method

The antibacterial activities of different crude extracts of the two plants on the test microorganisms using disck diffusion method were shown in Table 18. The data showed that the rations in susceptibility of the test organisms by the crude extracts ranging from inhibition

diameter of 0-11.83 mm. Accordingly, the test bacteria were not affected by extracts at 1.5 mg/disc, except *P. aeruginosa.*

In similar pattern the antimicrobial activities was also displayed *K. pnuemoniae* at concentration of 6 mg/disc followed by 1.5 mg/disc of crude extracts and *K. pnuemoniae* was the most resistant pathogen to all extracts. Whereas most of the pathogens were resistant to n-hexane crude extracts. Among the the crude extracts methanol and water extracts showed potent antibacterial activites at all concentration on *P. aeruginosa* and *S. aureus* with inhibition diameters of 6.6-11.83 mm and 6.6-11mm, respectively without showing significant differences between the two solvent extracts.

In all cases the solvent extracts showed better antibacterial activities than the negative control, but 1.5-3 times lower than the standard drugs (positive control). In agreement with this work, the previous showed that the methanol extract of *R. nepalensis* roots inhibited *S. aureus and E. coli* with bacterial inhibition of 10.5 mm and 10.0 mm, respectively at 1mg/disc (Ghosh *et al*., 2003)

In contrast to this study, Yadav *et al*., (2011) showed that the antibacterial activities of methanol extracts of the root of *R. nepalensis* were higher on *P. aeruginosa* (21 mm), *S. aureus* (29 mm) and *E. coli* (24 mm) at 1mg/ml. The lower bacterial inhibition of methanol extracts of the root of *R. nepalensis* on *P. aeruginosa, S. aureus* and *E. coli* could be due to the presence of chemotype differences between *R. nepalensis* plants in the previous and present study and the difference methods; in the present study the disc diffusion methods, but in the previous study agar well diffusion was used to determine antibacterial susceptibility.

In contrast to the present study, the previous related studies revealed that the crude methanol extracts of the leaves of *Rumex hastatus* showed comparatively higher bacterial inhibitions on *S. aureus* (21.3 ± 0.4 mm), *E. coli* (25.4±0.3 mm), *P. aeruginosa* (24.3 ± 0.5 mm) and *S. typhi* (22.5 ± 0.2 mm), respectively using agar well diffusion method at 100 mg/ml (Hazrat *et al*., 2013).

Table 18: Antibacterial activities of crude water, methanol and hexane extract of the root *R. nepalensis*

Bacteria strain	Solvent	Mean Bacterial inhibition in millimeter ± SEM			
		Extract concentration (mg/disc)			TE, C
		6	3	1.5	30µg
	MeOH	11.83±0.16	10.33±0.33	7.17±0.44	17.17±0.44
P. aeruginosa	n-Hex	-	-	-	18.12±0.13
	H_2O	11.00±0.29	10.17±0.17	6.6±0.16	18.06±0.32
	MeOH	11.66±0.33	8.99±0.13	8.6±0.30	24.83±0.44
	n-Hex	8.27±0.37	-	-	24.67±0.88
S. aureus	H_2O	11.00±0.29	9.1±0.46	8.5±0.29	24.33±0.33
	MeOH	6.83±0.17	6.5±0.00	-	20.33±0.33
E. coli	n-Hex	-	-	-	20.43±0.44
	H_2O	8.33±0.33	6.6±0.19	-	20.83±0.44
	MeOH	8.33±0.33	6.88±0.35		23.66±0.33
S. flexneri	n-Hex	-	-	-	24.05±0.30
	H_2O	8.23±0.79	6.88±0.58	-	23.57±0.34
	MeOH	7.06±0.29	-	-	24.67±0.34
S. typhimurium	n-Hex	7.3±0.32	-	-	24.40±0.30
	H_2O	-	-	-	34.46±0.29
K. pnuemoniae	MeOH	-	-	-	15.23±0.28
	n-Hex	-	-	-	16.34±0.16
	H_2O	-	-	-	15.6±0.28

Note: C = Chloroanphenicol; DMSO = Diethylsulfpoxide; H_2O = water; MeOH = methanol; n-Hex. = normal Hexane; SEM = Standared error of the mean; TE = Tetracycline, - = no bacterial inhibition

Similarly, the crude water extracts of the root of *R. nepalensis* inhibited *P. aeruginosa* (11.00±0.29 mm), *S. aureus* (11.00±0.29), *E. coli* (8.33±0.33), *S. flexneri* (8.23±0.79 mm) and had no inhibition on *S. typhimurium* and *K. pnuemoniae* at 6mg/disc diffusion method. On the other hand, the crude hexane extracts of the root of *R. nepalensis* inhibited *S. typhimurium* (7.3±0.32 mm), but this extract did not show inhibition on *P. aeruginosa, S. aureus, S. flexneri* and *K. pnuemoniae* at 6mg/disc by using disc diffusion method.

From these data, it is possible to explain that the active components of the root of *R. nepalensis* which inhibited *P. aeruginosa, S. aureus, E. coli* and *S. flexneri* are more probably dissolved in slightly polar compound (methanol) and polar compound (water) whereas *S. typhimurium* was inhibited by the components of the root of *R. nepalensis* which was dissolved by non polar solvent (hexane). The antibacterial activities of different solvent extracts of leaf of *R. nepalensis* on the test microorganisms are shown in Table 19. The data showed that most of the the extracts did not show antibacterial activities on the test microorganisms except the crude methanol extracts inhibiting *P. aeruginosa* and *S. aureus* with the inhibition diameter of 6.3-7 mm and 6.30-8.23mm at 3 and 6 mg/ disc, respectively.

In agreement with this study, the previous study Koochak *et al*., (2010) showed that the 80 % ethanol extracts of aerial parts of *Rumex obtusifolius* did not show any bacterial inhibition on *E. coli, K. pnuemoniae* and *S. typhimurium* at concentration of 400 and 500 mg/ml by using disc diffusion method. The water extracts also inhibited *S. aureus* with inhibition diameter of 7.66 mm at concentration of 6mg/disc. This result was in agreement with water extracts with no bacterial inhibition on *E. coli, S. aureus* and *P. aeruginosa* at 0.75mg/disc (Yildirım *et al*., 2001).

The antibacterial activities of the crude methanol, hexane and water extracts of the aerial parts of *C. asiatica* did not show any antibacterial activites of except the methanol extract that was able to inhibit *S. aureus* with the inhibition diameter of 7.33 (the data not shown). In contrast to this study, the water extracts of *C. asiatica* displayed with the inhibition diameter of 8 mm, 9 mm and 10 mm on *S. aureus* and *E. coli*, respectively (Arumugam *et al*., 2011).

Table 19: Antibacterial activities of crude water, methanol and hexane extract of the leaf of *R. nepalensis*

Bacteria strain	Solvent	Mean Bacterial inhibition in millimeter ± SEM			
		mg/disc			TE, C
		6	3	1.5	30µg
P. aeruginosa	MeOH	7.67±0.33	6.3±0.15	-	18.12±0.13
	n-Hex	-	-	-	18.62±0.16
	H_2O	-	-	-	20.32±0.21
S. aureus	MeOH	8.23±0.15	6.73±0.27	-	24.66±0.88
	n-Hex	-	-	-	25.00±0.05
	H_2O	7.66±0.33	-	-	24.34±0.51
E. coli	MeOH	-	-	-	19.66±0.34
	n-Hex	-	-	-	20.12±0.81
	H_2O	-	-	-	20.35±0.66
	MeOH	-	-	-	24.0±0.58
S. flexneri	n-Hex	-	-	-	23.8±0.22
	H_2O	-	-	-	23.6±0.64
S. typhimurium	MeOH	-	-	-	25.0±0.58
	n-Hex	-	-	-	24.78±0.89
	H_2O	-	-	-	24.6±0.15
K. pnuemoniae	MeOH	-	-	-	16.0±0.57
	n-Hex	-	-	-	15.3±0.88
	H_2O	-	-	-	15.8±0.56

Note: C = Chloroanphenicol; DMSO = Diethylsulfpoxide; H_2O = water; MeOH = methanol; n-Hex. = normal Hexane; SEM = Standared error of the mean; TE = Tetracycline., - = no bacterial inhibition

5.3.2 Antibacterial activities solvent fractions the roots of *R. nepalensis* by paper disc diffusion

Among the different extracts extracts, the methanol extracts of root *R. nepalensis* showed comparatively better bacterial inhibition and were fractionated and tested for their efficacy in antibacterial activities (Table 20). The data showed that most of the the solvent extract fractions did not show antibacterial activities on the test microorganisms except ethanol and water fractions. Accordingly, the ethanol fractions inhibited *P. aeruginosa* with inhibition diameter of 8.00 mm, 10.00 mm and 12.77 mm at 1.5, 3 and 6mg/well, respectively. The ethanol fractions

also inhibited *S. aureus*, *E. coli* and *S. flexneri* 13.00±0.29 mm, 6.9±0.86 mm and 11.00±0.33 mm, respectively.

