Evaluation of the antibacterial activity of the solvent fractions of the leaves of *Rhamnus prinoides* L'Herit (Rhamnaceae)

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This is to certify that the thesis prepared by Yalew Molla entitled "Evaluation of the antibacterial activity of the solvent fractions of the leaves of *Rhamnus prinoides* L'Herit (Rhamnaceae)" and submitted in partial fulfillment of the requirements for the degree of Master of Science in pharmacology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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ABSTRACT

Evaluation of the antibacterial activity of the solvent fractions of the leaves of *Rhamnus prinoides* L'Herit (Rhamnaceae)

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Infectious diseases are the critical problems of the world as a result of the emergence of different antimicrobial resistant microorganisms. Medicinal plants play great roles in the treatment of various infectious diseases. *Rhamnus prinoides* is one of the the medicinal plants used traditionally for treatment of bacterial infections. The antibacterial activity of the crude extract of the plant had been shown by a previous study, but this study was undertaken to further the claimed medicinal use of the plant by screening its solvent fractions for the said activity so that it could serve as a basis for subsequent studies. The solvent fractions of the plant were obtained by successive soxhlet extraction with solvents of increasing polarity, with chloroform and methanol, followed by maceration of the marc of methanol fraction with water. The antibacterial activity of the solvent fractions was evaluated on seven bacterial species using agar well diffusion method at different concentrations (780 mg/ml, 390 mg/ml and 195 mg/ml) in the presence of positive control and negative control. The minimum inhibitory concentration of the solvent fractions was determined by micro-broth dilution method using resazurin as indicator. Methanol and chloroform fractions revealed antibacterial activities against the growth of test bacterial strains with varying antibacterial spectrum and the susceptible bacterial species were *Staphylococcus aureus, Streptococcus pyogen, Streptococcus pneumoniae* and *Salmonella typhi*. The strains of *Pseudomonas aeruginosa* and *Escherichia coli* were

not sensitive to chloroform fraction. The average minimum inhibitory concentration value of the methanol and chloroform fractions ranged from 8.13 mg/ml to 32.5 mg/ml and from 8.13 mg/ml to 16.25 mg/ml, respectively. In conclusion, the methanol and chloroform fractions demonstrated significant antibacterial activities against the growth of pathogenic bacteria unlike the aqueous fraction.

Key words: Antibacterial activity, agar well diffusion, minimum inhibitory Concentration, *Staphylococcus aureus, Streptococcus pyogen, Streptococcus pneumoniae, Salmonella typhi Pseudomonas aeruginosa*, *Escherichia coli*.

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ABBREVIATIONS/ACRONYMS

ANOVA	Analysis of Variance
BHI	Brain Heart Infusion
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Units
CLSI	Clinical Laboratory Standard Institute
DMSO	Dimethyl Sulfoxide
ENT	Ear, Nose and Throat
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome
MBC	Minimum Bactericidal Concentrations
MDR	Multi-Drug Resistance
MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentration
SPSS	Statistical Package for the Social Sciences
TDR	Tropical Drug Research
USAID	United State Agency for International Development
WHO	World Health Organization

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1 INTRODUCTION

1.1 Overview of infectious diseases

Infectious diseases are among leading causes of death in the world, in the face of major medical advances (WHO, 2012; Xiang *et al*., 2008). In addition, infectious diseases are the key agents for aggravating poverty in the world and causes enormous health related burden through life long disability (TDR, 2012). According to the WHO death projection (2013), infectious diseases will remain to be the killer diseases with a level of about 13 million human deaths annually until at least in 2030 G.C. Although infectious diseases can affect people of all ages, they impose a particular burden on children of age below five years (TDR, 2004). The negative impacts of infectious diseases are highly observed in developing countries (Mulder *et al*., 2014). In high income countries, the deaths from infectious disease are mainly due to respiratory infections and Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome (HIV/AIDS) whereas in sub-sahran African countries, respiratory infections, diarrheal disease, HIV/AIDS, malaria and tuberculosis account for roughly similar proportions of total infectious disease deaths (TDR, 2004).

In addition to the problems of the known or established infections, epidemics of new and old infectious diseases periodically emerge which greatly magnify the global burden of infections (Morens *et al*., 2004). Emerging infectious diseases can be defined as infections that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range. To mention some examples, the emerging infectious disease includes HIV/AIDS, hemolytic uremic syndrome (caused by *Escherichia coli* O157:H7) (Morse, 1995) and Ebola (Barron and Leung, 2015).

Emerging infectious diseases are part of the infectious disease having a significant burden on global economies and public health (Jones *et al*., 2008). The contributing factors for emerging infectious diseases include ecological changes (those due to economic development and land use), human demographics behavior, international travel and trade, technology and industry, microbial adaptation and change, and break down in public health measures. These changes can lead to the emergence of new diseases, the re-emergence of diseases once controlled, and to the development of antimicrobial resistance (Cohen, 2000; Morse, 1995). The majority of emerging infectious events are caused by bacteria which can be associated with evolution of drug resistant microbes (Jones *et al*., 2008).

The incidence of bacterial infections can be affected by the occurrence of newly emerging disease. For example, the emergence of infectious disease like HIV/AIDS has caused the increase in the incidence of opportunistic infections in both the developed and developing countries of the world (Seddon and Bhagani, 2011). Among the opportunistic infections, bacterial infections are common since they occur when the natural host defenses like neutrophils are overwhelmed by disease causing agents like HIV (Malka *et al*., 2010).

Therefore, it is possible to implicate that researches focusing on the causes of infectious disease and how to effectively treat and prevent them from spreading have to be encouraged to effectively lift people out of infection and to build a better world for future generations.

1.2 Antimicrobial resistance

Antimicrobials are an extremely valuable resource across the spectrum of modern medicine to treat and prevent infectious diseases. Their development has been associated with dramatic reductions in communicable disease mortality and has facilitated technological advances in cancer therapy, transplantation, and surgery (Dixon and Duncan, 2014). However, after a certain time of antibacterial usage, some bacterial pathogens became unresponsive to many of the first effective drugs. For example, the resistance feature or evolution of *Staphylococcus aureus* had not been stopped to the first effective drug, benzylpenicillin, but continued to be resistant to different antibiotics. For example, this bacterium became resistant to methicillin and vancomycin by developing different mechanism of resistance such as production of a β-lactamase and increased cell wall production; which in turn decreased the usefulness of such drugs for serious staphylococcal infections (Howden *et al*., 2014; Livermore, 2000). In addition, antibacterial resource is threatened by the dwindling supply of new antimicrobials and the global increase in antimicrobial resistance (Dixon and Duncan, 2014).

Even though the antibiotic resistance is a global threat, its burden is higher in developing countries like Ethiopia because of the high prevalence of bacterial diseases and the presence of risk factors for its emergence and spread (Huynh *et al*., 2015). Rates of antimicrobial resistance among hospital and community pathogens have increased considerably. This is especially more common in clinic settings in which resistant strains are frequently found before they spread to the community (Canton *et al*., 2013). Hence, antibacterial resistant infections would be likely more common in immuno-compromising conditions like HIV/AIDS patients due to their frequent encounters with

the health care system, need for empiric antimicrobials, and immune dysfunction (McNeil, 2014). The increasing prevalence of hospital and community-acquired infections caused by multi drug- resistant (MDR) bacterial pathogens is the limiting option for effective antibiotic therapy. Moreover, this alarming spread of antimicrobial resistance has not been paralleled by the development of novel antimicrobials (Cassir *et al*., 2014).

Therefore, the ongoing explosion of antibiotic-resistant infections continues to be global health care problem with an equally alarming decline in the research and development of new antibiotics to deal with the threat (Cai *et al*., 2011; USAID, 2015). According to Centers for Disease Control and Prevention (CDC) estimation (2013), antibiotic resistant bacterial infections in United States caused more than two million people to be sickened and resulted in a death of at least 23,000 patients every year. In addition, antibiotic resistance has contributed to an increase in economic burden to United States, which may be as high as $20 billion in excess direct healthcare costs and $35 billion due to lost productivity of society.

In addition, even though it is difficult to quantify the consequences of bacterial resistance, its predictable consequence include increased morbidity, prolonged illness, a greater risk of complications and higher mortality rates. The increased disease morbidity has been associated with the increased outbreaks of bacterial infections like shigellosis, typhoid fever and pneumococcal infections. The economic burden of antibacterial resistance includes loss of productivity and increased health care cost of patients as a result of prolonged treatments, the use of expensive alternative antibiotics with serious

side effects, and extended hospital stays (Acar, 1997; Hunter and Reeves, 2002; WHO, 2012).

Generally, antibiotic resistance, with the current rising up rates of antimicrobial resistance, insufficient research and development activities; irrational use of antibiotics for humans and food animals; and uncoordinated overall international technical and non-technical efforts, could result in a time in which treatment of many of the infections is impossible (Spellberg *et al*., 2008). Thus, in order to address the impact of antimicrobial resistance on medical, social, and economical burdens, real and unreserved global coordinated efforts, paralleled with the development of new antibacterials, led by the health professionals have to be taken.

1.2.1 Causes of antimicrobial resistance

The emergence and spread of bacterial resistance have been driven by complex socioeconomic and human behavioral factors including misuse of antibiotics, the practice of unskilled practitioners and laypersons, and poor drug quality, particularly in developing countries (Okeke *et al*., 1999). In addition, the spread of resistant bacteria in hospitals and the community can be enhanced by overcrowding, lapses in hygiene or poor infection control practices (Michael *et al*., 2014; Rao, 1998).

The emergence of antibiotic resistance is primarily due to excessive and often unnecessary use of antibiotics in humans and veterinary medicine. The rates of bacterial resistance to the antibiotics have been increased proportionally with the volume of antibiotics used for different purposes; the more these drugs are used the quicker resistant strains emerge and spread (Austin *et al*., 1999; Wise *et al*., 1998) as a result of selective

resistance pressure induced by antibiotic overuse. For instance, the prevalence of penicillin resistant S*treptococcus pneumoniae* and S*treptococcus pyogen* has been associated with an increase in the outpatient antibiotic use in different countries (Albrich *et al.*, 2004). There are various factors which may influence an increase in irrational antibiotic use and hence antibacterial resistance, but lack of awareness and knowledge of the people to the antibiotics is the main factor which has strong impact on the non-adherence and inappropriate use of most antibacterials (Chan *et al*., 2012; McNulty *et al*., 2007). The use of counterfeit and substandard medications is the other component of irrational drug use aggravating the development of drug resistance as they may have insufficient or suboptimal dose to kill or inactivate microorganisms (Kelesidis *et al*., 2007).

