In vitro anti-bacterial activities of aqueous, ethyl acetate and methanol crude extracts of *Allophylus abyssinicus* and *Ligustrum vulgare* leaves on selected human pathogenic bacteria

By

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Declaration

I, the undersigned, declare that, this is an original work and has never been presented in any other university as well as research institutes and all the source material used for this thesis have been fully acknowledged.

Enat Mengistu

Signature _____

Date_____

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Acronyms /Abbreviations

AAU	Addis Ababa University
AMR	Antimicrobial Resistance
ATCC	American Type Culture Collection
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standard Institute
CNCS	College of Natural and Computational Sciences
DMCMB	Department of Microbial, Cellular and Molecular Biology
EMB	Ethyl methyl blue
EPHI	Ethiopian Public Health Institute
ExPEC	Extra-intestinal Pathogenic *E. coli*
InPEC	Intestinal Pathogenic *E. coli*
IRB	Institutional Review Board
LMICs	Low- and middle-income countries
MBC	Minimum Bactericidal Concentration
MDR	Multi-drug Resistance
MHA	Muller Hinton Agar
MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentration
MRSA	Methicilin Resistant *S. aureus*
OECD	Organization for Economic Cooperation and Development
PCV	Packed Cell Volume
rpm	rotation per minute
SD	Standard deviation
SPSS	Statistical Package for the Social Sciences
WHO	World Health Organization

Abstract

Bacterial diseases are a large burden of morbidity and mortality globally. Emergence of antimicrobial resistance is a major public health problem worldwide, particularly in low-and middle-income countries. Therefore, screening of energetic antimicrobial drugs from herbal sources is important. Thus, the aim of this study was to evaluate the antibacterial activity of two medicinal plants: *Allophylus abyssinicus* (Hochst.) Radlk and *Ligustrum vulgare* leaves which were collected from the premises of Addis Ababa University, College of Natural and computational Sciences. Plant samples were air dried in shade and ground into powder separately. Each powder was soaked in water, methanol and ethyl acetate in 1:10 solute-solvent ratio then, placed on a shaker for 72 hours. The filtered extracts were concentrated in a rotary evaporator and lyophilizer for alcoholic and aqueous extracts respectively. Antibacterial activity test was conducted against selected human pathogenic bacterial species (clinical and standard strains) using the agar-well diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) were determined using broth dilution method. Furthermore, the extracts phytochemical content was identified and safety of the plant was tested on Swiss albino mice. The methanol crude extracts of both plants had relatively higher antibacterial activity than aqueous extracts. Standard *Staphyllococcus aureus* strain was the most susceptible test organism in both methanol and aqueous extracts of both plants. But both clinical and standard strains of *Pseudomonas aeruginosa* were resistant to all extracts. The lowest MIC and MBC of both plants were also recorded for methanol extracts against the standard *Staphyllococcus aureus* strain. Flavonoids, terpenoids, alkaloids, steroids, saponins, tannins, steroidal glycosides and glycosides were detected in both plants. Both plants leaves extracts were not toxic up to 2000mg/kg on the test mice. Future antibacterial activity test on the fractionates of both plants are needed to obtain more promising results.

Keywords: *Allophylus abyssinicus*, Antibacterial activity, *Ligustrum vulgare*, Minimum bactericidal concentration, Minimum inhibitory concentration, Pathogenic bacteria

1. Introduction

Pathogenic bacteria are major global health threat. Serious bacterial infection-related complications affect over a million people every year with mortality rates ranging from 30-50% (Syal *et al*., 2017). Food-borne diseases are responsible for a large burden of morbidity and mortality in both high-income and low-and middle-income countries (LMICs) (Kirk *et al*., 2015). Food-borne diarrheal diseases accounted for about 230,000 deaths in 2010. Among these, *Salmenella typhi (S. typhi)* caused 52,000 deaths in the same year (WHO, 2015).

The sources of harm and health risks caused by water-borne pathogens are widely reported. In the U.S pathogens due to contamination is the foremost cause of water contamination. The U.S National Water Quality Inventory Report suggests that about 53% of the assessed rivers were impaired and a preponderance of them contaminated by pathogens (Pandey *et al*., 2014). Globally, more than 4 million people die of illnesses due to infections from microorganisms and most of these cases are caused by water contamination by microorganisms. There are many forms of water use in daily life, but the greatest threat to human life occurs when there is direct contact between water and human beings; for example bathing spots where sewage is mixed into the water, office buildings that treat and recycle waste water from toilets for reuse, and water works that use river water as the water supply source (Tersagh *et al*., 2018). Contamination of stream water by *Escherichia coli (E. coli)* and *Salmonella* spp. is not only a rising dilemma of low-income countries but also for the high-income countries. Stream water pollution is a major health problem worldwide resulting to nearly 1.7 billion cases of diarrheal diseases every year globally (WHO, 2013). Almost 90% of child deaths from diarrheal diseases are associated to contaminated water and India alone accounts for 24% of all deaths under 5 years old. Among the pathogens disseminated in water sources, enteric pathogens such as entero-toxigenic *E. coli*, *Shigella* spp., *Salmonella* spp., and so forth are the most often encountered that are accountable for group of diseases like diarrhea, dysentery, and enteric fever (Tersagh *et al*., 2018).