Similarly, the water fraction inhibited *P. aeruginosa*, *S. aureus*, *E. coli*and *S. flexneri* with bacterial inhibition of 12.77±0.17 mm, 12.33±0.44 mm, 8.2±0.33 mm and 10.9±0.06 mm respectively, however water and methanol fraction did not show bacterial inhibition on *S. typhimurium* and *K. pnuemoniae* at 6mg/disc. The inhibition of the ethanol and water fractions on pathogenic bacteria increased as the plant extract fractions increased from 1.5 mg/disc to 3 mg/disc and then to 6 mg/disc as presented in Table 20. But, the hexane and chloroform fractions did not show any inhibition on pathogenic bacteria. In contradiction with this study, the chloroform and hexane fractions of the whole parts of *R. hastatus* showed bacterial inhibition on *P. aeruginosa*, *S. aureus*, *E. coli* and *K. pnuemoniae* at 1mg/well (Kamal, *et al*., 2015).

In this work, the ethanol and water fractions at all concentrations (6 mg/ disc, 3 mg/ disc and 1.5 mg/ disc) showed significantly higher bacterial inhibition zone than the pathogenic bacteria (*P. aeruginosa*, *S. aureus*, *E. coli* and *S. flexneri*) treated with 2% DMSO. However, the ethanol and the water fractions showed significantly lower bacterial inhibition than the positive control (tetracycline and chloramphenicol). Ethanol and water extract fractions had antibacterial potentials especially pathogenic bacteria such as *P. aeruginosa*, *S. aureus*, *E. coli* and *S. flexneri.*

Table 20: Antibacterial activities of the hexane, chloroform, ethanol, water and methanol fractions obtained from crude extract of the roots of *R. nepalensis* by paper disc diffusion

Bacteria strain	Solvent	Mean Bacterial inhibition± SEM in millimeter			
		Extracts in mg/disc			TE, C
		6	3	1.5	30µg
	n-Hex	-	-	-	18.06±0.32
	CHCl3	-	-	-	18.12±0.13
P. aeruginosa	EtOH	12.17±0.12	10.93±0.03	10.33±0.17	23.67±0.33
	H_2O	12.77±0.17	10.00±0.12	8.00±0.58	23.33±0.88
	MeOH	-	-	-	20.33±0.33
	n-Hex	-	-	-	22.0±0.58
	CHCl3	-	-	-	23.45±0.34
S. aureus	EtOH	13.00±0.29	11.27±0.38	9.83±0.0.17	24.33±0.33
	H_2O	12.33±0.44	11.00±0.58	10.17±0.17	24.00±0.58
	MeOH	-	-	-	24.5±0.15
	n-Hex	-	-	-	19.44±0.17
	CHCl3	-	-	-	18.76±0.88
E. coli	EtOH	6.9±0.86	-	-	20.33±0.33
	H_2O	8.2±0.33	7.2±0.54	6.5±0.87	15.83±0.17
	MeOH	-	-	-	18.65±0.23
	n-Hex	-	-	-	23.34±0.44
S. flexneri	CHCl3	-	-	-	21.88±0.88
	EtOH	11.00±0.33	9.17±0.17	8.50±0.29	21.66±0.33
	H_2O	10.9±0.06	10.00±0.12	9.33±0.33	23.93±0.35
	MeOH	-	0	0	24.50±0.15
S. typhimurium	n-Hex	10.17±0.24	7.27±0.26	6.6±	24.00±0.29
	CHCl3	-	-	-	23.56±0.29
	EtOH	-	-	-	24.12±0.44
	H_2O	-	-	-	24.33±0.30
	MeOH	-	-	-	24.15±0.26
K. pnuemoniae	n-Hex	-	-	-	16.34±0.16
	CHCl3	-	-	-	15.6±0.28
	EtOH	-	-	-	16.0±0.57
	H_2O	-	-	-	16.2±.0.28
	MeOH	-	-	-	15.40±0.33

Note: C = Chloroanphenicol., CHCl3 = chloroform; DMSO = Diethylsulfpoxide., EtOH = ethanol; H_2O = water; MeOH = methanol; n-Hex = normal hexane; SEM = Standared error of the mean., TE = Tetracycline

5.3.3 Antibacterial susceptibility test determination of ethanol and water fraction of roots of *R. nepalensis* by using agar well diffusion method

The antibacterial activities of the two fractions (ethanol and water fraction) of the roots of *R. nepalensis* were done by agar well diffusion method to compare it with the antibacterial activities of the two fractions determined by disc diffusion method. As it is presented in Table 21, 6 mg/well, 3 mg/well and 1.5 mg/well of the ethanol fraction inhibited *P. aeruginosa* with bacterial inhibition of 19.33±0.33 mm, 18.23±0.12 mm and 17.1±0.12 mm, respectively.

The inhibition zone recorded from crude extracts were significantly higher than the negative control (2% DMSO) but were close inhibition diameters of ethanol fraction of the roots of *R. nepalensis* were not significantly different from the inhibition of *P. aeruginosa* by positive control, 30 µg/well of tetracycline and chloroquine. 6 mg/well, 3 mg/well and 1.5 mg/well of the ethanol fraction of the roots of *R. nepalensis* by agar well diffusion also showed significantly higher inhibition on *P. aeruginosa* than disc diffusion method at 6 mg/disc, 3 mg/disc and 1.5 mg/disc.

The ethanol fractions inhibited *S. aureus* with the inhibition values of 18.03±0.14 mm, 15.17±0.17 mm and 13.03±0.32 mm at 6 mg/well, 3 mg/well and 1.5 mg/well respectively. The above inhibitory values were significantly higher than the inhibitions on *S. aureus* by paper disc diffusion methods at 6 mg/disc, 3 mg/disc and 1.5 mg/disc. The ethanol fractions also showed the inhibitions on *S. flexneri* (12.77±0.15 mm) and on *S. typhimurium* (12.77±0.15 mm), without showing effects on *E. coli* and *K. pneumoniae* at all concentrations (at 6 mg/well, 3 mg/well and 1.5 mg/well).

From the above data it is possible to interpret that the bacterial inhibitions of the ethanol fraction of the roots of *R. nepalensis* on *P. aeruginosa, S. aureus, S. flexneri* and *S. typhimurium* increased as the concentration of the fractions of the roots of *R. nepalensis* doubled from 1.5 mg/well to 3 mg/well and to 6 mg/well by using agar well diffusion methods. In this work, the agar well diffusion method is the better method in the determination of antibacterial susceptibility compared to paper disc diffusion method. As a result of this, the ethanol fractions of the roots of *R. nepalensis* which was further fractionnated in column chromatography to have evaluated their antibacterial activities against the tested pathogenic by using the agar well diffusion method.

Table 21: Antibacterial activities of ethanol and water fraction of roots of *R. nepalensis* by using agar well diffusion method

Bacteria strain	Solvent	Bacterial inhibition in millimeter			
		Extracts in mg/well			TE, C
		6	3	1.5	30µg
P. aeruginosa	EtOH	19.33±0.33	18.23±0.12	17.1±0.1	18.33±0.33
	H_2O	16.1±0.15	13.3±0.17	11.06±0.07	19.43±0.28
	EtOH	18.03±0.14	15.17±0.17	13.03±0.32	21.8±0.44
S. aureus	H_2O	17.83±0.44	15.33±0.44	14.6±0.29	22.1±0.14
E. coli	EtOH	-	-	-	22.5±0.29
	H_2O	-	-	-	22.3±0.15
S. flexneri	EtOH	12.77±0.15	9.0±0.58	8.17±0.17	20.77±0.14
	H_2O	12.83±0.12	9.1±34	-	21.12±0.05
S. typhimurium	EtOH	8.33±0.33	6.67±0.33	-	23.0±0.58
	H_2O	-	-	-	22.82±033
K. pneumoniae	EtOH	-	-	-	15.06±0.07
	H_2O	-	-	-	15.0±0.15

Note: C = Chloroanphenicol. DMSO = dimthylsulfpoxide., EtOH = ethanol; H_2O = water; SEM = Standared error of the mean., TE = Tetracycline

5.3.4 The minimum inhibitory concentration (MIC) of cerude extracts and fractions of the roots of *R. nepalensis*

Different extracts and fractions of fractions of the roots of were further evaluated for the determination of MIC agar dilution methods as shown in Table 22. The data showed that the MIC of *S. aureus* and *P. aeruginosa* was 12.5 mg/ml followed by the MIC of *S. flexneri* and *S. typhimurium* 25 mg/ml and 50mg/ml, respectively. In the previous study, the butane fractions of *R. hastatus* have comparatively lower MIC on *S. aureus* (2.5 mg/ml), *P. aeruginosa* (0.1mg/ml) and had no MIC values on *E. coli* and *K. pnuemoniae* up to the highest concentration of 5mg/ml

(Sahreen *et al*., 2015). The MIC of *Rumex nervosus* for *S. aureus* and *Salmonella* were 1.56 mg/ml and 5 mg/ml, respectively (Abebe and Mekonnen, 2016).