On top of the contribution of irrational use of antibacterial by humans for bacterial resistance, inappropriate use in animals has shown to play for the prevalence of bacterial resistance. Even though the use of antibiotics in animals has its own medical reasons, its overuse for farm animals, especially for growth promotion and prevention of infection, contribute to the increased prevalence of antibiotic-resistant bacteria of human significance (Khachatourians, 1998**;** Mathew *et al*., 2007).

Self‐medication is another global practice and potential contributor to human pathogen resistance to antibiotics, with a risk of causing serious health hazards such as adverse reaction and prolonged suffering. Self-medication of antimicrobials without medical guidance may result in greater probability of inappropriate, incorrect, or undue therapy, delays in appropriate treatment; which in turn can contribute to the emergence of bacterial resistance and increased morbidity (Bennadi, 2014**)**. Self-medication with

antimicrobial agents has been associated with low income, lack of drug knowledge, advice from friends, previous experience, long waiting time in health settings and distance to the health facility (Ganesan *et al*., 2014; Ocan *et al*., 2014). The main sources of self medication without prescription are the different retail pharmacies (Ganesan *et al.*, 2014).

The extent of antibacterial resistance is not only attributed by the inappropriate use of antibiotics by patients but prescribers also contribute a lot. For example, bacterial selective pressure due to inappropriate glycopeptide and vancomycin prescribing has increased the prevalence of multidrug resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Shorr, 2007). Over prescribing of antibiotics is associated with an increased risk of adverse effects, more frequent re-attendance and increased medicalization of self-limiting conditions. Viral infections are the primary driving forces for over prescribing of antibiotics, particularly in primary health care. Among the different bacterial diseases, respiratory infections are the leading infection for antibiotic prescriptions (Lior and Bjerrum, 2014).

1.2.2 Molecular mechanisms of antibiotic resistance

Antibiotic resistance is the ways by which bacteria reduce the effectiveness of antibiotics to cure or prevent infections. Resistance mechanisms can be classified as innate or acquired.

Intrinsic or innate resistance mechanism is a natural characteristic or phenomenon of all isolates of particular bacterial species (Cox and Wright, 2013; MacGowan and Macnaughton, 2013). Intrinsic mechanisms are those specified by naturally occurring

genes found on the bacterial chromosome. The common intrinsic mechanism is mainly due to the presence of outer cell membrane in gram negative bacteria and the expression of efflux pumps by different bacteria (Alekshun and Levy, 2007; Cox and Wright, 2013). For instance, *Pseudomonas aeruginosa* is innately resistant to different bioactive compounds by expressing AmpC β-lactamase and efflux pumps, together with its outer membrane barrier (Strateva and Yordanov, 2009). The resistance of all gram-positive organisms to colistin and the resistance of *Enterobacteriaceae* to glycopeptides and linezolid are also inherent (MacGowan and Macnaughton, 2013).

Bacteria can also develop antibiotic resistance as a result of genetic changes. The genetic change accounting for antibacterial resistance can be obtained by chromosomal mutation or by resistant gene transfer from one microorganism to other, of the same or different species (MacGowan and Macnaughton, 2013; Tenover, 2006). Although chromosomal mutations contribute a lot for antibiotic resistance, acquired resistance is most commonly associated with horizontal transfer of mobile genetic elements from other bacteria in the environment (Alekshun and Levy, 2007). The common mobile genetic elements carrying the resistance gene are plasmids, transposons, and integrons. These genetic elements are transferred from one bacterium to the other by different methods. The methods include conjugation; which requires cell to cell contact between cells, transduction; which is bacteriophage-facilitated transfer of genetic information, and transformation; which is the uptake of free DNA from the environment (Alekshun and Levy, 2007; Kelly *et al*., 2009).

After a bacterium possesses resistant genes, it expresses and develops different biochemical resistance mechanisms to become insensitive to the antibiotics. Bacteria can use several biochemical types of resistance mechanisms, but the main ones are antibiotic

inactivation, target modification, changes in permeability and altering metabolic pathway (Giedraitiene *et al*., 2011; Tenover, 2006). For instance, altered target sites, β-lactamase production, decreased antibiotic penetration, and efflux pumps are the common mechanisms of resistance in gram-negative pathogens (Oliphant and Eroschenko, 2015). A single bacterium can develop antibiotic resistance either by using one of the resistance mechanisms or by combination of the above resistance mechanisms.

Enzymatic degradation or modification of antibiotic is the common mechanism of resistance in bacteria. Different bacteria resist the action of penicllins and cephalosporins by producing β-lactamse enzyme that breaks their β-lactam ring. *Pseudomonas aeruginosa* and *enterobacteriaceae* became insensitive to β-lactam antibiotics like cephalosporins by producing extended spectrum β-lactamase that destroys such antibiotics (Potron *et al*., 2015; Shaikh *et al*., 2015). Moreover, the action of different antibiotics is lost by the chemical modification mechanisms including acylation, phosphorylation, glycosylation, nucleotidylation, and ribosylation (Wright, 2005).

The interaction of antibiotic to its target site in bacteria is usually very specific and hence any small change in the targt site will hinder the action of antibacterial drugs. Target modification is a means of bacterial resistance in different bacteria to resist the action of antibacterial agents. For instance, β-lactam antibiotic resistance in *Streptococcus pneumoniae* is associated with the modification of penicillin binding protein, an enzyme used in the assembly of peptidoglycan in cell wall synthesis. In addition, the antibacterial action of quinolones has been reduced by the alteration of its target enzyme, DNA gyrase and Topoisomerase IV, in different bacteria like in *Pseudomonas aeruginosa* and

Staphylococcus aureus. This target modification of bacteria for quinolones is usually taken place by chromosomal mutations (Hooper, 2001; Lambert, 2005).

For antibacterial activity, most drugs have to enter to the interior component of the bacterial cell. But, bacteria can develop mechanisms that extrude drugs out of the cell before its entry. The permeability barriers or reduced antibiotic uptake and active efflux of drug molecules are the resistance mechanisms that have been associated with various infectious outbreaks of antibiotic resistant pathogens, especially gram negative bacteria (Kumar and Schweizer, 2005). For example, the efflux pump resistance mechanism has been detected in some pseudomonas species against the action of ciprofloxacin and levofloxacin (Sonnet *et al*., 2012).

The last mechanism of antibiotic resistance is by altering the drug target which can bypass the target metabolic pathways. To achieve this mechanism, bacteria produce an alternative protein (usually an enzyme) that have no a target site to the inhibition effect of antibiotics. For example, bacteria like *Escherichia coli* develop resistance to trimethoprim and sulphonamides by expressing non-sensitive dihydrofolate reductase and dihydropteroate synthase, respectively, or by over expressing of respective sensitive enzymes used in the folate metabolic pathways (Skold, 2001).

1.2.3 Strategies for combating antimicrobial resistance and bacterial infections

The manner of irrational antimicrobial use associated with resistance should be intervened by implementing rational use of antimicrobials and devising appropriate dosing regimens based on population-specific clinical outcomes (Essack, 2006). In

addition, regulation on over-the-counter availability of antibiotics, improving hand hygiene and improving infection prevention and control are also the critical approaches for the control and prevention of emergence and spread of antimicrobial resistance (Uchil *et al*., 2014).

In order to preserve the activity of currently available antibiotics for future generations, antibiotics should be prescribed when an infection is serious and is likely to respond significantly to treatment. Cautious prescribing will reduce the emergence of bacterial resistance as it will decrease the selective pressure on bacteria (Ferguson, 2004). Moreover, providing local resistance data (Schweizer *et al*., 2013), preparing guidelines for antimicrobial prescribing, training of prescribers, intensified surveillance on antibiotic use have great roles in combating antimicrobial resistance (Dixon and Duncan, 2014). On top of these, a comprehensive education program to the people has to be provided to change the public ideology of antimicrobial usage; especially on the consequences of irrational uses of antibiotic for their generations (Michael *et al*., 2014).

Generally, the rapid detection and control of spread of bacterial resistance; the development and use of rapid and innovative diagnostic tests for identification and characterization of resistant bacteria; development of new antibiotics and vaccines have to be put in the ground in order to alleviate the problems of bacterial resistance . These in turn need the collaborative efforts of all stakeholders involved in health and non-health settings including those involved in healthcare, public health, veterinary medicine, agriculture, food safety, academic, and industrial research (CDC, 2014; Cohen, 1992).

1.3 Treatment approaches for bacterial diseases

A Conventional approaches

Treatment of bacterial infections is becoming more difficult to treat as majority of the pathogenic bacteria become resistant at least to one antimicrobial drug which was effective previously for the causative pathogen. In spite of such difficulties, the commercially available antibacterial drugs play great roles for saving the life of patients with bacterial infections caused by susceptible bacteria (Carmeli, 2008). The common antibacterial agents are often classified according to their principal mechanism of action as cell wall synthesis inhibitors (example, β-lactams and glycopeptide agents), protein synthesis inhibitors (example, aminoglycosides, macrolides and tetracyclines), nucleic acid synthesis inhibitors (example, fluoroquinolones and rifampin), anti-metabolites (example, trimethoprim, sulfonamides) and those that disrupt bacterial membrane structure (example, polymyxins and daptomycin) (Tenover, 2006; Walsh, 2000).

Cell wall synthesis inhibitors, membrane disrupting agents, nucleic acid inhibitors and aminoglycosides are bactericidal, while most protein synthesis inhibitors and anti-metabolites are bacteriostatic. The bactericidal effect of cell wall synthesis inhibition is related with interference of the cell's osmotic defenses and causing it to absorb water and burst. Cell membrane disrupting agents cause lethality of bacterial cell by probably causing loss of vital metabolites (Crofton, 1969). Quinolones also produces bacteriocidal effect by binding DNA gyrase complexed with DNA, which drives double-strand DNA break formation and cell death (Drlica and Zhao, 1997). On top of their specific lethal effects, most bactericidal drugs have been suggested to cause death of bacteria by producing highly deleterious hydroxyl radicals as an end product of oxidative cellular

damage pathway (Kohanski *et al*., 2007). Protein synthesis inhibitors usually interfere with the synthesis of protein at an initiation phase and result in insufficient rather than warped proteins and so prevent the growth and proliferation of the bacteria without actually destroying them. Unlike other protein synthesis inhibitors, aminoglycosides have a lethal effect on bacteria as they cause mistranslation by acting both at initial and latter phase of protein synthesis and result in warped proteins in addition to the decrease in the rate of the protein synthesis (Crofton, 1969; Davis, 1987).

For the clinical purpose, bactericidal drugs are preferred when possible as compared to bacteriostatic agents. This is because of their rapid effect on bacteria, synergistic effectiveness when combined with other drugs against bacteria difficult to eliminate and less likely effect to leave residual organisms as the final elimination of the microorganism is not dependent on the host's defense mechanism (Crofton, 1969).