Antimicrobial resistance (AMR) is widely observed as one of the major public health concerns of the 21st century (Woolhouse *et al*., 2016). Globally, the emergence of drug resistant bacterial strains has been reported and this challenge limits the effectiveness of current drugs and significantly causing failure in the treatment of infectious diseases (Hancock, 2005). Several European reports show that, severe infections due to gram-negative bacteria, including multidrug-resistant (MDR)

strains that are resistant to multiple classes of antibiotics are significantly increasing (Angiles *et al*., 2014). Bacterial infections caused by MDR bacteria are a growing threat worldwide associated with greater morbidity and mortality at the same time increases healthcare costs *(*Roca *et al*., 2015*).*

The rate at which new drugs are developed is not keeping pace with the changing virulence and drug-resistance patterns of microbes. As a result, searching for alternative medicine against MDR pathogens has become an important concern. Herbal medicine is used for the medication of different bacterial diseases. Plant extracts and products have been reported to be valuable antimicrobial agents (Arya *et al*., 2010).

The historical utilization of plants as a health remedy both for human and animal is centuries old. It has been recognized that plants have the capacity to combat several types of diseases in ethnoveterinary medicines, a term generally used for folk skills, beliefs, knowledge, practices, methods related to animals' health and cure of various ailments in the rural areas (Aziz *et al*., 2018). Medicinal plants are widely used by all sections of the population with an estimated 7500 species of plants used by several ethnic communities (Sermakkani, 2018). Plant extracts from roots, barks, stems, leaves, flowers, fruits and seeds can be used to treat different types of infections (Khan *et al*., 2013; Ogbulie *et al*., 2007). Moreover, natural resources of vegetable origin represent an important source of drugs in the process of developing new pharmacologically active compounds (Vieira *et al*., 2014).

In Ethiopia, more than 80% of the population use traditional medicines to treat different types of infections (Bekele, 2007). In Eastern Ethiopia, various researchers have reported varieties of medicinal plants used against different ailments by traditional healers. The majority of the plants are very popular and known to be utilized and even marketed throughout the region for the management of skin and open wound infections (Bilal *et al*., 2017; Feyera *et al*., 2017; Alebie and Mehamed, 2016). Traditionally, the dried and powdered leaves of *Allophylus abyssinicus* (Hochst.) Radlk.) are either topically applied to treat wounds or taken orally to neutralize different inflammatory conditions. The leaves are also used for the treatment of swellings and sexually transmitted diseases (Yesuf and Asres, 2013). The boiled leaves or bark of *Ligustrum vulgare* helps to treat diarrhea, stomach ulcers, chronic bowel problems, chapped lips, sore mouths and throats, and a wash for skin problems (Foster and Johnson, 2008). However, experimental evidence is not available regarding the antibacterial activity of the leaves of *A. abyssinicus* (Hochst.) Radlk.and

Ligustrum vulgare against human pathogen bacteria in Ethiopia. Hence, this study was at aimed undertaking preliminary phytochemical screening, test toxicity on mice and *in-vitro* antibacterial activity of leaf crude extracts of these two plants. The antibacterial activity test was against the common pathogenic bacteria: *E. coli, S. typhi, S. aureus,* and *P. aeruginosa.*

1.1. Statements of the problem

Emergence of AMR is a major public health problem worldwide, particularly in low- and middle-income countries (LMICs); the effectiveness of currently available antibiotics is decreasing as a result of increasing resistant strains among clinical isolates (Amsalu *et al*., 2016). Globally there was greatest infectious disease burden and the considerable burden is from AMR particularly in Africa. The situation worsens due to second-line drugs which are more expensive than first-line ones and many newer, patent-protected drugs are not available in much in Africa compared to other continent. In Ethiopia, the misuse of antimicrobials, unskilled practitioners and drug consumers coupled with rapid spread of resistant bacteria and inadequate surveillance contributed to AMR (Tamiru and Woldu, 2017). The threat of AMR is growing at the highest rate and the situation is serious in LMICs due to gross abuse in the use of antimicrobials (Ayukekbong *et al*., 2017).