As it is described antibacterial susceptibility test, the higher the inhibition zones the ethanol fractions of the roots of *R. nepalensis,* the lower the MIC values on the corresponding pathogenic bacteria it had. From this data it can be drawn that the ethanol fractions contain potential secondary metabolites against *P. aeruginosa, S. aureus, S. flexneri* and *S. typhimurium,* whwereas the hexane crude extracts of the root had no MIC values up to the maximum concentration of 200 mg/ml. In agreement with this study, the methanol extracts was potent to (MIC range 1.56-25 mg/ml), but the hexane extracts of *R. obtusifolius* had no MIC values upto the maximum concentration of 50 mg/ml on *S. aureus, E. coli* and *S. typhi* (Harshow *et al*., 2010).

Table 22: MIC of ethanol fraction from crude methanol the roots of *R. nepalensis* on pathogenic bacteria

Bacteria	MIC at different concentration solvent extract and fractions				
	Concentration (mg/ml)				
	C MeOH	C H2O	C n.Hex	F EtOH	F H2O
S. aureus	25	50	-	12.5	25
P. aeruginosa	25	25	-	12.5	25
S. flexneri	50	50	-	25	25
E. coli	200	200	-	100	100
K. pnuemoniae	200	200	-	200	200
S. typhmurium	50	100	100	25	50

Note: C H2O = water crude extract, C MeOH = Methanol crude extract, C n.Hex + normal hexane crude extract, F EtOH = ethanol solvent fraction, F H2O MIC = Minimum inhibitory concentration, - = MIC not determined upto 200 mg/ml

Similarly, the MBC values of the ethanol fractions of the roots of *R. nepalensis* on *P. aeruginosa* and *S. aureus* was 25 mg/ml as shown Table 23. The MBC on the two test microorganisms was higher than the MIC indicating that the MIC values which inhibited the bacteria might not totally kill the bacteria at MIC. Thus the MBC of the fractions of the roots *R. nepalensis* is greater than the MIC on the pathogenic bacteria.

Table 23: MBC of ethanol fraction from crude methanol *R.nepalensis* root on pathogenic bacteria

Bacteria	MBC at different concentration solvent extract and fractions				
	Concentration (mg/ml)				
	C MeOH	C H2O	C n. Hex	F EtOH	F H2O
S. aureus	50	100	-	25	25
P. aeruginosa	50	50	-	25	25
S. flexneri	100	100	-	50	50
E. coli	400	400	-	200	200
K. pnuemoniae	400	400	-	400	400
S. typhmurium	100	200	200	100	100

C H2O = water crude extract, C MeOH = Methanol crude extract, C n.Hex + normal hexane crude extract, F EtOH = ethanol solvent fraction, F H2O MBC = Minimum bactericidal concentration

5.3.5 Antibacterial susceptibility of column Chromatographic fractions and refractions of the roots of *R. nepalensis* by using agar well diffusion method

The ethanol fraction of the roots of *R. nepalensis* was further fractionnated by Column chromatography (CC) within three solvent systems (Chloroform, ethyl acetate and ethyl acetate: methanol) as shown in Table 24. The data showed that chloroform and ethylacetate CC fractions did not show any inhibition in all test microorganisms. However the acetate: methanol CC fractions inhibited *P.aeroginesa* (16.23±0.15mm), *S. aureus* (15.93±0.52mm), *S. flexneri* (14.17±0.17 mm) and *S. typhimurium* (8.0±0.58mm) at 3.6 mg/well by using agar well diffusion method.

But this Chromatographic fraction did not show inhibition on *E. coli* and *K. pneumoniae* as shown in Table 24. In agreement with this study, the ethyl acetate fraction of *Rumex tingitanus* leaves

inhibt *S. aureus* with inhibition diameter of 14.7 mm at 5mg/ml, but in contrast to this study the column chromatographic fraction did not inhibit *P.aeroginesa, E. coli* and *K. pneumoniae* at 5mg/ml (Mhalla *et al*., 2017).

The acetate: methanol column Chromatographic fractions of the roots of *R. nepalensis* which had 5 undefined compounds on TLC profile showed lower bacterial inhibitions than ethanol fractions of the roots of *R. nepalensis*. This may be due to the high effectiveness of the mixed components in ethanol fractions that could have had synergetic antimicrobial activities. On the other hand, the acetate: methanol column Chromatographic fractions reduced its components compared to solvent fraction (ethanol fraction).

Table 24: Antibacterial activities of Column Chromatographic fractions and refraction in different solvents of root of *R. nepalensis* by using agar well diffusion method

Bacteria strain	Mean bacterial inhibition in millimeter ±SEM						
	CC fractin in mg/well)			CC re-fractin in (mg/well)			TE, C
	$CHCl_3$ (0.24)	EtOAc (1.2)	EtOAc: MeOH (3.6)	$CHCl_3$ (0.045)	n-Hex (0.09)	MeOH (0.75)	30µg
P. aeruginosa	-	-	16.23±0.15	-	-	15.27±0.37	18.1±0.06
S. aureus	-	-	15.93±0.52	-	-	13.67±0.33	24.83±0.17
E. coli	-	-	-	-	-	0	20.43±0.29
S. flexneri	-	-	11.17±0.17	-	-	11.0±0.58	24.8±0.42
S. typhimurium	-	-	8.0±0.58	-	-	10.0±0.57	19.4±0.31
K. pneumoniae	-	-	-	-	-	0	12.67±0.42

Note: C = Chloroanphenicol., $CHCl_3$ = chloroform; DMSO = Dimethyl sulphoxide; EtOAc = ethyl acetate; MeOH = methanol; SEM = Standared error of the mean., TE = Tetracycline

The Chromatographic fractions (ethyl acetate: methanol) of the roots of *R. nepalensis* were further re-fractionnated by CC in hexane, chloroform and methanol. The CC re-fraction was evaluated on pathogenic bacteria at the concentration prepared based on proportions of the yield obtained from ethyl acetate: methanol CC fractions.

In this work, the methanol column Chromatographicre-fractions inhibited *P. aeruginosa* (15.27±0.37mm), *S. aureus* (13.67±0.33mm), *S. flexneri* (11.0±0.58 mm) and *S. typhimurium* (10.0±0.57 mm) at the concentration of 0.75 mg/well by using agar well diffusion method, whereas the chloroform and hexane re-fractions obtained from column Chromatographicre-fractions had no bacterial inhibition at 0.05mg/well and 0.09 mg/well respectively on all bacteria used in this work as shown in Table 22.

On the other hand, the positive control, tetracycline inhibited *P. aeruginosa* (20.0±0.58 mm), *S. aureus* (23.97±0.33 mm), *E. coli* (21.0±0.58 mm) and *K. pnuemoniae* (12.66±0.33 mm). The positive control drug, chloramphenicol also inhibited *S. flexneri* (24.47±0.29 mm) and *S. typhimurium* (22.73±0.18 mm). On the other hand, the negative control, 2 % DMSO, had inhibition on all pathogenic bacteria used in this study.

The inhibition zone of the standards drug chloroamphenicol and tetracycline (30 μg) was interpreted in such a way that its bacterial inhibition is resistant (≤12mm), intermediate (13-17 mm) and sensitive (≥18mm) as described in Bauer *et al*., (1966). Bases on this interpretation, *P. aeruginosa, S. aureus* and *E. coli* were sensitive to the standard drug, tetracycline. Similarly *S. flexneri* and *S. typhimurium* were sensitive to chloroamphenicol, but *K. pneumoniae* was resistant to tetracycline. In agreement with this study, the acetylacetate-ethanol in (1:1 v/v) column chromatographic fraction inhibited *Streptococcus mutans* with bacterial inhibition of 15 mm at 10 mg/well (Aldana *et al*., 2013).

The highest bacterial inhibition on *P. aeruginosa* (19.33±0.33 mm), *S. aureus* (18.03±0.14 mm) and S. flexneri (12.77±0.15 mm) were obtained from ethanol fractions of the roots of *R. nepalensis* at 6 mg/well, but the bacterial activities of the ethyl acetate: methanol CC fractions obtained from ethanol fractions of the roots of *R. nepalensis* reduced on *P. aeruginosa* (16.23±0.15mm), *S. aureus* (15.93±0.52mm) and S. flexneri (11.17±0.17mm) at 3.6 mg/well. Similarly the bacterial inhibitions of the methanol CC re-fractions which were obtained from ethyl acetate: methanol CC fractions reduced on *P. aeruginosa* (15.27±0.37mm), *S. aureus* (13.67±0.33mm) and *S. flexneri* (11.0±0.58 mm) at 0.75 mg/well.