In order to treat the infectious disorders effectively and to reduce the emergence of bacterial resistance, the practice of antimicrobial therapy should consider the following principles of chemotherapy. These includes susceptibility of the bacteria to concentrations of the antimicrobial agent at the site of infection, the dose and route of administration of the drug, the duration of treatment and the immune status of the host for microbial clearance. In addition, initial empirical therapy to severely diseased patients should be adequately broad spectrum and adequately dosed by considering safety and the pharmacokinetics of the drug in individual patients (Carmeli, 2008; Levison, 2000).

B Traditional approaches of managing bacterial infections using medicinal plants

Herbal medicines have been used for many years dating back as far as 3000 B.C. Despite enormous advances in conventional medicines, traditional medicines have been encouraged by WHO partly because some conventional drugs have failed to prove effective, have serious side effects, or cannot cure certain new illnesses such as AIDS (Kareru *et al*., 2007). It has been estimated that about 65% of the world's population have incorporated medicinal agents into their primary modality of health care (Fabricant and Farnsworth, 2001). In Africa, 90% of the population relies on traditional healers to meet their primary healthcare needs (Kareru *et al*., 2007), which indicate the presence of many medicinal plants with a potential source of new drugs.

The antimicrobial value of medicinal plants relies on some chemical substances produced by these plants: these chemicals called "secondary metabolites" and include alkaloids, terpenoids, flavonoids, tannins and phenolic compounds, essential oils, lectins and polypeptides, polyacetylenes etc (Cowan, 1999).

Herbal products from medicinal plants are preferred because of less testing time, higher safety, efficiency, cultural acceptability and lesser side effects. On top of these, the chemical compounds present in herbal products are part of the physiological functions of living organisms and hence they are believed to have better compatibility with the human body (Prasad *et al.*, 2012). However, the use of traditional plants may be associated with the problems of scarcity of valuable medicinal plants, lack of standardization of methods of preparation, poor storage conditions and incertitude in some traditional health

practitioners, which affect the efficacy and the practice of traditional medicine (Njume and Goduka, 2012).

The crude extracts of different medicinal plants such as *Rhamnus prinoides* leaf extract (Berhanu, 2014), *Calpurnea aurea* leaf extract (Umer *et al*., 2013), *Peterollobium stellatum* root extract *(*Andualem *et al*., 2014), *Datura stramonium*, *Croton macrostachyus,* and *Acokanthera schimperi* extracts *(*Taye *et al*., 2011), etc have been scientifically evaluated and approved for their antimicrobial activity against the growth of different pathogenic bacteria from which novel bioactive drugs could be obtained or derived by devising an appropriate scientific studies.

1.4 The Experimental plant

Rhamnus prinoides L'Herit (Rhamnaceae) is an evergreen shrub or small tree to 7 meter which is ecologically widespread and locally cultivated from medium to high altitudes (1000-3200m) in Moist and Wet Kolla, Weyna Dega and Moist Dega agroclimatic zones of Ethiopia. It is widely planted in gardens (Bekele *et al*., 1993). The plant is not only grows in Ethiopia but also it is widely distributed in different countries of the world including Cameron, Eritrea, South Sudan, through east Africa to South Africa, Angola, and Arabia. In Ethiopia, *Rhamnus prinoides* occurs in Tigray, Gondar, Wollo, Arsi, Gojjam, Welega, Ilubabor, Kefa, Gamo gofa, Sidamo, Bale and Harerge regions. The genus, Rhamnus consists of 150 species (Gebre-Egziabher *et al.*, 1989). In Ethiopia, *Rhamnus prinoides* is commonly known as Gesho (Amharic, Tigrigna and Afan Oromo), Gishe (Guragegna) and Geshu (Agewgna) (Bekele *et al*., 1993).

Rhamnus prinoides has different ethnomedicinal uses in different countries of Africa. In Kenya, traditionally, the roots and leaves of the plant are used in the management of Ear, Nose and Throat (ENT) infections (Njoroge and Bussmann, 2006), gonorrhea, malaria and brucellosis (Muthee *et al*., 2011), fever and snakebites (Fratkin, 1996). In South Africa, the plant has been used as psychoactive agent in the healing of different mental disorders (Sobiecki *et al*., 2002).

In Ethiopia, *Rhamnus prinoides* is used to add flavour to the local drinks, *tella* and *tej* brewed from fermented barley, sorghum or finger millet (Bekele *et al*., 1993; d'Avigdor *et al*., 2014; Gebre-Egziabher *et al.*, 1989). Apart from this, *Rhamnus prinoides* have traditional medicinal uses in the treatment of different disorders. As presented by different ethnobotanical studies, the plant is used to treat tonsillitis, by chewing fresh leaves (Birhanu, 2013; Enyew *et al*., 2014; Kidane *et al*., 2014; Megersa *et al*., 2013) and by applying drops of crushed fruits or shoots nasally (Gebeyehu *et al*., 2014), and uvulitis (Andemariam, 2010; Yirga, 2010). In addition, the different parts of *Rhamnus prinoides* has been used in the management of scabies (applying dried leaf powder) (Gebeyehu *et al*., 2014), hepatitis (taking juices of young shoots) (d'Avigdor *et al*., 2014), tinea capitis (rubing its seed on the affected part), itching/skin rash (Teklay *et al*., 2013), 'chiffea' (Eczema) (applying leaf paste with butter as ointment) (Teklehaymanot and Giday, 2007), ringworm (fresh ripened fruits with the latex of *Croton macrostachyus*) (Enyew *et al*., 2014) and dandruff (Mesfin *et al*., 2013). Moreover, the leaf of the *Rhamnus prinoides* is used for the management of waterborne and related diseases (Siyum and Woyessa, 2013).

The plant is not only traditionally used for human disorders but also used for treating animals as ethnoveterinary agent. For example, the leaf is used for treatment of animal diarrhea and intestinal parasites (after pounded and mixed with water) (Bekele and Mussa, 2009), hepatitis (fresh or dried leaf with other medicinal plants) (Yineger *et al*., 2007) and Leech infestation (pounded leaf) (Bekele *et al*., 2012).

Ethnopharmacological studies revealed that the methanolic extracts of leaves or the root barks of *Rhamnus prinoides* has a pharmacological activities against chloroquine tolerant *Plasmodium berghei* NK65 in mice, either alone or in combination with chloroquine (Muregi *et al*., 2007). Another *in vitro* study presented that the methanolic or aqueous extracts of the root barks of the plant endowed with an antiplasmodial activity against chloroquine sensitive and resistant *Plasmodium falciparum* (Muregi *et al*., 2003). Apart from this, aqueous extract of roots of *Rhamnus prinoides* has been shown to have a potential effect on Alzheimer's disease in addition to its anti-inflammatory and antioxidant activities (Crowch and Okello, 2009). Furthermore, the role of extracts of the leaves and shoots of *Rhamnus prinoides* as bittering and antibacterial activities has been presented in the different phases of 'tella' brewing processes (Berhanu, 2014).

The phytochemical studies indicated that the fruits of *Rhamnus prinoides* contained the anthracene derivatives including physcion, emodin, emodinanthrone, emodin bianthrone and prinoidin (Abegaz and Dagne, 1988). In addition, the leaves of the plant contained different compounds including naphthalenic derivatives (Geshodin, musizin, and sorigenin), anthracene derivatives (physcion, emodin, β-chrysophanol,) and flavonoid derivatives (rhamnocitrin, rhamnazin, and quercetin) (Abegaz and Kebede, 1995).

Figure 1: Photograph of the *Rhamnus prinoides*

1.5 Rationale for the study

Bacterial diseases are the major cause of morbidity and mortality in the world, particularly in developing countries. The current levels of antibiotic consumption, often clinically unnecessary, have led to a steady increase in drug resistance, particularly to antibiotics used in treating high prevalence diseases and hence the effectiveness of many antimicrobial drugs is lost almost as quickly as scientists discover them (Lino and Deogracious, 2006; Rojas *et al*., 2003). Moreover, the empirical use of antibiotics is

associated with the risk of adverse reactions, harmful eradication of normal intestinal flora, and the increase of antimicrobial resistance (Manatsathit *et al*., 2002). On top of these, there is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action due to an alarming increase in the incidence of new and reemerging infectious diseases (Rojas *et al*., 2003).

The treatment of bacterial infections with traditional medicinal plants is common in the world. However, in most of the cases, these practices are handed down from generation to generation empirically without knowing the plausible mechanisms, safety, and efficacy of herbal treatments (Mujumdar *et al*., 2001). In view of this, scientific studies have to be conducted on the traditional medicinal plants to overcome the global problem of antimicrobial resistance and for the purpose of developing a new, effective and safe antimicrobial drug. Even though the preliminary antibacterial activity of the study plant has been conducted previously (Berhanu, 2014), it was necessary to further study its antibacterial activity by using solvent fractions, which in turn could simplify the isolation and identification of active principle(s) responsible for the antibacterial activity of the plant.

2 OBJECTIVES

2.1 General objective

- To evaluate the *in vitro* antibacterial activities of solvent fractions of the leaves of *Rhamnus prinoides* on slected bacterial species.

2.2 Specific objectives

- To assess the antibacterial activities of solvent fractions of the leaves of *Rhamnus prinoides* on selected bacterial species using agar well diffusion method.
- To determine the minimum inhibitory concentration of solvent fractions of leaves of *Rhamnus prinoides* on selected bacterial species using broth dilution method.
- To determine the minimum bactericidal concentration of solvent fractions of leaves of *Rhamnus prinoides* on the selected bacterial Species.
- To qualitatively determine the phytochemical constituents of the solvent fractions of the leaves of *Rhamnus prinoides*.

3 MATERIALS AND METHODS

3.1 Drugs, reagents and bacteriological media

The solvents used for extracting the plant material were distilled water, absolute methanol (Carlo Erba Reagents, Italy) and chloroform (Carlo Erba Reagents, Italy). The standard antibiotic discs that were used in the antibacterial activity tests include cefoxitin 30µg/disc (Benex Limited Shannon, Ireland, United States of America (USA)), ciprofloxacin 5µg/disc (Becton, Dickinson and Company, Sparks, USA) and ampicillin 10µg/disc (Oxoid Ltd, Basingstoke, Hampshire, England). In addition, other agents like a ready made 0.5 McFarland standard (Remel, Lenexa Kansas 66215, USA), Dimethyl Sulfoxide (DMSO) (Riedel-de Haen, Honeywell, Germany), sterile physiological saline (Albert David Limited, India) and resazurin sodium salt (Serva Feinbiochemica, Heidelberg, New York) were used during the study. The bacteriological media that were used in the study includes Mueller Hinton agar (MHA) (Oxoid Ltd, Basingstoke, Hampshire, England), Muller Hinton Broth (MHB) (Oxoid Ltd, Basingstoke, Hampshire, England), Brain Heart Infusion (BHI) (Difco Laboratories, Detroit Michigan, USA), blood agar base (Himedia Laboratories Pvt. Ltd, India), nutrient agar (Himedia Laboratories Pvt. Ltd, India) and nutrient broth (Himedia Laboratories Pvt. Ltd, India). All chemicals and reagents used were of laboratory grade.