Medicinal plants have been used in treating human diseases for thousands of years. They are rich in a wide variety of secondary metabolites that include tannins, saponin, terpenoids, alkaloids and flavonoids used for antimicrobial, antioxidant and anti-inflammatory properties (Cowan, 2001). However, many of these compounds obtained from plants are currently available as unregulated crude botanical preparations in herbal medicine and their use by the public is increasing rapidly (Shinkafi and Dauda, 2013).Therefore, screening of energetic antimicrobial drugs from herbal sources is important as many previous studies demonstrated that herbal remedies are promising antimicrobial activities. Hence, the purpose of this study was to assess the safety at *in-vivo* level and antibacterial activities at *in-vitro* level of the leaves of *A. abyssinicus* (Hochst.) Radlk and *L. vulgare.*

1.2. Objectives

1.2.1. General objective

The general objective of this study was to evaluate *in-vitro* antibacterial activities of the leaves of *A. abyssinicus* (Hochst.) Radlk and *L. vulgare* of aqueous, ethyl acetate and methanol crude extracts on selected human pathogenic bacteria.

1.2.2. Specific objectives

1. To determine antibacterial activity of *A. abyssinicus* (Hochst.) Radlkand *L. vulgare* leaves crude extracts of different solvents based on minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC).
2. To identify the secondary metabolites of the two plants extracts using qualitative phytochemical test.
3. To examine the oral acute toxicity of the two plants extracts on Swiss albino mice.

1.3. Hypothesis

It was hypothesized that aqueous, ethyl acetate and methanol crude extracts of *A. abyssinicus* (Hochst.) Radlk and *L. vulgare* leaves have *in-vitro* antibacterial effect on selected pathogenic bacteria with no acute toxicity on Swiss albino mice.

2. Literature review

2.1. Challenges of pathogenic microorganisms

World Health Organization (WHO) stated that microorganisms like fungi, bacteria, viruses and parasites are the causes of infectious diseases. One of greatest the challenge in the world is the continuing impact of infectious diseases. Infectious diseases are closely dependent on the nature and complexity of human behavior. A few of the current global trends that make challenging our relationship with infectious diseases are: climate change and vector-borne diseases, antimicrobial resistance, increasing numbers of immune compromised patients, emerging threats (Fauci and Morens, 2012). A large burden of morbidity and mortality in both high-income and LMICs are caused by food-borne diseases. More than 200 diseases can be transmitted to people through the ingestion of contaminated food with microorganisms (bacteria, viruses, fungi and parasites) or with chemicals (Kirk *et al*, 2015). Those bacteria, viruses, parasites and fungi that are resistant to drugs cause 700,000 deaths each year globally (Bloom *et al*., 2017).

In bacterial pathogens, AMR is a global challenge associated with high morbidity and mortality (Frieri *et al*., 2016; Lim *et al*., 2016). Antibiotic resistance is a persistently evolving occurrence; a threat to infection and disease control. For this reason, it complicates patient management and treatment strategy and delays hospital stays of the patient (WHO, 2018). Rates of antibiotic resistant gram-negative bacteria infections continue to rise globally; effective therapeutic options against these infections are severely limited. Each year in Europe, approximately 400,000 patients with hospital-acquired infections present with a resistant strain (Nathwani *et al*., 2014). MDR patterns in gram-positive and gram-negative bacteria have resulted in difficult to treat or even untreatable infections with conventional antimicrobials. Because of early identification of causative microorganisms and their antimicrobial susceptibility patterns in patients with bacteremia and other serious infections is lacking in many healthcare settings, broad-spectrum antibiotics are liberally and mostly unnecessarily used. This causes dramatic increases in emerging resistance and when coupled with poor practice of infection control, resistant bacteria can easily be disseminated to the other patients and the environment (Akova, 2016). Each year, in the United States, issues related to antibacterial resistance are projected to result in over 23,000 deaths, 2 million illnesses, as well as costs amounting to 20 billion dollars and 25,000 death in the European Union (Lim *et al*., 2016; WHO, 2014). These illnesses cause an additional healthcare cost of $20 billion and a productivity loss of $35 billion to the U.S. economy (Aslam *et al*., 2018). While AMR occurs naturally, the

emergence and spread of new resistance mechanisms may have been greatly accelerated by the overuse and misuse of antimicrobials (Tadesse *et al*., 2017). In many countries, antibiotics are given without professional oversight and are inappropriately used in both people and animals; important examples of such misuse include the consumption of antibiotics by people with common viral infections or when given to farm-raised fish or livestock as growth promoters (Hay *et al*., 2018).