From this it can be interpreted that ethanol fractions of the roots showed higher antibacterial activities of the *P. aeruginosa*, *S. aureus* and *S. flexneri* compared to its methanol crude extracts. This can be due to non- active components of the roots of *R. nepalensis* removed by non polar solvents such as hexane and chloroform and the active components may be retained in ethanol solvents, but the reduction of antibacterial activities in column Chromatographic fractions and refractions of the roots of *R. nepalensis* can be interpreted in terms of the higher synergetic effects in the ethanol fractions compared column Chromatographic fractions and re-fractions.

5.3.6 Antifungal sensitivity determination of crude methanol, hexane and water extract roots and leaf of *R. nepalensis* and *C. asiatica* by agar well dilution

The antifungal sensitivity of crude methanol, hexane and water extract of the roots, leaves of *R. nepalensis* and the aerial parts of *C. asiatica* was determined by agar well dilution method. The crude methanol extracts of the roots of *R. nepalensis* inhibited *C. albicns* (12.97± 0.03 mm at the concentration of 6 mg/well as shown in Table 25, whereas the positive control, 30 µgm/ well

of Ketokonazole had significantly higher fungal inhibitions on *C. albicns* (19.97±0.08 mm) than the crude methanol extracts of the roots of *R. nepalensis* on *C. asiatica* at 6 mg/well.

In contradiction with this result Buli *et al*., (2015) revealed that there was no fungal inhibition of water extracts of *Lippia adoensis* var. koseret, but with fungal inhibition (8.33 mm) of methanol extracts at 80 mg/ml using disc diffusion method. Oshim *et al*., (2016) also revealed that the methanol extract of *Vernonia amygdalina* leaves had no fungal inhibition at 200 mg/ml. In this study, the water extracts of the roots of *R. nepalensis* showed fungal inhibitions on *C. albicans* (12.73±0.18 mm) at 6 mg/well. In contrast to methanol and water extracts, the hexane extracts of the roots of *R. nepalensis* had significantly lower inhibition on *C. albicans* (6.27±0.15).

The crude methanol extracts of leaves of *R. nepalensis* on *C. albicans* was 7.17±0.160 mm at 6mg/well which is significantly less that the inhibition (20.34±0.13 mm) of positive control, 30 µgm/ well of ketokonazole on *C. albicans*, but the inhibition of the extract was significantly higher than the negative control (2% DMSO). Similarly, the water extracts of the leaves of *R. nepalensis* inhibited *C. albicans* (6.9±0.32 mm) which is significantly less the inhibition of 30 µgm/ well on the on *C. asiatica* and significantly higher than the negative control (2% DMSO).

The methanol extracts of the aerial parts of *C. asiatica* inhibited *C. albicans* at 6 mg/well. These inhibitory values of the methanol extracts were significantly lower than the positive control (ketokonazole). Similarly the water extracts of the aerial parts of *C. asiatica* inhibited *C. albicans* fungal inhibition of 6.73±0.33 mm 6 mg/well. The hexane extracts of *C. asiatica* did not show inhibition in this research. In the previous studies, 200 mg/ml of the leaves of was known to inhibit *C. albicans* 27.3 ±0.0 % of its growth (Lalitha *et al*., 2013) which is less than

the percentage inhibition (33.68%) of the water extracts of aerial parts of C. asiatica in the present work.

From this data it is possible to explain that the crude methanol extracts of the roots of *R. nepalensis* had comparatively higher antifungal activities than the hexane, water extracts of leaves of *R. nepalensis* and the aerial parts of *C. asiatica*. Its fungal inhibition increased as the concentration of extracts of the roots, leaves of *R. nepalensis* and the aerial parts of parts of *C. asiatica* doubled. Since the methanol extracts of the roots of *R. nepalensis* showed better anifungal activities, the root extracts were further fractionnated by hexane, chloroform, ethanol and methanol.

Table 25: Antifungal sensitivity determination crude water, methanol and hexane extract roots and leaf of *R. nepalensis* and *C. asiatica* by agar well dilution

	Plant	Solvent	Mean fungal inhibition in millimeter ± SEM			
			Extracts in mg/well			Ketokonazole
			6	3	1.5	0.03mg/well
	R. nepalensis root	MeOH	12.97±0.03	11.63±0.31	10.37±0.32	19.97±0.08
		n-Hex	6.27±0.15	-	-	20.16±0.49
		H_2O	12.73±0.18	10.83±0.17	9.5±0.29	19.43±0.29
C.albicns	*R. nepalensis* leaf	MeOH	7.17±0.160	6.83±0.17	6.27±0.22	20.34±0.13
		n-Hex	-	-	-	19.76±0.33
		H_2O	6.9±0.32	6.5±0.15	-	21.27±0.98
	C. asiatica aerial	MeOH	6.9±0.15	6.57±0.23	-	20.67±0.88
		n-Hex	-	-	-	20.44±0.31
		H_2O	6.73±0.33	-	-	19.98±0.43

Note: DMSO = dimethylsulfpoxide. H_2O; MeOH = methanol; n-Hex = normal hexane SEM = Standared error of the mean

5.3.7 Antifungal sensitivity of solvent fractions of roots of *R. nepalensis* by using agar well diffusion methods

The antifungal properties of the better performing crude extract, the roots of the roots of *R. nepalensis,* was further fractionnated by hexane, chloroform, ethanol water and methanol based on their polarity increment. The ethanol fractions of the roots of *R. nepalensis* inhibited *C. albicans* (14.1±0.2mm) at 6mg/well as shown in Table 25.

This value was significantly higher than the negative control, 2% DMSO, but it was lower than the positive control, 30µg Ketokonazole. Similarly the inhibition of the ethanol fractions of the roots of *R. nepalensis* on *C. albicans* was not significantly different from its positive control, 30µg Ketokonazole. The water extracts inhibited *C. albicans* with the inhibition diameter of 6.73±0.33 mm at 6 mg/well as it is shown in Table 26. This inhibition was lower than the corresponding inhibition of the ethanol the roots of *R. nepalensis* however the changes were not significant. However, the hexane, chloroform and methanol fractions of the roots of *R. nepalensis* did not show inhibitions on the test pathogen.

From this it is possible to say that the anifungal potential of the rots of *R. nepalensis* were dissolved first in methanol and further magnified by ethanol fraction whereas the non polar solvents were no good enough to be used as solvent for the antifungal activities of the roots of *R. nepalensis.* The ethanol fractions of the rots of *R. nepalensis* had comparatively higher fungal inhibition than its crude methanol extracts.

5.3.8 MIC and MFC fractions of roots of *R. nepalensis* against *C. albicans*

The MIC and MFC values of dfferent solvent fractions of roots of *R. nepalensis* were shown in Table 26. Accordingly the MIC varied within the ranges from 12.5 to 200 mg/ml. The ethanol

and water fraction had the lowest MIC value (12.5 mg/ml) which is in agreement with Ginovyan *et al*., (2017) that revealed MIC values (10 mg/ml) of water extracts of *Rumex obtusifolius*. But the n-haxane and methanol fractions had no MIC values upto the highest concentration (200 mg/ml) against *C. albicans* indicating that the n-hexane and methanol do not readily dissolve the the active compounds against *C. albicans.* Furthermore MIC the solvent fractions (ethanol, water and chloroform) of roots of *R. nepalensis* in this study were lower than its corresponding MFC values.

Table 26: Antifungal activities of hexane, chloroform, and ethanol water and methanol fraction of roots of *R. nepalensis* by using agar well diffusion methods

Fungal species	Solvent	Mean fungal inhibition in millimeter ± SEM				MIC mg/ml	MBC mg/ml
		Extracts in mg/well			Ketokonazole 30µg		
		6	3	1.5			
C. albicans	n-Hex	-	-	-	15.34±0.76	-	-
	$CHCl_3$	-	-	-	14.76±0.87	200	400
	EtOH	14.1±0.2	12.47±0.29	10.93±0.23	15.93±0.34	12.5	25
	H_2O	13.47±0.09	11.1±0.1	10.27±0.37	15.64±0.41	12.5	50
	MeOH	-	-	-	15.45±.46	-	-

Note: $CHCl_3$ = chloroform; DMSO = dimethylsulfoxide., EtOH = ethanol; H_2O = water; MeOH = methanol; n-Hex = normal hexane; SEM = Standared error of the mean

5.3.9 Antifungal test determination of CC fractions and refractions of the roots of *R. nepalensis*

The ethanol fractions showing good antifungal activities was again fraction by using column chromatography in three solvents system (Chloroform, ethyl acetate and ethyl acetate: methanol). The ethyl acetate: methanol column Chromatographic fractions of the roots of *R. nepalensis* inhibited *C. albicns* (15.67±0.46 mm) as shown in the Table 27. This inhibitory value is slightly higher than the ethanol fractions of the roots of *R. nepalensis,* whereas the chloroform

and ethyl acetate isolates did not show any fungal inhibition at at all concentrations as shown in Table 27.