3.2 Collection and authentication of the plant material

The leaves of *Rhamnus prinoides* was collected from the cultivated garden of a family in October 2014, around Debre Markos Town, East Gojjam Zone, Amhara Region, 300 km away from Addis Ababa, Ethiopia. Identification of the plant specimens was done by a taxonomist at the National Herbarium, Department of Biology, College of Natural and

Computational Sciences, Addis Ababa University, and the specimen was deposited for future reference with a voucher specimen number of YM002.

3.3 Plant extraction

The leaf of *Rhamnus prinoides* was thoroughly washed gently with tap water to remove dirt and soil. The leaves of the plant was dried under shade and then, ground into a coarse size using mortar and pestle. Then, the coarse powder of the leaves of the plant was subjected to crude extraction, but for solvent fractionation, the coarse powder was further powdered, sieved and used for fractionation purpose.

Crude extraction was carried out by maceration protocol. Briefly, one hundred fifty gram of coarsely powdered material was weighed and soaked in a flask containing 200 ml of 80% methanol in water for a period of 3 days with occasional shaking using a shaker (Bibby scientific limited stone Staffo Reshire, United Kingdom) at room temperature. The extract was filtered using gauze and then with Whatman No 1 filter paper (Schleicher and Schuell Microscience Gmbh, Germany). The residue was re-macerated for the second and third times with fresh solvent, for a total of 6 days in order to obtain a better yield. After filtration, the three extracted solutions were combined and concentrated using a rota vapor (Buchi Rota-vapor R-200, Switzerland) with a temperature not exceeding 40°C to remove methanol. Then, the concentrated filtrate was frozen in a deep freezer and dried in lyophilizer (Operan, Korea vacuum limited, Korea) to remove its aqueous content. Finally, the dried extract was packed in a closed vessel and stored in deep freezer until required for the experiment.

The solvent fractionation was conducted by using both Soxhlet and maceration techniques. Soxhlet extraction was carried out by sequential extraction of the powdered leaves in solvents of increasing polarity viz. chloroform and methanol as described by different studies (Kumar *et al*., 2012**;** Mohammed and Teshale, 2012; Rahman *et al*., 2011). First, fifty gram of the powdered plant material was weighed and placed in the extraction timble of the soxhlet apparatus. Then, about 200 ml of chloroform was added into the flask of the soxhlet apparatus set up. Then, the chloroform was heated with a temperature not exceeding 40°c to evaporate and condense into plant powder containing timble. This extraction process was continued exhaustivley until clear solution in the timble was siphoned into the solvent flask. Then, the chloroform fraction was filtered with Whatman No. 1 filter paper and concentrated using the rotary evaporator under reduced pressure. The remaining chloroform was removed by placing in oven adjusted at a temperature of 40°c. The extraction process was repeated until the desired quantity of chloroform fraction was obtained.

The marc of the chloroform based extraction was collected and dried at room temperature to remove chloroform. The dried left marc was extracted using absolute methanol following the same procedure as described for chloroform extraction to get the methanol fraction. Finally, the marc of methanol fraction was collected and dried at room temperature. Then, the whole dried marc was macerated in a flask with distilled water and allowed to stand at room temperature for a period of 3 days with occasional shaking. Then, it was filtered with gauze, then through whatman No.1 filter paper. The marc was re-macerated for the second and third times, for a total of 6 days in order to obtain a better yield. After combining the filtrates, the combined filtrate was deep frozen in a

refrigerator and then dried in a lyophilizer (Operan, Korea vacuum limited, Korea) to remove water. Then, the dried fractions of each solvent were weighed and packed in a closed vessel, and stored in a deep freezer until used for the antibacterial activity study. The percentage yield of the crude extract, aqueous fraction, methanol fraction, and chloroform fraction were 17.5%, 4.32%, 8.74% and 6.21%, respectively.

For preparing the desired solutions of the leaves of plant, each dried crude extract and solvent fractions was reconstituted in sterilized 20% DMSO (for crude extract and the methanol fraction), 70% DMSO (for the chloroform fraction) or sterilized distilled water (for aqueous fraction) at appropriate concentration to be used for the antibacterial activity test.

3.4 Phytochemical screening

The qualitative phytochemical investigations of the crude extract, and chloroform, methanol and aqueous fractions of leaves of *Rhamnus prinoides* were carried out using standard tests as described below.

Test for terpenoids (Salkowski test)

To 0.25 g of each of the crude and solvent fractions of *Rhamnus prinoides* leaves, 2 ml of chloroform was added. Then, 3 ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids (Ayoola *et al*., 2008).

Test for saponins

To 0.25 g of the crude extract and each fraction, 5 ml of distilled water was added in a test tube. Then, the solution was shaken vigorously and observed for a stable persistent froth. Formation of froth indicated the presence of saponins (Ayoola *et al*., 2008).

Test for tannins

About 0.25 g of each fraction and crude extract was boiled in 10 ml of water in a test tube and then filtered. The addition of a few drops of 0.1% ferric chloride to the filtrate resulting in blue, blue-black, green or blue-green coloration or precipitation was taken as evidence for the presence of tannins (Ayoola *et al*., 2008).

Test for flavonoids

About 10ml of ethyl acetate was added to 0.25 g of the crude extract and each fraction and heated on a water bath for 3 min. The mixture was cooled and filtered. Then, about 4 ml of the filtrate was taken and shaken with 1 ml of dilute ammonia solution. The layers were allowed to separate and the yellow color in the ammonical layer indicated the presence of flavonoids (Ayoola *et al*., 2008).

Test for cardiac glycosides (Keller-Killiani test)

To 0.25 g of the crude extract and each fraction diluted to 5 ml in water, 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was under lied with 1 ml of concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer (Ayoola *et al*., 2008).

Test for alkaloids

About 0.25 g of the crude extract and each solvent fraction was stirred with 5 ml of 1% HCl on a steam bath. One mililiter of the filtrate was treated with a few drops of Mayer's reagent and another 1 ml was similarly treated with Dragendorff's reagent. Turbidity or precipitation with both reagents was taken as preliminary evidence for the presence of alkaloids (Ayoola *et al*., 2008).

Test for anthraquinones (Borntrager's Test)

About 0.5 g of sample of each plant extract was shaken with 5 ml of chloroform and filtered. A 10% ammonium hydroxide solution (5ml) was added to the filtrate, and the mixture was shaken. The presence of a pink, red or violet color in the ammonical phase was taken as an indication of the presence of anthraquinones (Aiyelaagbe and Osamudiamen, 2009).

Test for polyphenols

To 5 ml of the aqueous solution of the crude extract and solvent fractions, 1 ml of $FeCl_3$ (1%) and 1 ml $K_3(Fe(CN)_6)$ (1%) were added. The appearance of fresh redish blue color indicated the presence of polyphenols (Farhan *et al*., 2012).

3.5 Inoculum preparation and standardization

Standard and clinical isolates of different bacterial strains including *Escherichia coli (E. coli)* (American Type Culture Collection (ATCC) 25922), *E. coli* (clinical isolate), *Pseudomonas aeruginosa (P. aeruginosa)* (ATCC 27853), *P. aeruginosa* (Clinical isolate), *Staphylococcus aureus (S. aureus)* (ATCC 25923), *S. aureus* (clinical isolate), *Shigella flexneri (S. fleneri)* (ATCC 12022), *S. fleneri* (clinical isolates), *Streptococcus*

pneumoniae (S. pneumoniae) (ATCC 49619), *S. pneumoniae* (clinical isolate), *Streptococcus pyogen (S. pyogen)* (ATCC 19615), *S. pyogen* (clinical isolate) and *Salmonella typhi (S. typhi)* (ATCC 13062) were obtained from Ethiopian Public Health Institution (EPHI). These bacterial strains were chemically well identified in the microbiology laboratory of EPHI. The bacteria were selected based on the availability and by considering the likely bacterial strains that can cause bacterial infections for which the experimental plant is indicated traditionally. Then, nutrient agar and 5% sheep blood agar (for fastidous streptococcus species that require enriched media) were prepared following the manufacturer's protocol. After cooling the media to about 45°c, it was poured to a prelabelled sterile petridishes aseptically and allowed time for the congealing of the agar. Then, standard and clinical isolates of pathogenic bacteria were inoculated and spread on the respective prepared agar using inoculating wire loop following aseptic condition in a Safety Cabinet (Bioair instruments, Eurolone® Company, Italy) and incubated for 24 h at 37°c.

The bacterial turbidity of each of bacterium was prepared and standardized by following the guideline of Clinical and Laboratory Standard Institute (CLSI) (CLSI, 2012). The bacterial suspension in a broth was prepared by the growth method as follows. After preparing nutrient broth in distilled water, 5 ml of the broth was transferred to test tubes and autoclaved (Astell Scientific Limited, England), 3–5 well isolated colonies of the same morphological type of each bacterium were picked up by wire loop from fresh agar plates of bacterial culture and aseptically transferred into prelabelled test tubes containing the sterile nutrient broth and incubated for about six hours. The turbidity of the inoculum tube was adjusted visually by either adding bacterial colonies or by adding sterile normal

saline solution to that of the already prepared 0.5 McFarland standard which is assumed to contain a bacterial concentration of $1x10^8$ CFU/ml. The adjustment and comparison of turbidity of inoculum tube and that of 0.5 McFarland standard was performed by visually observing them with naked eye against a 0.5 McFarland turbidity equivalence standard card with white background and contrasting black lines in the presence of adequate light.