In LMICs, infectious diseases remain the leading cause of death in children fewer than 5. The infectious disease accounts for over 76% of fewer than 5 children deaths in Africa and an estimated 36% of neonatal deaths globally (Huynh *et al*., 2015). The emergence of AMR is the most important health concern in LMICs due to antibiotic use is increasing with rising incomes, affordable antimicrobials and the lack of stewardship in hospital and poor control of over-the-counter sales. This is driving the emergence and spread of MDR pathogens in community and hospital settings (Lim *et al*., 2016). According to WHO pathogens priority list: *P. aeruginosa, E. coli, S. aureus,* and *Salmonella* spp. are categorized as MDR strains which cause different bacterial infections in human throughout the world (WHO, 2017). Although food-borne diseases are a major public health problem across the globe, the problem is severe in LMICs due to difficulties in securing optimal hygienic food handling practices. In LMICs, up to 70% of cases of diarrheal disease are associated with the consumption of contaminated food due to the health status of the food handlers, personal hygiene, and knowledge and practice of food hygiene (Mengist *et al*., 2018; Souse, 2008). Diarrheal diseases caused bacterial enteric infections such as *Salmonella* spp. and pathogenic *E. coli* account for an estimated 10% of childhood deaths in Sub-Saharan Africa. These bacterial pathogens are also associated with linear growth faltering, which has cognitive, economic, and health consequences which extended into adulthood (Brander *et al*., 2017).

Bacterial infections caused by MDR are major cause of morbidity and mortality in LMICs including Ethiopia. AMR is a major problem in both hospital and community acquired infections (Mulu *et al*., 2017). Low-income countries like Ethiopia are the least to regulate drug distribution and utilization quality which leads to the emergence of AMR pathogens (Shimalis *et al*., 2015). Food-borne disease is one of the most common illnesses and a leading cause of death among people of all ages in Ethiopia. The major contributing factors for exposure to food-borne pathogens are lack of surveillance of food-borne pathogens, poor hygiene and sub-standard slaughter practices in the abattoirs, and the widespread cultural practice of raw meat consumption (Dulo *et al*., 2015).

2.2. Major human pathogenic bacteria

2.2.1. *Escherichia coli*

Escherichia coli are a gram-negative, rod-shaped, facultative anaerobic bacterium, non-spore forming, usually motile by flagella widely distributed in the gastrointestinal tract (Percival and William, 2014). This microorganism was first described by Theodor Escherich in 1885 (Shulman *et al*., 2007). It is measuring only about 1micrometer long and 0.35micrometer wide (Blount, 2015). Most *E. coli* strains harmlessly colonize the gastrointestinal tract of humans and animals as a normal flora (Lim *et al*., 2010).

Although most *E. coli* strains are harmless, certain strains are pathogenic and cause of acute urinary tract infections, sepsis, neonatal meningitis and is a general cause of traveler's diarrhea, a dysentery-like disease affecting humans and haemorrhagic colitis often referred to as bloody diarrhea and watery diarrhea which can lead to death (Cho *et al*., 2018; Percival and William, 2014). Pathogenic *E. coli* is mainly divided into two groups depending on the disease location: extra intestinal pathogenic *E. coli* (ExPEC) which is mainly associated with neonatal meningitis *E. coli* (NMEC) and urinary tract infections *E. coli* (UPEC) in adults and intestinal pathogenic *E. coli* (InPEC) related to diarrheal disease (Rojas-Lopez *et al*., 2018). ExPEC strains are responsible for an estimated 40,000 deaths and at least $2.6 billion of expenses in healthcare treatment in the U.S alone (Ramirez-Castillo *et al*., 2018). InPEC are also subdivided in to six pathotypes: enterotoxigenic *E. coli* (ETEC), Shigatoxin producing/enterohemorrhagic *E. coli* (STEC/EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E.coli* (EAEC) and diffusely adherent E. coli (DAEC) (Russo and Johnson, 2000). EPEC is a major cause of infantile diarrhea in low-income countries (Hu and Torres, 2015).

Infections with pathogenic *E. coli* strains are usually considered an indication of poor hygiene. In LMICs, 63% of children with persistent diarrhea tested are positive for *E. coli* strains (Fletcher *et al*., 2013). AMR in *E. coli* has been characterized by changes in morphology (Liu *et al.*, 2018). MDR in *E. coli* has become a worrying issue that is increasingly observed in human and also in veterinary medicine globally (Poire *et al*., 2018; Rasheed *et al*., 2014). Antibiotic resistance rates in *E. coli* are rapidly increasing, especially concerning to the use of fluoroquinolones and third- and fourth-generation cephalosporin due to the spreading of extended-spectrum β-lactamases (ESBL). Treatment for *E. coli* infection has been increasingly complicated by the emergence of resistance to most first-line antimicrobial agents (Rasheed *et al*., 2014).