Table 27: Antifungal activities Column Chromatographic fractions *R. nepalensis* by agar well diffusion methods

	Mean fungal inhibition in millimeter ± SEM						
Fungal	CC extracts fraction in (mg/well)			CC extracts re-fraction in (mg/well)			Ketokonazole
type	$CHCl_3$(0.24)	EtOAc (1.2)	EtOAc: MeOH (3.6)	$CHCl_3$ (0.18)	n-Hex (0.27)	MeOH (2.7)	30µg/ well
C. albicans	6.97±0.26	-	15.67±0.46	-	-	15.33±0.22	21.08±0.17

Note: $CHCl_3$ = chloroform; DMSO = dimethylsulphoxide; EtOAc = ethyl acetate; MeOH = methanol; SEM = Standared error of the mean

5.3.10 Antifungal test determination of CC re-fractions of the roots of *R. nepalensis*

The column Chromatographic fractions that showed higher antifungal activities, ethyl acetate: methanol was further fractionnated by CC for fungal assay. The methanol CC re-fractions inhibited *C. albicns* (15.33±0.22 mm) (Table 27), whereas the chloroform and hexane isolates had no fungal inhibition at concentration of 0.75 mg/well as it is shown in Table 27. As it is shown in Table 26 and 27 the fungal inhibition increased as the concentration of the solvent extract, fraction of the roots of *R. nepalensis* increased.

The fungal inhibition of crude methanol extracts of the roots of *R. nepalensis* at 6 mg/well was comparatively lower than its ethanol fractions. As the roots of *R. nepalensis* fractionnated through solvent-solvent fractionation and through column chromatography fractionation, its antifungal potential slightly increased on *C. albicns.* This can be interpreted as the secondary metabolites which inhibit *C. albicns* were probably dissolved in ethanol and water and magnified in ethanol fractions.

5.4 Conclusions

In this work, among the crude extracts, the crude methanol extracts of roots of *R. nepalensis* performed higher antimicrobial potentials on the test pathogenic bacteria and fungus were further fractionnated by using column chromatography method. The data showed the agar well diffusion method was better than in the determination of bacterial and fungal susceptibility test compared to the paper disc diffusion method.

In this study, the ethanol fraction of the roots of *R. nepalensis* at the concentration of 6mg/well performed higher antibacterial potentials on *P. aeruginosa, S. aureus* and *S. flexneri* than the antibacterial activities of its crude methanol and its column chromatographic fraction and re-fractions. On the other hand, the crude hexane extracts of the roots of *R. nepalensis* showed higher inhibitory activities on *S. typhimurium.* On the contrary, the crude extracts, solvent fractions and the CC fractions of and refraction the root had no inhibitory activities on *K. pneumonia. R. nepalensis* has medicinal properties and is widely distributed in Ethiopia, and its secondary metabolities could be dissolved polar solvents such as methanol and ethanol and polar solvent (water).

6. GENERAL CONCLUSIONS AND RECOMMENDATIONS

Rumex nepalensis and *Centella asiatica* were tested for antiplamodial, anitibacterial, antifungal, acute toxicity and also subjected to phytochemical analysis. In this work, the crude methanol, water and hexane extracts of the roots, leaves of R. *nepalensis* and aerial parts of *C. asiatica* showed plasmodial suppression *in vivo* in albino mice. Similarly in antibacterial and antifungal susceptibility tests, the crude methanol, water and hexane extracts of the roots of R. *nepalensis*, the leaves of R. *nepalensis* and aerial parts of *C. asiatica* were active against the test pathogenic bacteria (*E. coli, P. aeruginosa, S. flexneri and S. typhimurium, S. aureus*) and fungus (*C. albicans*). As a result methanol extracts of the roots *R. nepalensis* which had higher antiplasmodial and antimicrobial activities was further fractionnated.

In this work the antibacterial susceptibility test carried out by agar well diffusion was more revealing antibacterial susceptibility than than test done by paper disc diffusion method on the tested pathogenic bacteria. The ethanol fractition of the roots *R. nepalensis* showed higher plasmodium suppression, bacterial and fungal inhibition. The ethyl acetate: methanol CC had significantly higher *Plasmodium* suppression (73.66%) in mice at 300 mg/kg BW and significantly reduced the loss of BW and PCV values the infected mice. Similarly 150 mg/kg BW methanol re-fraction showed the highest percent suppression (79.66) of *Plasmodium in vivo* in mice.

From the above data it can be concluded that the percent suppression of *P. berghei* in mice increased as the roots of *R. nepalensis* were further fractionnated by using solvent fraction (ethanol), CC (ethyl acetate: methanol) and re-fractionnated by using methanol as eluating solvent. Whereas the ethanol fraction of the roots of *R. nepalensis* performed higher antibacterial

potentials on *P. aeruginosa, S. aureus* and *S. flexneri* at the concentration of 6 mg/well than the antibacterial activities of its crude methanol and its CC fraction and re-fractions.

In oral acute toxicity test, crude methanol and hexane extracts of the root, the leaf extracts of *R. nepalensis* and the aerial parts of *C. asiatica* treated mice did not show observable clinical signs and there were no death within 14 days at at the highest oral dose of 5000 mg/kg BW. The extracts of the two plants did not affect the BW of the mice at highest oral doses of 5000 mg/kg BW within 14 days follow up. Hence the two plants were not toxic to the albino mice at or lower 5000 mg/kg BW.

To possibly isolate the responsible active compounds of the roots, leaves of R. *nepalensis* and aerial parts of *C. asiatica* phytochemical screening was used and of ethanol fractition of the roots *R. nepalensis* was further analyzed by CC and TLC. The phytovhemical screening tests of the two plants revaled the presence of major secondary metabolites which include alkaloids, anthraquinone, flavonoids, glycosides, triterpnoids and saponins. In addition to the above metabolites, *R. nepalensis* contained tannin. The CC and TLC techniques identified the presence of emodin and chrysophanol in the roots of *R. nepalensis*, but the CC and TLC techniques alone did not identify the five unknown compounds. Therefore more material was required to go on further isolation technique.

It can be recommended that there is the need to conduct detail phytochemistry research on the two plants to isolate the responsible metabolites in the compound level. The crude extracts of *R. nepalensis* and *C. asiatica* were not toxic to albino mice at highes oral dose of 5000 mg/kg BW. Therefore the two medicinal plants, especially *R. nepalensis* which is available in different

agronomic region of Ethiopia should be considered for further compound isolation and identifications.

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List of Appendices

Appendix 1: Ethical Clearance

COLLEGE OF NATURAL & COMPUTATIONAL SCIENCES
Addis Ababa University

OFFICE OF THE DEAN
የዲን ጽ/ቤት

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CNSDO/ 475/08/2016
August 4, 2016

To Whom It may Concern

The College of Natural & Computational Science Institutional Review Board (CNS-IRB) Committee in its meeting held on 22/07/2016 Minute No. IRB/022/2016 has examined the PhD. project proposal entitled **"Evaluation of the antiplasmodial, antibacterial and antifungal properties of the medicinal plants Rumex nepalensis Spreng and Centella Asiatica L.",** by Asmamaw Habtamu from the Microbial Cellular and Molecular Biology.

The proposal is approved for implementation.