3.6 Antibacterial activity assay

3.6.1 Agar well diffusion

The antibacterial agar well diffusion assay was conducted by following the methods described previously (Andualem *et al*., 2014; Taye *et al*., 2011). Bacterial broth culture was prepared to a density of 10^8 CFU/ml of 0.5 McFarland standard as stated in section 3.5. Briefly, nutrient broth was prepared according to the manufactuer's procedure in test tubes and was incubated at 37°C for 24 h for sterility testing. Then, few colonies (3-5) of similar morphology of the respective bacteria were transferred with a sterile inoculating loop from freshly prepared agar innocula to the sterile 5 ml nutrient broth aseptically and this liquid culture was then incubated until adequate growth of turbidity equivalent to McFarland 0.5 standard was obtained. The aliquot of inoculum of the respective bacteria were streaked on the sterile MHA plates (prepared according to the manufacturer's guideline) or MHA with 5% sheep blood (for streptococcus species to enrich the medium with nutrient) in 150 millimeter (mm) diameter sterile petri dish using a sterile swab in such a way as to ensure thorough coverage of the plates and a uniform thick lawn of growth following incubation. To ensure even distribution of inocula, the plate was rotated approximately 60° each time and finally rim of the agar was swabbed. Then, the plated media were allowed to dry at room temperature for 30 minutes. On each plate, four

equidistant wells were made with a 6 mm diameter sterilized cork borer and labelled (Taye *et al*., 2011). The corresponding wells were filled with 100 µl of 780 mg/ml, 390 mg/ml and 195 mg/ml of the solutions of the crude and each of the solvent fractions. These concentrations were determined based on the data obtained from the previous study (Berhanu, 2014). In addition, the commercial antibiotic discs of ampicillin 10 µg/disc (for streptococcus species), cefoxitin 30 µg/disc (for *E. coli* and *S. aureus* species) and ciprofloxacin 5 µg/disc (for other bacterial strains) were used as a positive control. The positive controls were selected based on the susceptibility of the bacterium used (CLSI, 2012). The solvents of the crude and each fraction were used as a negative control. Then, the plates were left undisturbed for about 2 h at room temperature (Wasihun *et al*., 2014), for giving time of pre-diffusion on the inoculated agar. Finally, the plates were incubated at 37°C for 24 h. After incubation, the resulting diameters of zones of inhibition, including the diameter of the well, were measured using a ruler and reported in mm. The experiment was performed in three independent tests for each bacterium and the mean of zones of inhibition was calculated for each extract and the standard antibiotics.

3.6.2 Determination of the Minimum Inhibitory Concentration (MIC)

The crude extract and solvent fractions that showed antibacterial activity by agar well diffusion method were subjected to serial microbroth dilution technique to determine their MIC by using resazurin as a cell growth indicator, as described by previous studies (Gahlaut and Chhillar, 2013; Rouis *et al*., 2013). Under aseptic conditions, 96 well microtitre plates (Greiner Bio-One, Germany) were used for resazurin based microtitre dilution assay. The first column of microtiter plate was filled with 100 µl stock solution

(390 mg/ml) of the crude or each active solvent fraction of test material except the last well in which equal amount of the respective solvent was added. Then, all the wells of microtitre plates were filled with sterilized 100 µl of MHB (CLSI, 2012) or BHI (for streptococcus species) (Bertanha *et al*., 2013). Two fold serial dilution of the extract or solvent fraction (throughout the row until the 10^{th} column) was carried out by evenly mixing and transferring 100 µl test material from wells of first row to the subsequent wells along the next column of the same row using micropipette. Then, 100 µl of mixed solution of the test material and the broth was removed from the 10^{th} column so that each well contained 100 µl of test material in serially descending concentrations. The 11^{th} and the 12^{th} column were used as the growth control for labeled bacterium in which the 100 µl of the solvents of the extract or that of the solvent fraction was added and diluted down the same column upto 7^{th} row instead of the test plant material. The resazurin solution was prepared by dissolving 0.01 gm of the resazurin powder 100 mL of autoclaved distilled water. Then, 30 µl of 0.01 % w/v resazurin color indicator solution (Bertanha *et al*., 2013) was added and mixed in each well.

The bacterial suspension was prepared according to CLSI guideline (CLSI, 2012) so that the bacterial concentration was made to be approximately 5×10^6 CFU/mL by diluting the 0.5 Macfarland standard turbidity equivalent bacterial suspensions in the ratio of 1:20 in the respective broth. Briefly, within 15 minutes of standardization of bacterial suspension, 20 µl of diluted bacterial suspension was added to each well except 7^{th} and 8^{th} row which were reserved as extract color contrast control and sterility control, respectively, to achieve a final concentration of 6×10^5 CFU/mL. Subsequently, the serial dilution procedures, after addition of the colorant and the bacterial suspension, gave rise

to a final concentration of the crude extract and solvent fractions ranging from 130 mg/ml to 0.26 mg/ml.

To avoid the dehydration of bacterial culture, each plate was wrapped loosely with parafilm (American National Can ™, Greenwich) to ensure that bacteria did not become dehydrated. In the experiment, each microtitre plate had a set of three controls: (a) a column with all solutions with the exception of the test extract or fraction (replaced by the respective solvent) was used as growth control, (b) a row with all solutions except bacterial suspension (replaced by 20 µl of MHB or the BHI) and the extract or fraction (replaced by the respective solvent) was used as sterility control, (c) a row with all solutions except the bacterial inoculum, which was replaced by 20 µl of MHB or the BHI, was used as color contrast control. Finally, the plates were incubated in temperature controlled incubator at 37° C for 24 h.

As the indicator dye accepts electrons in living cell, it changes from the oxidized, blue state (resazurin) to the reduced, pink state (resorufin) which in turn can further be reduced to hydroresorufin (colorless state) (Gahlaut and Chhillar, 2013). After incubation, the color change in the well was then observed visually. Any color change observed from purple to pink or colorless was taken as positive growth of bacteria. The lowest concentration of plant leaf extract at which no color change occurred was recorded as the MIC value. All the experiments were performed in triplicates for each bacterium. The average value was taken for the MIC of test plant material.

3.6.3 Determination of Minimum Bactericidal Concentration (MBC)

The MBC is defined as the lowest concentration where no bacterial growth is observed. This was determined by aseptically sub-culturing the contents of wells from the MIC results for individual bacterium to antimicrobial free agar as described in different studies (Nikolic *et al*, 2014; Powthong *et al*., 2012; Rouis *et al*., 2013). In this technique, the contents of all wells containing a concentration of test material above the MIC value from each triplicate, in the MIC determination test, was streaked using a sterile wire loop on MHA or MHA supplemented with 5% sheep blood (for streptococcus species) aseptically and incubated at 37ºC for 24 h. The lowest concentration of the extract which showed no bacterial growth after incubation was observed for each triplicate and noted as the MBC. The average value was taken for the MBC of test material against each bacterium.

3.7 Statistical analysis

The experimental data are expressed as mean ± Standard Error of the Mean (SEM). Data are analyzed using the Statistical Package for the Social Sciences (SPSS), version 16.0 software. The statistical differences of the mean zone of inhibition of crude extract and solvent fractions for individual bacterium was carried out by employing one way analysis of variance (ANOVA) followed by Tukey Post Hoc Multiple Comparison test at a significance level of $P<0.05$. The MIC and MBC are analyzed using descriptive statistics using SPSS software. Moreover, the concentration dependent antibacterial activities of the crude extract, chloroform and methanol fractions for each bacterium were determined by linear regression analysis using the SPSS software.

4. RESULTS

4.1 Antibacterial activity

According to the agar well diffusion test, the growth of all test bacterial strains were inhibited by the tested concentrations of the crude (80% methanol) extract of the plant in concentration dependent manner, with R^2 values ranging from 0.813 (for clinical isolates of *P. aeruginosa)* to 0.999 (for standard strains of *E. coli*). Similarly, The R^2 values of the mean zone of inhibition of methanol fraction varied from 0.911 (for standard strain of *P. aeruginosa*) to 0.999 (for standard strains of *S. typhi* and clinical isolate of *S. pyogen*) whereas that of chloroform ranged from 0.792 (for standard strain of *S. aureus*) to 0.994 (for standard strains of *S. flexneri*). But, the observed zone of inhibition of the crude extract, methanol and chloroform fractions at the tested concentrations were statistically different compared to that of their respective positive control ($p<0.05$) against all test bacteria (Table 1 and Table 2).

Among the test bacteria, gram positive bacterial species of *S. aureus, S. pneumoniae* and *S. pyogen* were more susceptible than that of the gram negative bacterial species at the corresponding tested concentrations of the crude extract, especially at 780 mg/ml and 390 mg/ml. As depicted in Table 1, the most susceptible bacterium at 780 mg/ml was standard strains of *S. aureus* followed by clinical isolates of *S. aureus* and standard strains of *S. pyogen* with a mean zone of inhibition of 22 mm, 21.67 mm and 20.33 mm, respectively.

Moreover, the zone of inhibition of crude extract at 195 mg/ml was significantly different ($p<0.05$) compared to that of its zone of inhibition at 780 mg/ml against the growth of each test bacterium with the exception of clinical isolates of *P. aeruginosa.*

The zone of inhibition of the crude extract was greater than that of the methanol fraction at equal concentrations against the growth of each test bacterium, with a significant difference ($p<0.05$) against staphylococcus species (at all tested concentrations), *S. pyogen* strains (at 780 mg/ml and 390 mg/ml) and clinical isolate of *S. pneumoniae* (at 780 mg/ml). Similarly, the zone of inhibition of the chloroform fraction was less than that of the crude extract at their comparable concentarions against the growth of each of susceptible bacterial strain, especially with a statistically significant difference at 780 mg/ml to all test bacterial strains ($p<0.05$) (Table 1 and 2).

Similar to the crude extract, solvent fractions of the leaves of the *Rhamnus prinoides* were subjected to antibacterial activity tests to determine which polarity gradients of the plant are active against the susceptible bacteria to the crude leaf extract. Among the solvent fractions, the aqueous fraction was devoid of an antibacterial activity against any of the test bacterium. However, the methanol and the chloroform fractions were endowed with antibacterial activities with varying antibacterial spectrum as depicted in Table 1 and Table 2.

The antibacterial activity pattern of the methanol fraction seemed to be in line with that of the crude extracts of the *Rhamnus prinoides,* especially in terms of its antibacterial spectrum even though they had been extracted by different methods. The most susceptible bacterium against methanol fraction was standard strains of *S. aureus* followed by standard strains of *S. pneumoniae* with a maximum mean zone of inhibition of 16.17 mm and 16 mm, respectively, at 780 mg/ml concentration (Table 1). Furthermore, the zones of inhibition of the methanol fraction at 195 mg/ml were

significantly different from that of its activity at 780 mg/ml concentration against each of the test bacterium ($P<0.05$).

Similarly, gram positive bacteria were more susceptible than that of the gram negative ones at equal concentrations of chloroform fraction. The most sensitive bacterium against chloroform fraction at 780 mg/ml concentration was standard strains of *S. pneumoniae* followed by standard strains of *S. pyogen* and clinically isolated strains of *S. pneumoniae* with a maximum mean zone of inhibition of 15.67 mm, 15 mm and 15 mm, respectively, (Table 1). The zones of inhibition of chloroform fraction at 195 mg/ml were also significantly different ($P<0.05$) compared to that of its activity at 780 mg/ml against each of the test bacterium for which it was able to inhibit their growth with the exception of clinically isolated strains of *S. flexneri*.

Table 1: Zone of inhibition (in mm) of the different concentrations of crude extract and solvent fractions of the leaves of *Rhamnus prinoides* against gram positive bacteria.