2.2.2. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an aerobic, motile, gram-negative rod that belongs to the family Pseudomonadaceae (Siddiqua *et al*., 2018). It can be commonly found in soil, plants and hospital reservoirs of water, including showers, sinks and toilet water (Lopez- Garcia *et al*., 2018). They are often MDR due to intrinsic and acquired determinants; it can survive with low levels of nutrients and grow in temperatures ranging from 4 to 42^{o}C (Siddiqua *et al*., 2018).

It causes different infections such as urinary tract, respiratory system, soft tissue, bone and joint, gastrointestinal and a variety of systemic infections, dermatitis and bacteremia particularly in patients with severe burns, cancer and AIDS (Andualam, 2012). *P. aeruginosa*is also a leading cause of nosocomial infections, pneumonias in particular and responsible for 10% of all hospital-acquired infections in US (Aloush *et al*., 2006). Nosocomial pneumonia caused by *P. aeruginosa* has a mortality rate ranging from 13-50%, lengthens hospital stays, and adds approximately $40,000 in excess cost per patient in US (Curran *et al*., 2018). Resistant *P. aeruginosa* infections are associated with high mortality, morbidity and increased resource utilization and costs (Nathwani *et al*., 2014). *P. aeruginosa* demonstrates increased resistance to various antimicrobials. MDR *P. aeruginosa* is resistant to three or more anti-microbial; carbapenems, fluoroquinolones, penicillins /cephalosporins and aminoglycosides (Hassuna *et al*., 2015). WHO has recently reported carbapenem-resistant *P. aeruginosa*as one of bacterial species in which there is a critical need for the development of new antibiotics to treat infections (Tacconelli *et al*., 2018). Increasing rates MDR *P. aeruginosa* among hospitalized patients is a major public health problem and infections are associated with poor outcomes including increased resource utilization, costs, morbidity and mortality (Raman *et al*., 2018). According to the Centers for Disease Control and Prevention (CDC 2018), MDR *P. aeruginosa*can be fatal for patients in critical care. In the U.S, an estimated 51,000 healthcare-associated *P. aeruginosa* infections occur each year and more than 6,000 (13%) of these are MDR, with roughly 400 deaths per year.

The mechanism of resistance in MDR *P. aeruginosa*is through the production of several enzymes which inactivate beta-lactams and carbapenems such as extended spectrum beta lactamases (ESBLs) and metallo-β-lactamases (MBLs) (Vahdani *et al*., 2012).

2.2.3. *Salmonella typhi*

*Salmonella typhi*is a gram negative, a rod shape, facultative anaerobic, a non–sporing and a non–capsulated bacteria. It is an etiological agent of typhoid fever, which is called ‘enteric fever’ for

typhoid *Salmonella* (Eng *et al*., 2018). Globally typhoid fever is responsible for 21 million illness incidents and over 140,000 deaths each year (John *et al*., 2018). Transmission of *S. typhi* occurs through ingestion of faecally-contaminated food or water. Untreated typhoid fever may result in severe illness including the complications of gastrointestinal bleeding, bowel perforation and sometimes death (De Jong *et al*., 2012). The incidence of typhoid fever is very low in high-income areas like Europe and North America, whereas many in LMICs like Africa, Latin America and South or Southeast Asia the disease is endemic (Yan *et al*., 2016). Despite its long-standing presence in human history, typhoid fever is still a major public health concern of morbidity and mortality with an estimated 26.9 million cases and approximately 200,000 deaths mostly children in low-income countries (Als *et al*., 2018). It is also among the common food-borne pathogenic bacterium which is responsible for the major causes of diarrhea in humans (Jelalu *et al.*, 2015).

In many parts of the world, the changing approaches of appearance and the development of MDR have made enteric fever progressively difficult to diagnose and treat (Lugito and Cucunawangsih, 2017). *S. typhi* isolates has been increasingly reported as resistance to the antimicrobial agents such as trimethoprim–sulfamethoxazole, chloramphenicol and amoxicillin (Elshayeb *et al*., 2017).

2.2.4. *Staphylococcus aureus*

Staphylococcus aureus is gram-positive, catalase-positive, oxidase-negative, non-motile and non-spore forming facultative anaerobes belongs to the family Staphylococcacace. It is the leading bacterium in the normal flora of humans that causes a variety of clinical infections (Engemann *et al*., 2003). *S. aureus*is a desiccation tolerant organism with the ability to survive in potentially dry and stressful environments such as the human nose, skin and inanimate surfaces such as clothing and surfaces (Chaibebjamong and Foster, 2011). These characteristics favor growth of the organism in many food products. *S. aureus* can remain viable on hands and environmental surfaces for extended durations after initial contact (Kadariya *et al*., 2014).