With regards,

Shibru Temesgen /Dr.
Dean , College of Natural & Computational Science

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Please Quote our reference number in you correspondence

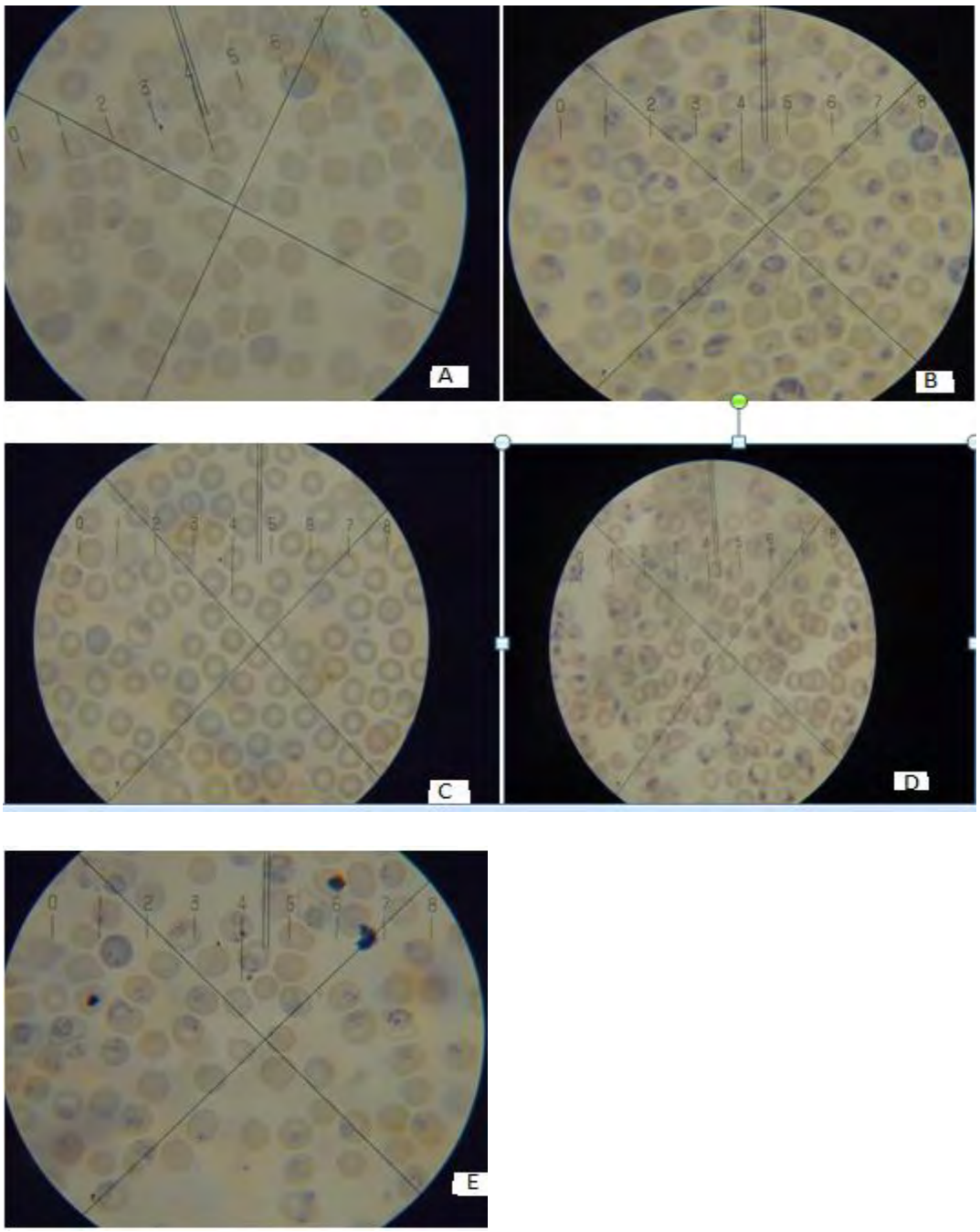
"Examine all things; hold fast that which is good"

"ሁሉን መርምሩ መልካሙን ያዙ"

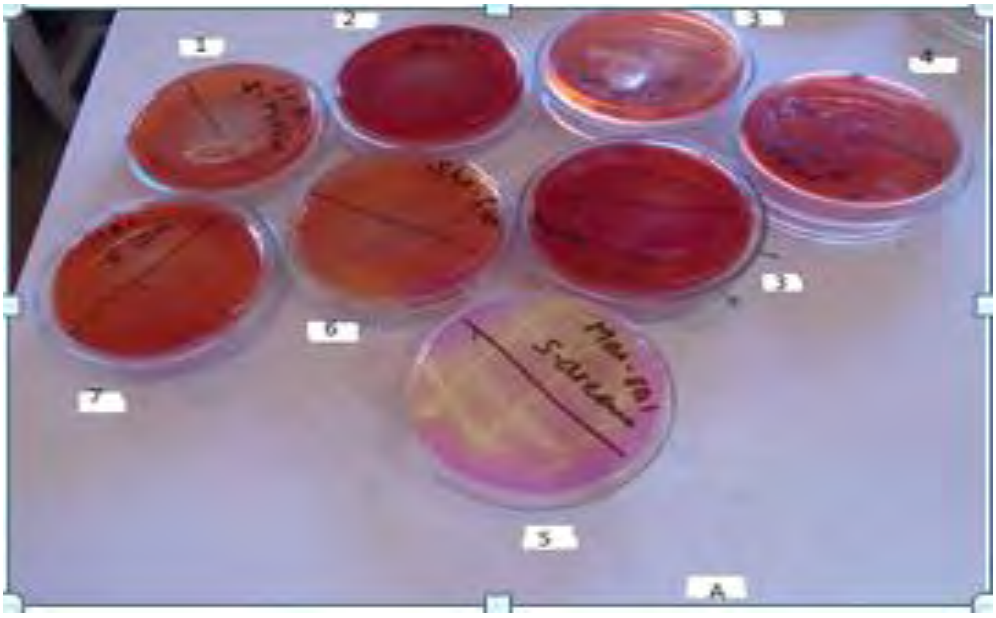
Appendix 2: Some pictures of the research work

Collection *of R. nepalensis* and *C. asiatica* plant from Dejen District (A)

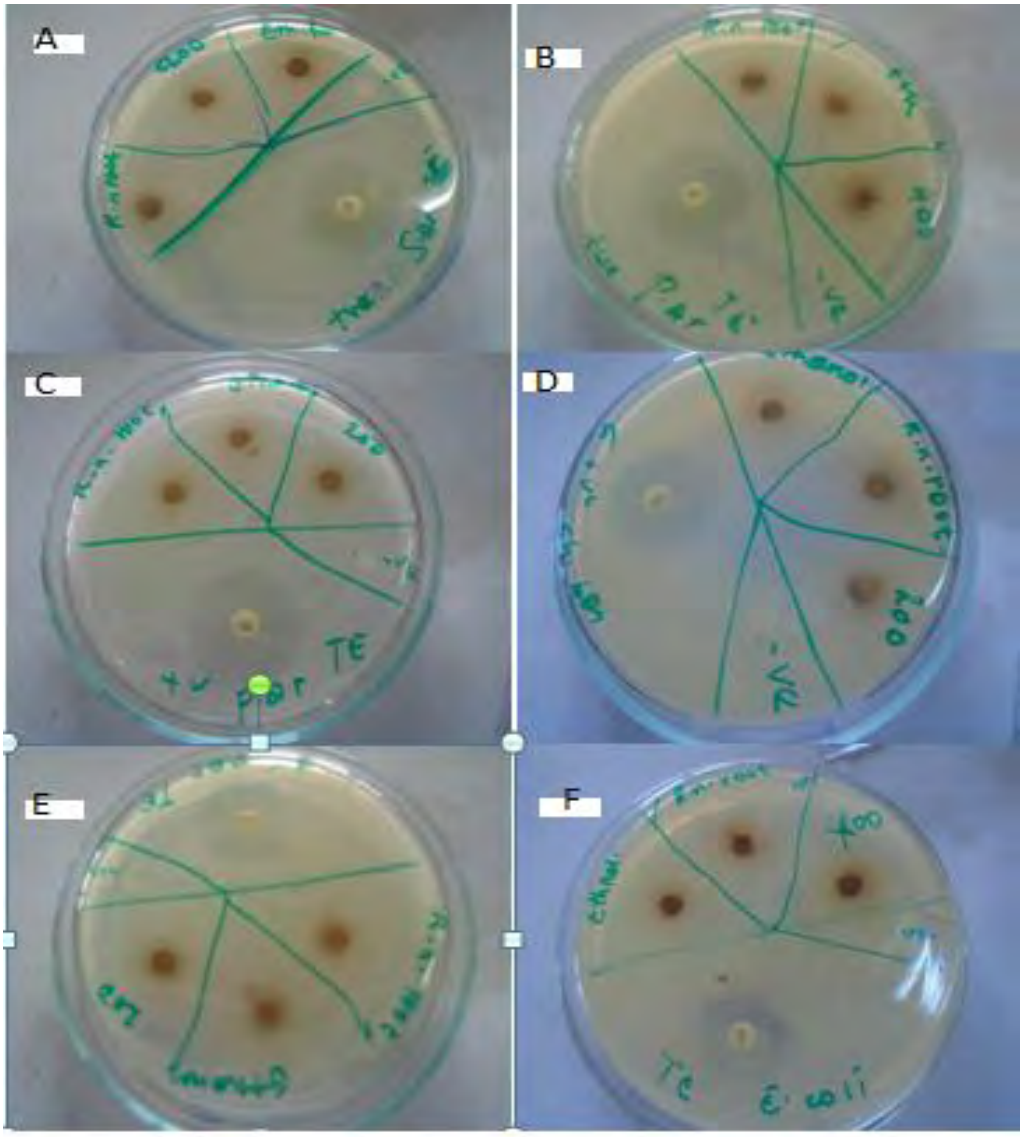
Extract administration (A), blood taking from mice (B), smear preparation (C), PCV preparation (D)