Category of test	Concentration	Bacteria					
		S. aureus		*S. pyogen*		*S. pneumoniae*	
		Stand	Clinic	Stand	Clinic	Stand	Clinic
Crude	195 mg/ml	16.67±0.88^{a3d2}	16.67±0.33^{a3c1d3}	13.67±0.88^{a3c2d3}	12.33±0.33^{a3c1d2}	13.33±0.33^{a3d2}	11.67±0.33^{a3c2d3}
	390 mg/ml	19.67±0.88^{a3}	19.00±0.58^{a3d1}	18.33±0.67^{a3}	16.33±0.67^{a3}	16.00±1.00^{a2}	15.33±0.33^{a3d2}
	780 mg/ml	22.00±0.58^{a2}	21.67±0.33^{a2}	20.33±0.33^{a3}	18.67±0.33^{a3}	19.33±1.20^{a1}	18.00±0.00^{a3}
	Cef 30µg/disc*	28.33±0.58	26.33±0.67	NT	NT	NT	NT
	Amp10µg/disc*	NT	NT	27.33±0.67	26.33±1.20	24.00±0.58	23.00±0.58
Methanol	195 mg/ml	12.00±0.58^{a3d2e2}	11.67±0.33 ^{a3d2e3}	12.67± 0.33^{a3d1}	11.67±0.33^{a3d2}	11.67±0 .88^{a3d2}	11.33±0.33^{a3d3}
	390 mg/ml	14.00±0.58^{a3f3}	13.33± 0.33^{a3f3}	14.00±0.58^{a3f2}	12.67±0.33^{a3d1f3}	13.67±0 .33^{a3}	13.67± 0.33^{a3d1}
	780 mg/ml	16.17±0.44^{a3g3}	15.33± 0.67^{a3g3}	15.00± 0.58^{a3g3}	15.00± 0.58^{a3g3}	16.00±0.58^{a3}	15.33±0.33^{a3g2}
	Cef 30µg/disc*	27.67± 0.33	26.33±0 .33	NT	NT	NT	NT
	Amp10µg/disc*	NT	NT	28.00±0.00	26.33±0 .67	25.00±0 .58	23.67±0.33
Chloroform	195 mg/ml	11.67±0.33^{a3c1d2e3}	11.67±0.33^{a3d1e3}	12.00±0.58 ^{a3d2}	10.67±0.33^{a3d2}	11.33±0.67^{a3d2}	11.00±0.00^{a3d2}
	390 mg/ml	13.67±0.33^{a3f3}	13.33±0.33^{a3f3}	13.33±0.33 ^{a3f3}	12.00±0.58 ^{a3f3}	13.33±0.33^{a3d1}	13.00±0.58^{a3f2}
	780 mg/ml	14.33±0.33^{a3g3}	14.00±0.58^{a3g3}	15.00±0.58 ^{a3g3}	13.67±0.58^{a3g3}	15.67±0.33^{a3g1}	15.00±0.58^{a3g3}
	Cef 30µg/disc*	27.00±0.57	26.33±0.33	NT	NT	NT	NT
	Amp10µg/disc*	NT	NT	27.67±0.33	27.00±0.58	25.00±0.58	23.67±0.33

Values are expressed as Mean ± S.E.M (n=3), analysis was performed with One-Way ANOVA followed by Tukey test; ^{a} compared to positive control, ^{b} to 195mg/ml, ^{c} to 390mg/ml, ^{d} to 780mg/ml, ^{e} to crude 195mg/ml, ^{f} to crude 390mg/ml, ^{g} to crude 780mg/ml; $^{1}P<0.05$, $^{2}P<0.01$, $^{3}P<0.001$. The negative control has shown no antibacterial activity. * = positive controls, Stand= standard (ATCC) strains, Clinic. = clinically isolated strains, NT=not tested, Cef= Cefoxitin, Amp= ampicillin, Cip=Ciprofloxacin.

Among the gram negative bacteria, standard strains of *S. typhi* was most susceptible followed by that of standard strains of *S. flexneri* at 780 mg/ml of the crude extract with the maximum mean zone of inhibition of 17.67 mm and 17 mm, respectively. However, the strains of *P. aeruginosa* and *E. coli* were relatively less susceptible compared to the other bacterial species at comparable concentration level of the crude extract (Table 2).

Similarly, the methanol and chloroform fraction were endowed with growth inhibitory effect against the test gram negative bacterial strains with varying antibacterial spectrum. The zone of inhibition methanol fraction at 780mg/ml against *S. typhi* was also greater than that of its activity against the other gram negative test bacterial strains (Table 2). The less susceptible bacteria for methanol fraction, at equal or comparable concentrations, were the strains of *E. coli* and *P. aeruginosa* compared to other gram negative bacterial test strains, even compared to the gram positive bacterial strains

The chloroform fraction did not show antibacterial activities against the strains of *E. coli* and *P. aeruginosa* regardless of its tested concentrations. However, the chloroform fraction was shown to have nearly comparable growth inhibitory effect against the strains of *S. flexneri* and S. typhi at its the comparable concentrations (Table 2).

Generally, the antibacterial activities of the chloroform fraction in terms of its zone of inhibition were slightly lower than that of the methanol fraction against each bacterial strain for which it was active, especially at 780 mg/ml and 390 mg/ml concentrations even though the difference at the corresponding concentration were not statistically significant.

Table 2: Zone of inhibition (in mm) of the different concentrations of crude and solvent fractions of the leaves of *Rhamnus prinoides* against gram negative bacteria.

Category of test	Concentration	Bacteria			
		S. flexneri		*E. coli*	
		Stand	Clinic	Stand	clinic
Crude	195 mg/ml	11.67±0.33^{a3c1d3}	11.33±0.33 ^{a3c1d2}	11.67±.88^{a3d1}	11.33±0.67^{a3d2}
	390 mg/ml	14.00±0.00^{a3d2}	13.67±0.33 ^{a3d1}	12.67±0.33^{a3}	12.67±0.33^{a3}
	780 mg/ml	17.00±0.58^{a3}	16.00±0.58 ^{a3}	15.00±0.00^{a3}	14.50±0.29 ^{a3}
	Cef 30µg/disc*	NT	NT	28.33±0.88	27.00±0.58
	Cip 5µg/disc*	26.33±0.67	25.00± 0.58	NT	NT
Methanol	195 mg/ml	11.00±0.00^{a3d2}	10.67±0.67^{a3d2}	10.33±0.33^{a3d3}	9.00±0.58^{a3c2d3}
	390 mg/ml	12.50±0.76^{a3}	12.67±0.33^{a3}	12.00±0.58^{a3d1}	11.67± 0.33^{a3d2}
	780 mg/ml	15.00± 0.58^{a3}	14.67±0.33^{a3}	14.50± 0.29^{a3}	14.33± 0.33^{a3}
	Cef 30µg/disc*	NT	NT	27.67±0.33	26.67 ±0.33
	Cip 5µg/disc*	27.00± 0.58	26.33± 0.67	NT	NT
Chloroform	195 mg/ml	10.00±0.58^{a3d2}	10.33±0.33^{a3}	---	---
	390 mg/ml	11.33±0.33^{a3f1}	11.67±0.67^{a3}	----	---
	780 mg/ml	13.33±0.33 ^{a3g2}	12.33±0.33^{a3g2}	---	---
	Cip 5µg/disc*	25.00±0.58	24.67±0.33	NT	NT

Values are expressed as Mean ± S.E.M (n=3), analysis was performed with One-Way ANOVA followed by Tukey test; ^{a} compared positive control, ^{b} to 195mg/ml, ^{c} to 390mg/ml, ^{d} to 780mg/ml, ^{e} to crude 195mg/ml, ^{f} to crude 390mg/ml, ^{g} to crude 780mg/ml; $^{1}P<0.05$, $^{2}P<0.01$, $^{3}P<0.001$. The negative control has shown no antibacterial activity. * = positive controls, Stand= standard (ATCC) strains whereas Clinic. = clinically isolated strains, NT=not tested, Cef = Cefoxitin, Cip=Ciprofloxacin, --- = no activitiy

Table 2: continued

Category of test	Concentration	Bacteria		
		P. aeruginosa		*S. typhi*
		Stand	Clinic	stand
Crude	195 mg/ml	11.67±0.33[a3c1d2]	10.67±0.88[a3]	13.33±0.33[a3d2]
	390 mg/ml	13.33±0.33[a3]	13.00±0.58[a3]	14.00±0.58[a3d2]
	780 mg/ml	14.33±0.33 [a3]	14.00±0.58[a3]	17.67±0.67[a3]
	Cip 5µg/disc[*]	25.67±0.33	24.33±0.88	30.00±0.58
Methanol	195 mg/ml	10.33±0.33[a3d1]	10.33±0.33[a3c1d2]	11.00± 0.58[a3d2]
	390 mg/ml	12.00±1.00[a3]	12.33±0.33[a3]	12.67±0.88[a3d1]
	780 mg/ml	13.67±0.33[a3]	13.67±0.33[a3]	15.67±0.33[a3]
	Cip 5µg/disc[*]	24.33±0.33	24.00±0.58	31.00± 0.58
Chloroform	195 mg/ml	---	---	10.33±0.33[a3d2e1]
	390 mg/ml	---	---	11.67±0.33[a3]
	780 mg/ml	---	---	13.00±0.58[a3g3]
	Cip 5µg/disc[*]	NT	NT	30.33±0.33

Values are expressed as Mean ± S.E.M (n=3), analysis was performed with One-Way ANOVA followed by Tukey test; [a] compared to positive control, [b] to 195mg/ml, [c] to 390mg/ml, [d] to 780mg/ml, [e] to crude 195mg/ml, [f] to crude 390mg/ml, [g] to crude 780mg/ml; [1]P<0.05, [2]P<0.01, [3]P<0.001. The negative control has shown no antibacterial activity [*] = positive control, Stand= standard (ATCC) strains whereas Clinic = clinically isolated strains, Cip=Ciprofloxacin, --- = no activity.

4.2 Minimum inhibitory concentration of crude extract and solvent fractions

As presented in Table 3, the MIC figures of the crude extract were in agreement with its preliminary antibacterial activities i.e the more susceptible is the bacterium, the lower is the concentration of the extract required for growth inhibition in most of the test bacteria. The crude extract of the plant was more potent against gram positive bacteria than that of gram negative bacteria with the exception of *S. pneumoniae*. The maximum MIC (less diluted) obtained was 8.13 mg/ml (against *E. coli*, *P. aeruginosa* and *S. pneumoniae species*) and the minimum MIC (highest dilution) was 2.03 mg/ml (against Staphylococcus species). Moreover, unlike the slight differences between the figures of zone of inhibition of the clinically isolated and standard strains within the same species, the MIC values were equal for the strains of the same species with the exception of *S. flexneri* for which the MIC of the clinical isolate is slightly higher (5.42 mg/ml) than that of its standard ones (4.06 mg/ml).