S. aureus causes a wide range of infections commonly involving the skin, soft tissue, bone, joints and infections associated with indwelling catheters or prosthetic devices, which is leading cause of boils, cellulitis, abscesses, wound infections, toxic shock syndrome, pneumonia and food poisoning (Tong *et al*., 2015). About 30% of the populations are asymptomatically colonized by *S. aureus*; mostly in the anterior areas. Infections with *S. aureus* are common both in the community and health care facilities range from mild skin infections to soft tissue and bone infections to severe life

threatening pneumonias and bacteremia's (Venkatesh, 2018). Additionally, *S. aureus* is a leading cause of bacteremia in industrialized nations (Hassoun *et al*., 2017). *S. aureus* causes bacteremia in children with significant morbidity and mortality; but, the epidemiology in children is not well characterized (McMullan*et al*., 2016). Methicillin resistant *S. aureus* (MRSA) is a bacterium that has developed resistance to most antibiotics such as methicillin, gentamycin, fucidic acid and clindamycin that are commonly used for *Staphylococcus* infections unfortunately, leading to failure of treatment. The two major strains of MRSA are known to be hospital-acquired (HA-MRSA) and community-acquired (CAMRSA). Hospital-acquired-MRSA includes cases in which the patient has a recent hospitalization, receives dialysis or resides in a long-term care facility (Rajaa and Nahed, 2012).

Although the burden of MRSA is a major public health concern globally; the overall epidemiology of MDR strains is neither coordinated nor harmonized, particularly in low-income countries including Ethiopia (Eshetie *et al*., 2016). According to the study conducted at Yekatit 12 Hospital, Addis Ababa, showed isolation rate of *S. aureus* from 14.3% of clinical specimens, of which over 50% of the isolates were MDR and 17.5% were MRSA (Dilnessa and Bitew, 2016).

2.3. Antimicrobial activities of medicinal plants

The origin of healing with plants is started in the mankind's history. Medicinal plants are traditionally used as remedies for the treatment of various diseases, including asthma, gastrointestinal symptoms, skin disorders, respiratory and urinary problems, hepatic and cardiovascular disease worldwide (Egamberdieva *et al*., 2017; Joseph *et al*., 2013). Traditional medicine practitioners through ancient indigenous technology and by a series of "trial and error" over time have successively applied various plant parts to treat diverse sicknesses (Adamu *et al*., 2017).

Medicinal plants are abundant source of antimicrobial molecules. In high-income countries, it is estimated that 80% of individuals use traditional medicine which possess products obtained from medicinal plants (Jangra *et al.,* 2018; Abera, 2014). Also, in many high-income countries a large proportion of the population relies on traditional practitioner of medicinal plants in order to meet health care need (Oyebode *et al*., 2016). The use of medicinal plants is growing rapidly throughout the world. People in LMICs are dependent on herbal drugs for their primary healthcare and over 25% of prescribed medicines in high-come countries are derived from wild plant species (Chen *et*

al., 2016). A wide range of medicinal plants extracts are used to treat several infections as they have potential antimicrobial activity. For the reason, some of these bioactive molecules are screened and traded in market as raw material for many herbal industries (Javid *et al.,* 2015).

Medicinal plants are sources of diverse molecules, many of which display antimicrobial and antioxidant properties which protect human body from both pathogens and cellular oxidation (Rahma *et al*., 2015). The use of medicinal plants for human healthcare is well documented in India, China, Egypt and Arab world (Chandra *et al*., 2015).

Ethiopia is endowed with a diverse biological resources including about 6, 500 species of higher plants, with approximately 12% endemic, hence making it one of the six plant biodiversity rich regions and have been used as a source of traditional medicine to treat different human and livestock ailments (Abera, 2014). About 80% of human and 90% of livestock population depend on traditional medicine in the country and the majority of the population that lives in the rural and the poor people in urban areas rely mainly on traditional medicines to meet their primary health care needs (Biranu *et al*., 2015). Medicinal plants used as antimicrobial agents were acting by different mechanisms to avoid the development of MDR bacteria (Al-Snafi, 2016). Medicinal plants contain several phytochemicals such as flavonoids, alkaloids, tannins and terpenoids which possess antimicrobial and antioxidant properties (Altamani *et al*., 2017; Yaqoob *et al*., 2016; Mujeeb *et al*., 2014). Since earliest times, many plants have been known to exert healing properties against human infections due to their contents of secondary metabolites, which in more recent times have been found to act as antimicrobial agents against MDR gram-negative and gram-positive human pathogenic bacteria (Borges *et al*., 2015).