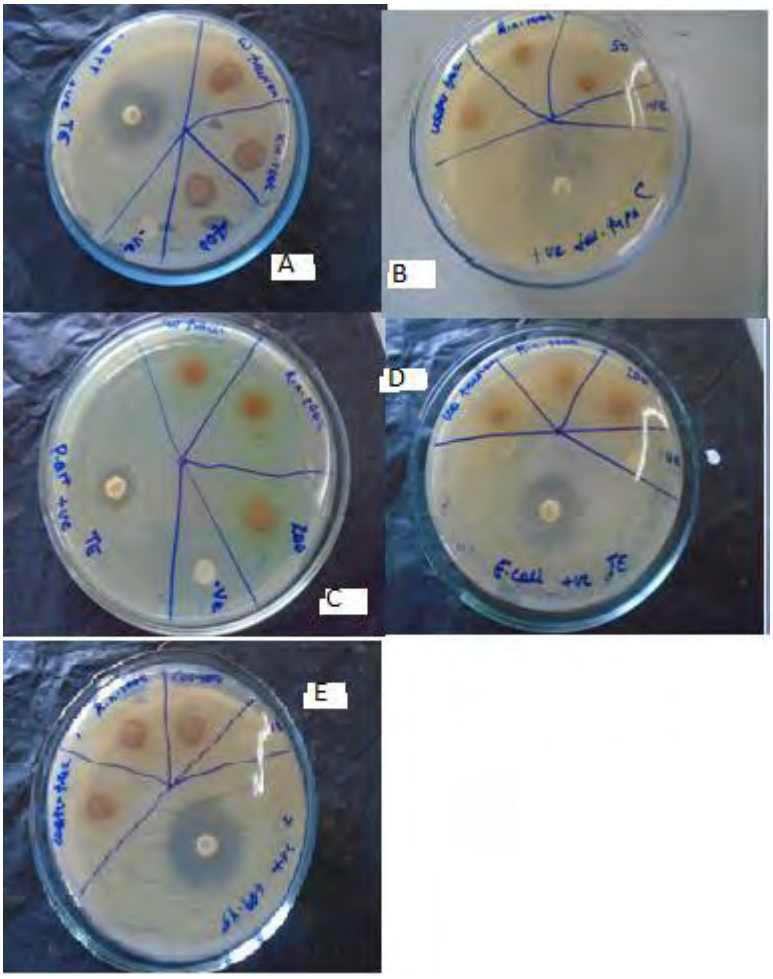
Blood smear of chloroquine (A), negative control (B), methoanol CC re-fraction (C), hexane CC re-fraction (D), chloroform CC re-fraction (E) treated mice.



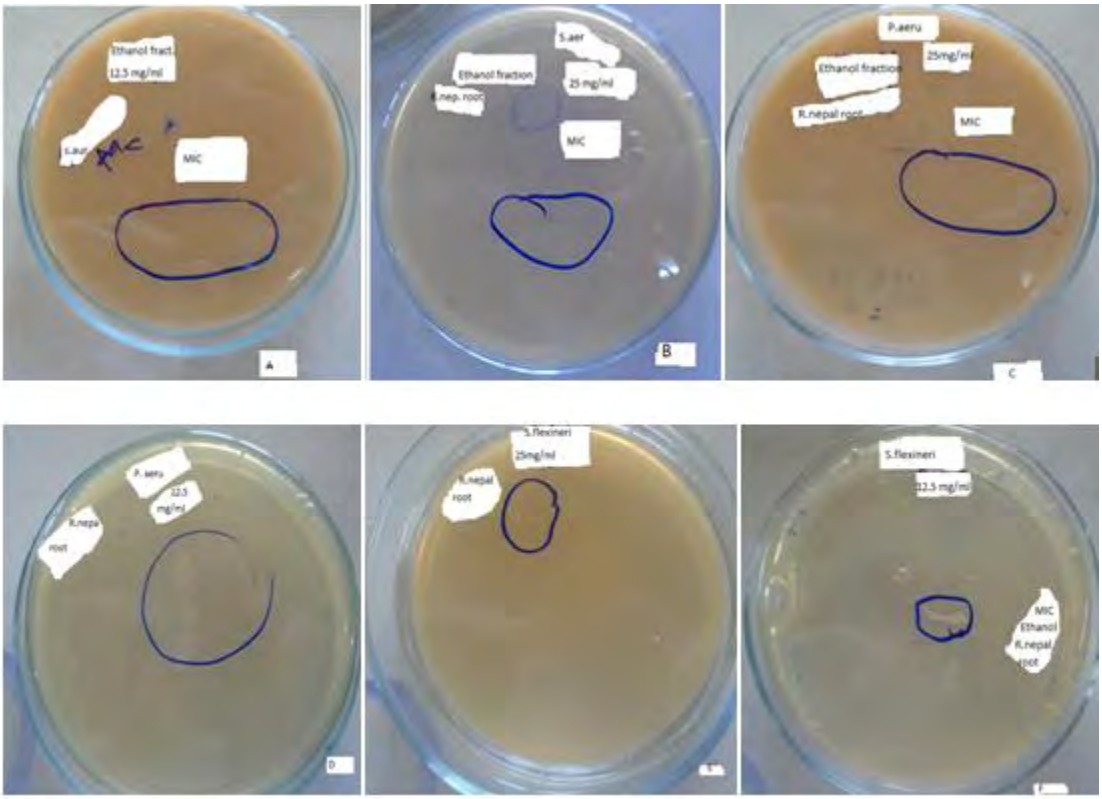
Bacterial culture on selected agar (A) *S. typhimurium* (1, 3), *K. pneumoniae* (2), *P. aeruginosa* (4), *S. flexneri* (6), *E. coli* (7)., bacterial growth in incubator (B).



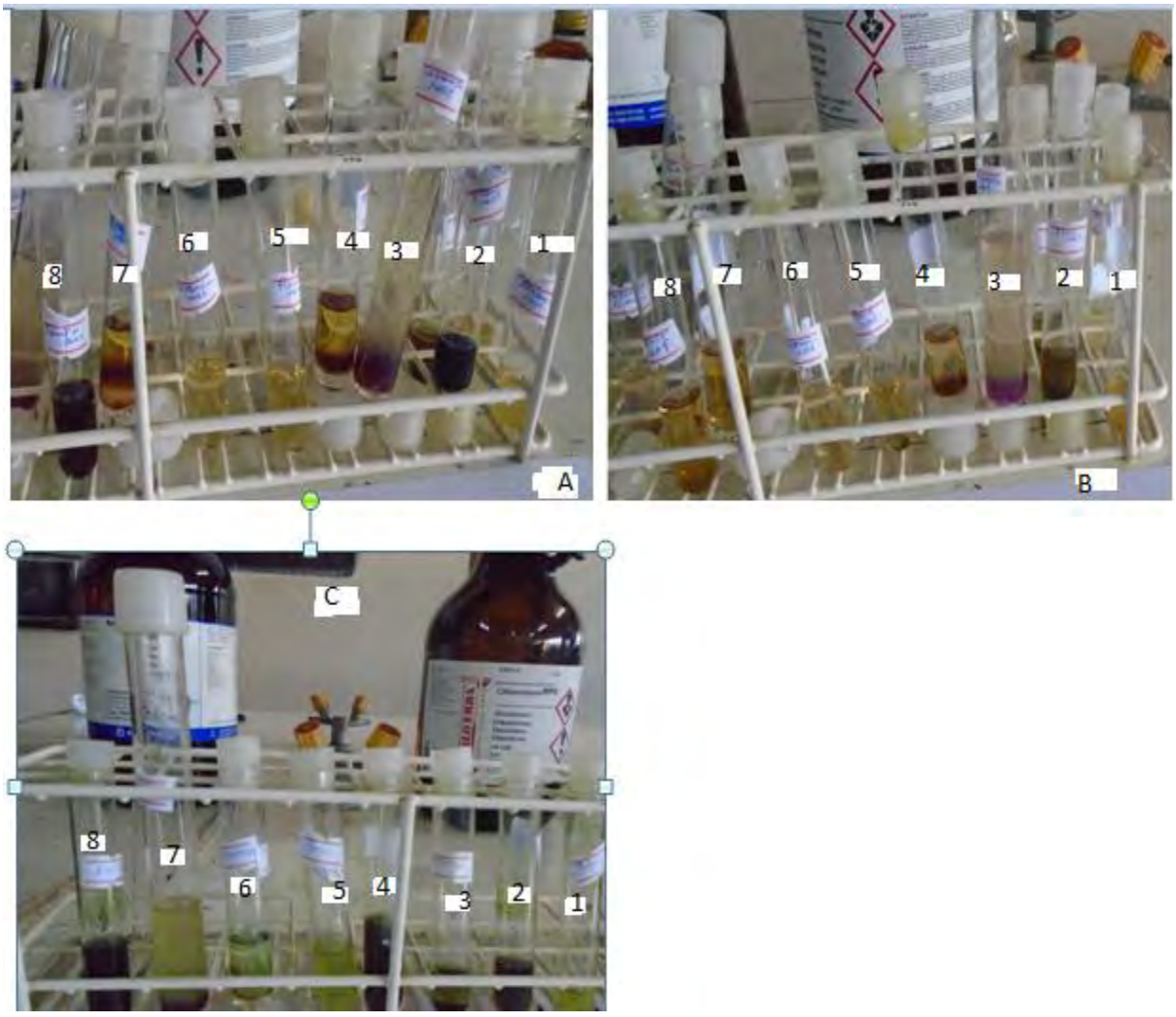
Inhibition zone of of ethanol fraction of *R. nepalensis* on *S.aerus* (A), *P. aeruginosa* (B,C), *S. flexneri* (D), *E. coli* (F), *S. typhimurium* (G) by disc diffusion method