When the MIC value of the crude extract and that of the active solvent fractions are compared, the maximum MIC figure of the crude extract became the minimum MIC for that of solvent fractions in all of the tested bacterial strains. As indicated in Table 3, in the case of the methanol fraction, the maximum MIC was 32.5 mg/ml (against *E. coli* species) and the minimum MIC value was 8.13 mg/ml (against *S. aureus* and *S. pyogen* species). The MIC figures of the chloroform fraction ranged from 8.13 mg/ml to 16.25 mg/ml in all bacterial species for which it was active. For the same bacterial species tested, the MIC of the two active solvent fractions were equal with the exception of

clinically isolated strains of *S. aureus* and *S. pyogen* for which the methanol fraction was more potent than the chloroform fraction.

Table 3: The MIC (in mg/ml) of the crude extract and the solvent fractions of the leaves of *Rhamnus prinoides* against gram positive and gram negative bacteria

Bacteria		Crude extract	Solvent fractions	
			Methanol fraction	Chloroform fraction
		MIC	MIC	MIC
S. aureus	Stand.	2.03±0.00	8.13±0.00	8.13±0.00
	Clinc.	2.03±0.00	8.13±0.00	16.25±0.00
S. pyogen	Stand.	4.06±0.00	8.13±0.00	8.13±0.00
	Clinc.	4.06±0.00	8.13±0.00	16.25±0.00
S. pneumoniae	Stand.	8.13±0.00	16.25±0.00	16.25±0.00
	Clinc.	8.13±0.00	16.25±0.00	16.25±0.00
S. flexneri	Stand.	4.06±0.00	16.25±0.00	16.25±0.00
	Clinc.	5.42±1.36	16.25±0.00	16.25±0.00
E. coli	Stand.	8.13±0.00	32.50±0.00	---
	Clinc.	8.13±0.00	32.50±0.00	---
P. aeruginosa	Stand.	8.13±0.00	16.25±0.00	---
	Clinic	8.13±0.00	16.25±0.00	---
S. typhi	Stand	4.06±0.00	16.25±0.00	16.25±0.00

MIC= Minimum Inhibitory Concentration, the values are the average of triplicate tests. Stand. = standard (ATCC) strains whereas Clinic. = clinically isolated strains

4.3 Minimum bactericidal concentration of crude extract and solvent fractions

Based on the MBC determination method, the crude extract and active solvent fractions of the *Rhamnus prinoides* were bactericidal even though the crude extract showed bactericidal activity at lower concentrations. As indicated in Table 3 and 4, the corresponding mean MIC value and MBC value of the crude, methanol fraction and

chloroform fraction were equal against the growth of the majority of the bacterial strains that belong to the same species.

As depicted in Table 4, similar to the MIC value profile, the maximum mean MBC (least dilution) was 16.25 mg/ml (against both strains of *S. pneumoniae*) and the minimum mean MBC (highest dilution) of the crude extract of the study plant was 2.03 mg/ml (against standard strains of Staphylococcus species). The corresponding values of the methanol fraction were 65 mg/ml (against clinical isolate of *E. coli*) and 13.54 mg/ml (against standard strain of *S. pyogen*). Similarly, the range for the mean MBC values of the chloroform fraction was narrow, having a range from 32.5 mg/ml to 16.25 mg/ml against the growth of the bacterial species which were susceptible in its antibacterial activity testing experiment.

Taken together, the crude extract was more potent and killed the bacteria at lower concentration compared to that of the methanol and chloroform fractions.

Table 4: The MBC (in mg/ml) of the crude extract and the solvent fractions of *Rhamnus prinoides* against gram positive and gram negative bacteria.

Bacteria		Crude extract	Solvent fractions	
			Methanol fraction	Chloroform fraction
		MBC	MBC	MBC
S. aureus	Stand.	2.03±0.00	16.25±0.00	16.25±0.00
	Clinc.	4.06±0.00	16.25±0.00	16.25±0.00
S. pyogen	Stand.	4.06±0.00	13.54±2.71	16.25±0.00
	Clinc.	4.06±0.00	16.25±0.00	16.25±0.00
S. pneumoniae	Stand.	8.13±0.00	32.50±0.00	32.50±0.00
	Clinc.	16.25±0.00	32.50±0.00	32.50±0.00
S. flexneri	Stand.	8.13±0.00	32.50±0.00	16.25±0.00
	Clinc.	8.13±0.00	32.50±0.00	32.50±0.00
E. coli	Stand.	8.13±0.00	32.50±0.00	---
	Clinc.	8.13±0.00	65.00±0.00	---
P. aeruginosa	Stand.	8.13±0.00	32.50±0.00	---
	Clinic.	8.13±0.00	32.50±0.00	---
S. typhi	Stand	4.06±0.00	16.25±0.00	16.25±0.00

MBC=Minimum Bactericidal Concentration, the values are the average of triplicate tests. Stand. = standard (ATCC) strains whereas Clinic. = clinically isolated strains

4.4 Phytochemical constituents of the crude extract and solvent fractions

The result of phytochemical screening test is shown in Table 5. According to the qualitative phytochemical screening study, the crude extract of the leaf of *Rhamnus prinoides* was found to be positive for the presence of all of the tested secondary metabolites except for cardiac glycosides, whereas the methanol solvent fraction was positive for the presence of alkaloids, tannins, flavonoids, saponins, polyphenols and terpenoids. The chloroform fraction was confirmed for the presence of tannins,

polyphenols, terpenoids, anthraquinones and flavonoids while the aqueous fraction contained only saponins.

Table 5: Preliminary phytochemical investigation of the crude extract and the solvent fractions of leaves of *Rhamnus prinoides* using chemical test methods

Metabolites tested	Crude extract	Solvent fractions		
		Chloroform fraction	Methanol fraction	Aqueous fraction
Alkaloids	+	-	+	-
Saponins	+	-	+	+
Tannins	+	+	+	-
Anthraquinones	+	+	-	-
Polyphenols	+	+	+	-
Terpenoids	+	+	+	-
Flavonoids	+	+	+	-
Cardiac glycosides	-	-	-	-

+ = present, - =absent

5. DISCUSSION

Rhamnus prinoides is one of the medicinal plants used for the treatment of various disorders including bacterial infections. The present study was undertaken to determine on which fractions do the constiuents of the leaves of *Rhamnus prinoides* responsible for its antibacterial activitity are concentrated. In addition, even though the antibacterial activity of the crude extract of the plant had been studied by a previous work in Gondar, Ethiopia (Berhanu, 2014), the crude extract was also evaluated in this study to assure its antibacterial activity as there was differences in geographical areas of plant collection, bacterial stains used and the parts of the plant used for extraction purpose.

According to the present study, the result of the antibacterial activity test indicated that the crude extract was found to have greater antibacterial effect against all the test bacteria than that of the active solvent fractions. But, in the case of the solvent fractions, only methanol and chloroform fractions were shown to have antibacterial activities with varying degrees of antibacterial activity spectrum. The zones of inhibition of the bioactive fractions were lower than that of the crude extract at equal concentrations against the individual strains of test bacteria. The reason could be due to the differences in the composition and concentrations of the secondary metabolites in the crude extract, and methanol fraction and chloroform fraction. For instance, majority of the metabolites were detected in the crude extract, as can be depicted in the preliminary phytochemical screening result, unlike in the case of the methanol and chloroform fractions in which the bioactive secondary metabolites had been partitioned in the successive solvents used for extraction. Therefore, the enhanced antibacterial activity of the crude extract might be due to the synergistic or additive effect of the secondary metabolites that were relatively

partitioned in the solvent fractionation which in turn caused the decrease in antibacterial activity observed in the methanol and chloroform fractions. In addition, their antibacterial activity difference may be related to the difference in the method of extraction and the type of solvent used for extraction purpose.

In spite of having antibacterial activities, the mean zone of inhibition of the crude and the active solvent fractions at all test concentrations were not statistically comparable to that of their respective positive control for each of the susceptible bacterium. This might be due to the less concentration of the active principles in the crude extract, methanol and chloroform fractions against the test bacteria.

The crude extract and the methanol fraction had similar antibacterial activity profile in terms of antibacterial activity coverage and their activity was higher in the gram positive bacteria than that of the majority of gram negative test bacteria at their comparable concentraation. The mean zone of inhibition of chloroform fraction at its comparable concentration against gram positive bacteria was also greater than that of gram negative bacteria. Obviously, it is not surprising that gram negative bacteria were less susceptible for the crude and the active solvent fractions of the study plant as they have an outer membrane. Therefore, the activity difference among the gram negative and gram positive bacteria could be because of the partial penetration of the bioactive phytochemicals through the lipopolysaccharide rich outer cell membrane in the cell wall of gram negative bacteria (Nikaido and Vaara, 1985) unlike in the cell wall of the gram positive bacteria with less effective permeability barrier (Scherrer and Gerhardt, 1971).

The antibacterial activity of solvent fractions indicated that the methanol and chloroform fractions have been found to inhibit the test bacteria though the chloroform fraction was not active against the clinical and standard strains of *P. aeruginosa* and *E. coli*. In addition, the clinical and standard strains of *P. aeruginosa* and *E. coli* were the least susceptible bacteria for the methanol fraction at equal concentrations compared to the other gram positive test bacteria, even compared to the other gram negative test bacteria. The possible reason for the less susceptible nature of these bacterial species could be due to the differences in the resistance mechanisms to the bioactive compounds detected in each solvent fraction among the test bacteria in addition to their outer cell membrane permeability barrier. For example, *P. aeruginosa* and *E. cloi* have the inherent ability of producing different resistance mechanisms like efflux pump (Iyer and Erwin, 2015) or biofilm formation (Carmen *et al*., 2004) which could hinder the antibacterial activity of the bioactive compounds detected in the chloroform fraction.

Furthermore, the differences in the antibacterial spectrum of the methanol and the chloroform fraction against the test bacteria might be linked to the differences in the composition and/or the concentration of the secondary metabolites in the respective fractions. For instance, unlike the chloroform fraction, the presence of alkaloids and saponins in methanol fraction could directly or indirectly enhance its bacterial growth inhibitory effects on gram negative bacteria including *E. coli* and *P. aeruginosa* which were not responsive to the chloroform fraction (Maatalah *et al*., 2012).

The zones of inhibition of the methanol fraction and that of the chloroform fraction at the comparable concentration were not statistically different for all bacteria against which both fractions were active. This implies that both fractions were comparably bioactive for

inhibiting the growth of the susceptible bacteria. However, the slightly lower zone of inhibition of chloroform fraction against its susceptible bacteria, at equal concentrations, than the corresponding effect of the methanol fraction may be associated with the less diffusible components of the chloroform fraction on the aqueous surfaces of the agar plate (Pauli *et al*., 2005; Sanchez *et al*., 2010) and their differences in the composition and concentrations of bioactive secondary metabolites.