2.4. Selected medicinal plant species

2.4.1. *Allophylus abyssinicus* (Hochst.) Radlk.

Allophylus abyssinicus (Hochst.)Radlk.is the genus *Allophylus* and family Sapindacea. It is medium sized to large tree. Bark is smooth greyish-green; the mature trunk up to 1 meter across and often fluted (Fig. 1). Leaves are compound with 3 on a stalk to 12 centimeters, edges slightly toothed and hairs only in the vein axils below tip pointed. The leaflets have short stalks and the big central one 9-18 centimeters long. Flowers are yellow-white in much-branched heads to 20 centimeters. Fruit are bunches of rounded soft red berries about 7 millimeters across and very small seeds inside. The

genus *Allophylus* which has about 255 species is an important medicinal plant with a great ethnopharmacological value (Chavan and Gaikwad, 2016)

In Ethiopia, the wood of *A. abyssinicus* (Hochst.) Radlk.is used for the manufacture of farm tools and yokes, sometimes for joinery and boxes as well as firewood and charcoal. The leaves are used as an anthelmintic and the fruits to cure venereal diseases in Ethiopia. In Uganda it is considerably useful as a shade tree for crops. In Kenya roots are grated and eaten in small quantity to treat coughs and rheumatism (Bosch, 2011). The leaves of *A. abyssinicus* (Hochst.) Radlk. (Sapindaceae) are used for the treatment of wounds, burns, skin diseases and to arrest bleeding in the Ethiopian folk medicine (Yusuf and Asres, 2013).

Figure1: *A. abyssinicus*

2.4.2. *Ligustrum vulgare*

Ligustrum vulgare L. (*L. vulgare*) (Wild privet) belongs to the genus *Ligustrum L.* and family Oleaceae, according to different classifications, which contains, between 37 and 50 species, divided into five sections. Most of these species are native to Asia and only a few species are present in Europe, Australia, North America or North Africa (Qin, 2009; Starr *et al*., 2003). These species are small trees or shrubs (Fig. 2) widely planted for hedge, shade, shelter and garden purposes. Wild privet (*L. vulgare*) is generally recognized as a useful shrub species across its wide natural distribution range (Enescu *et al*., 2015). It is one of the most used shrub species in establishing several types of protective forest shelterbelts or windbreak (Spacilova and Stredova, 2014; Malschi *et al*., 2010). *L. vulgare* leaf was well known in the Mediterranean historical medicine and has been used for treatment of oropharyngeal inflammations or as antirheumatic, diuretic and hypotensive

agents in folk medicine in southern Europe (Czerwinska *et al.*, 2013). In the folk medicine of Azerbaijan, the leaves of common privet are used to treat hypertension (Hammermann *et al*., 1971). Moreover, water infusions from leaves of *L. vulgare* have shown a high antimutagenic effect (Nagy *et al*., 2009). Several extracts from this plant have also been proven to act as a dual angiotensin-converting enzyme (Kiss *et al*., 2008).

Figure 2: *L. vulgare*

2.5. Phytochemical screening

Phytochemicals are the chemicals that are present naturally in plants which can cure diseases without causing any harm to human beings (Banu and Cathrine, 2015). Phytochemicals are non-nutritive chemical compounds that occur naturally on plants and have diverse protective properties against human pathogens (Minakshi *et al*., 2016). The medicinal value of a plant is depending on the bioactive constituents of the plant, which shows various physiological effects on human body. Through phytochemical screening one could detect the various important compounds which could be used as the base of modern drugs for curing various diseases (Sheikh *et al*., 2013). The medicinal effects of plant materials typically result from the combination of secondary products such as alkaloids, steroids, tannins, glycosides, volatile oils, fixed oils, resins, phenols and flavonoids which are deposited in their specific parts such as leaves, flowers, bark, seeds, fruits, root (Joseph *et al*., 2013).

The idea that plants are a reservoir of biologically active compounds with therapeutic properties has been well established since ancient time, they have been used worldwide for the treatment of various ailments including asthma, gastro-intestinal problems, skin disorders, respiratory and urinary complications, hepatic and cardiovascular disease (Tian *et al*., 2014). Now a days, these phytochemicals become more common due to their countless medicinal uses and their vital roles against number of diseases such as arthritis, cancer, antioxidant, antibacterial, antifungals, anti-diabetic, anti-inflammatory and radio protective activity; due to these properties they are largely used for medicinal purpose (Banu and Cathrine, 2015; Joseph *et al.*, 2013).