Inhbition zone of of water fraction of *R. nepalensis* on *S. aerus* (A), *S. typhimurium* (B) *P. aeruginosa* (C), *E. coli* (D), *S. flexneri* (E)) by disc diffusion method



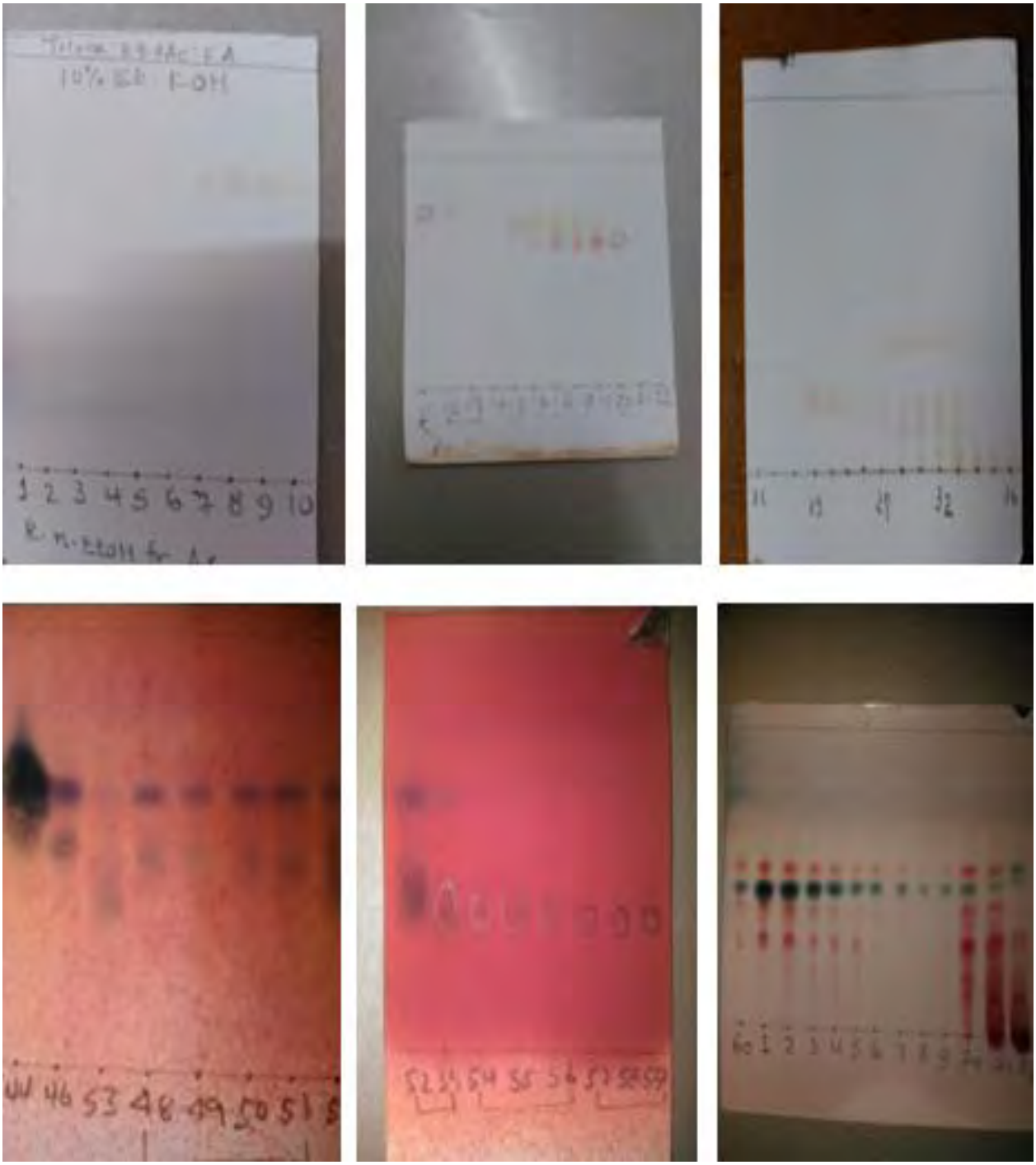
MIC of ethanol fraction of *R. nepalensis* at 12.5 mg/ml on *S. aureus* (A). 25 mg/ml *S. aureus* (B), 25 mg /ml *P. aeruginosa* (C), 12.5 mg /ml *P.aeruginos,* 25 mg /ml *S. flexneri* (D), 12.5 mg /ml *S. flexneri* (E).



Phytochemical screening tests of the methanol extracts of roots of *R. nepalensis* (A), leaves of *R. nepalensis* (B), *C. asiatica* (C), alkaloids (1), flavonoids (2), glycosides (3), triterpnoids (4), resin (5), saponin (6), steroid (7) tannin (8)

frns	Solvent	Vol.	frns	Solvent	Vol.	frns	Solvent	Vol.
1	Hex:CHCl3		24	2:8	30 ml	47	EtAc-EtOH	30 ml
2	10:0	30 ml	25	2:8	30 ml	48	9:1	30 ml
3	10:0	30 ml	26	2:8	30 ml	49	9:1	30 ml
4	9:1	30 ml	27	1:9	30 ml	50	8:2	30 ml
5	9:1	30 ml	28	1:9	30 ml	51	8:2	30 ml
6	9:1	30 ml	29	1:9	30 ml	52	7:3	30 ml
7	8:2	30 ml	30	CHCl3:EtOAc	30 ml	53	7:3	30 ml
8	8:2	30 ml	31	10:0	30 ml	54	6:4	30 ml
9	8:2	30 ml	32	10:0	30 ml	55	6:4	30 ml
10	7:3	30 ml	33	9:1	30 ml	56	5:5	30 ml
11	7:3	30 ml	34	9:1	30 ml	57	5:5	30 ml
12	7:3	30 ml	35	8:2	30 ml	58	6:4	30 ml
13	6:4	30 ml	36	8:2	30 ml	59	6:4	30 ml
14	6:4	30 ml	37	7:3	30 ml	60	3:7	30 ml
15	6:4	30 ml	38	7:3	30 ml	61	3:7	30 ml
16	5:5	30 ml	39	6:4	30 ml	62	2:8	30 ml
17	5:5	30 ml	40	6:4	30 ml	63	2:8	30 ml
18	5:5	30 ml	41	5:5	60 ml	64	1:9	30 ml
19	4:6	30 ml	42	4:6	60 ml	65	1:9	30 ml
20	4:6	30 ml	43	3:7	60 ml	66	1:9	30 ml
21	3:7	30 ml	44	2:8	60 ml	67	0:10	30 ml
22	3:7	30 ml	45	1:9	60 ml	68	0:10	30 ml
23	3:7	30 ml	46	0:10	60 ml	69	0:10	30 ml

Column Chromatography of ethanol extract of roots of *R. nepalensis*



TLC profile of CC fraction of of the ethanol fractions of the roots of *R. nepalensis* btained in tolune: Ethyl acetate: Formic acid (5:4:1) solvent system.

Appendix 3

Declaration

I, the undersigned declare that this Dissertation is my original work and it has not been presented in other universities, colleges or institutes for a degree or other purpose. All sources of the materials used have been duly acknowledged.

Name: Asmamaw Habtamu Signature  Date Feb. 16, 2017

This work has been done under my supervision

Name Yalemtsehay Mekonnen (Prof) signature  Date Feb. 17, 2017

Prepared based on AAU's Thesis Writing, Examination and Grading Guidelines