Unlike the methanol and chloroform fractions, the aqueous fraction was found to be devoid of antibacterial activities against all the test bacterial strains regardless of the tested concentrations used. Such type of result in the present study is concordant with other similar study following the same solvent fractionation principles in which the aqueous fraction was not having antibacterial activities (Abeysinghe *et al*., 2011**)**. The reason for the absence of antibacterial effects in the aqueous fraction might be due to the localization of the bioactive secondary metabolites of the plant in the chloroform and methanol fractions as the plant had been extracted in sequential solvents of increasing polarity. The localization of bioactive metabolites in the chloroform and methanol fractions can be supported by the fact that most of the secondary metabolites of the medicinal plants have aromatic rings and hence could be extracted by the organic solvents used sufficiently before being extracted by water (Cowan, 1999). In addition, the absence of antibacterial activity of the aqueous fraction might be further strengthened and associated with the absence of almost all of the secondary metabolites tested with the exception of saponins in aqueous fraction, as displayed in the preliminary phytochemical screening test (Table 5).

The antibacterial screening findings in terms of zone of inhibition of the crude and the active solvent fractions of *Rhamnus prinoides* against the respective susceptible bacteria were inversely proportional to their values of MIC and MBC, with some exception like for *S. pneumoniae*, that is, the more susceptible the bacteria to the crude extract or the solvent fraction, the less is its corresponding MIC and MBC values, suggesting the reproducibility and consistency of the experiments. In addition, in almost all findings, the MBC value was equals to or one dilution factor greater than that of the MIC value for each individual bacterium in the micro-broth dilution tests of crude extract and the active solvent fractions, which might indicate the sensitivity of the dilution method in detecting the minimum bacterial turbidity that indicated the growth of bacteria than that of the visual inspection method in which ambiguity of determining the MIC value is common (Ncube *et al*., 2008).

However, the MIC values of clinical isolate of *S. flexneri* for the crude extract and that of clinical isolate of *S. aureus* and *S. pyogen* for the chloroform fraction were greater than the corresponding values of the standard strains of the same species. In addition, the MBC values of clinical isolates of *S. aureus* and *S. pneumoniae* for crude extract; *S. pyogen* and *E. coli* for methanol fraction*;* and *S. flexneri for chloroform* were greater than the corresponding values of the standard strains of the same species. These differences in the potency of the crude extract and the active solvent fractions against the strains of the same bacterial species might be associated to the susceptibility differences between the strains in which the clinical isolates could have a higher chance of developing a resistance mechanism of decreasing the access of the bioactive metabolites to the target

sites since they had been isolated from the clinic settings in which resistant strains are common (Canton *et al*., 2013).

Groups of phytochemical compounds commonly associated with combating microbial resistance and having antimicrobial activity in medicinal plants are flavonoids, alkaloids, tannins, tritepenoids, essential oils, saponins, glycosides and phenols (Neog *et al*., 2013; Wright, 2005). Even though, at this point in time, it is difficult to judge the mechanism of actions of the bioactivity of the crude and the bioactive solvent fractions of the study plant, it is plausible to speculate their antibacterial effect based on the different mode of action of the bioactive phytochemicals detected in phytochemical analysis of this study.

Hence, the antibacterial activities of flavonoids isolated from medicinal plants have long been studied by different studies. For instance, different structural derivatives or congeners of flavonoids like quercetin, naringenin (Rauha *et al*., 2000), apigenin, luteolin (Sato *et al*., 2000), crycristagallin, and orientanol B (Tanaka *et al*., 2002) have been found to have antimicrobial activities against the growth of different bacteria. According to the previous work (Abegaz and Kebede, 1995), the present plant contained flavonoids like quercetin and rhamnazin which strengthen the obtained positive result for the presence of falvonoids in this study. Therefore, the presence of flavonoids in crude extract, chloroform faction and methanol fraction of the leaf of *Rhamnus prinoides* could contribute their own share for the observed antibacterial activities, especially against gram positive bacteria. The possible mechanism of action for the antibacterial effects of flavonoids includes the damage or disruption of the cell membranes and inhibition of the synthesis of nucleic acids which can lead to the death of the susceptible bacterium (Dzoyem *et al*., 2013).

Polyphenols are the other phytochemicals that might involve in the antibacterial activities of medicinal plants used for different infectious diseases. The extracts of phenolic compounds (isolated from *Carum carvi*) have been found to have a growth inhibition effect against different bacteria (*E. coli, S. aureus and Salmonella typhimurium*) (Thippeswamy *et al*., 2013). Moreover, polyphenols isolated from oolong tea have been reported to have antibacterial activities on streptococci species with a mechanism of decreasing the adherence of the growing cells of test organisms on the mucosal wall of human body tracts (Brantner *et al.,* 1996; Nakahara *et al*., 1993*)*. In addition, polyphenols can disturb the metabolic function of microorganisms by forming heavy soluble complexes with enzymes. Thus, the antibacterial activity of the crude extract, the methanol fraction and chloroform fraction of the present plant could be associated with the mentioned mode of action of polyphenols and with their possible attack on the cell walls of bacteria (Brantner *et al.,* 1996).

Tannins are the other compounds that have been found to have antimicrobial activities against the growth of bacteria. The antibacterial role of tannin constituents in green tea leaf extract has been shown to inhibit and decrease the load and growth of aerobic, mouth cavity colonizing bacteria (Moghbel *et al.,* 2011). In addition, different derivatives of tannins such as catechin, ellagetannin and gallotannin have also been found to be bioactive compounds against the growth of *S. aureus* (Akiyama *et al*., 2001; Min *et al*., 2008). Therefore, the antibacterial activity findings of *Rhamnus prinoides* could be due to the presence tannins in the crude extract, the methanol and chloroform fractions. The antibacterial mechanism for tannins might be due to its membarane damaging effects and

inhibition of metabolic pathways of bacteria like oxidative phosphorylation which could lead to death of the microorganism (Funatogawa *et al*., 2004; Scalbert, 1991).

Terpenoids are the other class of compounds known to have antimicrobial activities. The terpenoid fractions isolated from *Luffa cylindrical* (Nagarajan *et al*., 2010) and *Elephantopus scaber* (Jasmine *et al.,* 2007) were found to have antibacterial activities against various pathogenic microbes including *S. aureus, E. coli* and *P. aeruginosa* with varying selectivity. Therefore, the antibacterial activities of terpenoids detected in the crude extract, chloroform fraction and methanol fraction of the study plant might be linked to the disruption of cytoplasmic cell membarane (Brehm-Stecher and Johnson, 2003).

Moreover, the antibacterial activities of medicinal plants are associated with their alkaloid components. For example, the alkaloidal fractions extracted from the leaf of *Prosopis juliflora* have been found to be bioactive phytochemicals which inhibited the growth of both gram positive and gram negative test bacterial strains (Singh *et al*., 2011). In addition, different alkaloid compounds like bisbenzylisoquinoline alkaloids (tetrandrine, demethyltetrandrine) (Zuo *et al*., 2011) and dictamnine isolated from *Hortia oreadica* (Severino *et al*., 2009) have shown to have antibacterial activity against the growth of bacteria. Therefore, the presence of alkaloids in the crude and methanol fraction could contribute for their respective antibacterial activities observed in the present study. The possible mechanism of antibacterial activities of alkaloid compounds might be disruption of cell membranes or inhibition of the protein synthesis of bacteria (Sathyabama and Kingsley, 2013).

According to the qualitative test of this study and previous work (Abegaz and Kebede, 1995), anthrquinones have been detected in the extracts of the leaves of *Rhamnus prinoides*. Thus, the antibacterial activities of the crude and the chloroform fractions of the present study might be related to the presence of anthraquinones. This antibacterial action of anthraquinones has been strengthened by growth inhibitory effect of different anthraquinone compounds such as emodin (isolated from *Rheum officinale)* (Liu *et al*., 2012) and 1, 8-dihydroxy-anthraquinone (isolated from *Porphyra haitanensis*) (Wei *et al*., 2015). The plausible antibacterial activity of anthraquinones have been associated with their interaction with the bacterial cell wall and cell membrane components which can lead to the death of the bacteria as a result of leakage of cytoplasmic components and loss of cell integrity (Wei *et al*., 2015).

Finally, saponins have also been considered to have antibacterial activities. Most literatures found that saponin fractions or isolated saponin compounds have antibacterial activities. For instance, the saponin purified fractions isolated from *sorghum bicolor* (Soetan *et al*., 2006) and *Acacia aroma (*Mattana *et al*., 2010) were found to have antibacterial activity against the growth of *S. aureus.*

Though the phytochemical screening test for saponins in aqueous fraction was positive, it did not demonstrate a growth inhibition effect against any of the test bacterium. The possible reason for this could be due to absence of inhibitory concentration of saponins to inhibit growth of bacteria or their effect is observable in the presence of other bioactive compounds that can act in synergistic or additive mechanism.

Generally, even though there are variable degrees of sensitivities of the test bacteria, antibacterial activity screening results are still indicative of the potential of the leaves of *Rhamnus prinoides* as effective medicaments in the treatment of infections caused by the test bacteria. This effect of the leaves of *Rhamnus prinoides* might be attributed to either the individual class of compounds present in the crude and the active solvent fractions or to the synergistic effect that each class of compound exerted to give the observed antibacterial activity findings.

6. CONCLUSION

The present study revealed that the chloroform fraction and the methanol fraction of the leaves of *Rhamnus prinoides* have antibacterial activities against the growth of the selected pathogenic bacteria with varying antibacterial spectrum. Therefore, the study provides scientific basis on the traditional claimed use of the medicinal plant for the treatment of bacterial infections like tonsillitis, water born disease, or others which are probably caused by the susceptible bacteria. The antibacterial activities of the plant might be linked with the presence of non-polar and/or intermediately polar bioactive secondary metabolites in the chloroform and methanol fractions including alkaloids, terpenoids, anthraquinones, tannins, polyphenols, saponins and flavonoids that can act either individually or synergistically. But, in addition to those bioactive secondary metabolites covered by this study, other bioactive principles which were not addressed might contribute their own share for the antibacterial activities of the crude extract and the active solvent fractions of the study plant.

7. SUGGESTIONS FOR FUTURE WORK

- Further studies should be conducted to isolate, purify and identify bioactive principle(s) responsible for the antibacterial activities of the plant.
- Mechanistic studies for the responsible antibacterial agent of the study plant have to be conducted for the antibacrerial activity.
- In *vivo* antibacteri*al* studies of the crude and active solvent fractions should be conducted to confirm the antibacterial effectiveness of the plant.
- The acute, sub-chronic and chronic toxicological studies should be done for the safety of the extracts of the plant.
- At last, the antibacterial activities of the plant should also be done on other bacterial species which were not addressed by this study.

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