2.6. Toxicity of medicinal plants

Although plants may look harmless enough, but they can harbor some of the most deadly poisons which have been responsible for human deaths throughout history. Therefore, Medicinal plants not only have medicinal and nutritional values but also they are toxic to the normal physiology of animals. Plants produce secondary metabolites as defense against animals, parasites, bacteria and viruses (Robber and Huxtable, 1992). The general perception that herbal remedies or drugs are very safe and devoid of adverse effects is not only untrue, but also misleading. Herbs have been shown to be capable of producing a wide range of undesirable or adverse reactions, some of which are capable of causing serious injuries, life-threatening conditions and even death (Ernst, 2002). Even though there is none which is not a poison, the right dose differentiates remedy from poison (Wong and Irwin, 2013). The plants having medicinal activity should have low toxicity because of their long-term use in humans. However, various medicinal plants used in folklore medicines have been reported to exhibit toxic effects depending on their origin and nature (Brima, 2017).

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

3. Materials and Methods

3.1. Plant materials collection and identification

Fresh leaves of *Allophylus abyssinicus* (Hochst.) Radlk. (local name "Embis" in Amharic or "Tatesa"in Afan Oromo) and *Ligustrum vulgare* (local name "Yemefakya enchet in Amharic or "Muka riga" in Afan Oromo) were collected from AAU, Arat Killo Campass and authenticated by Mr. Melaku Wondafrash in the National Herbarium, Department of Plant Biology and Biodiversity Conservation, AAU. Then the specimens of *A. abyssinicus* (Hochst.) Radlk.and*L. vulgare* were deposited with voucher number EM001and EM002, respectively.

3.2. Extract preparation

The plants samples were washed twice with tap water and once with sterile distilled water to eliminate adhering dust and any foreign particles and allowed to dry shade in Biomedical Research Laboratory of DMCMB at CNCS, AAU at room temperature for about 20 days. The dried samples were grinded with an electric grinder, sieved with a fine mesh of 500micrometer sieve size and stored at room temperature until considered for extraction.

The extracts of *A. abyssinicus* (Hochst.) Radlk. and *L. vulgare* were prepared separately using aqueous (distilled water), methanol and ethyl acetate solvents. The sieved powder forms of *A. abyssinicus* (Hochst.) Radlk.(100g) and *L. vulgare* (100g) were extracted with 1000ml of sterile distilled water separately in 500ml Erlenmeyer flask on a rotary shaker (GFL, model 3020, Germany) with 120rpm at room temperature for 72 hours as done by (Chonoko and Rufai, 2011). The extracts were first filtered using four-fold muslin cloth then by filter paper Whatman No.1 (Whatman Ltd., England) and the extracts were deposited under deep freeze for 24hours; the extracts were separated by using lyophilizer (CHRIST ALPHA1-4, Germany) at -40°C with vacuum pressure and the crude extracts were placed at -20°C until used.

About 100g powders of *A. abyssinicus* (Hochst.) Radlk. were soaked in 1000ml methanol (99.8%) and other 100g were also soaked in 1000ml ethyl acetate (99.0%) in separate 500ml Erlenmeyer flask on a rotary shaker (GFL, model 3020, Germany) with 120rpm at room temperature for 72 hours according to (Chonoko and Rufai, 2011). The extracts were first filtered using four-fold muslin cloth then by filter paper Whatman No.1 (Whatman Ltd., England). The final filtrates were concentrated in a rotary evaporator (BUCHI type TRE121; Switzerland) with 60rpm at a temperature of 45°C.

About 100g powders of *L. vulgare* were soaked in 1000ml methanol (99.8%) and 100g of powder *L .vulgare* were also soaked in 1000ml ethyl acetate (99.0%) in separate 500ml Erlenmeyer flask on a rotary shaker (GFL, model 3020, Germany) with 120rpm at room temperature for 72 hours according to (Chonoko and Rufai, 2011). The final filtrates were concentrated in a rotary evaporator (BUCHI type TRE121; Switzerland) with 60 rpm at a temperature of 45°C.

The extracts of ethyl acetate and methanol were further dried in oven at 45°C and finally the percentage yield of each extracts were preserved at -20ºC for further tests.

3.3. Phytochemical detection

The water and methanol crude extracts of leaves of *A. abyssinicus* (Hochst.) Radlk.and*L. vulgare* were screened for the presence of bioactive compounds like phenol, flavonoid, tannins, steroids, terpenoids, steriodal glcosides, alkaloids, saponins, resin and glycosides in accordance to standard phytochemical methods.

Alkaloid test

In this test, the extracts were dissolved in dilute (2%) Hydrochloric acid and filter with Whatsman filter paper. The filtrates were treated with Dragendorff reagent. Reddish brown precipitate was observed (Chandra *et al*., 2015).

Phenol test

To determine the presence of phenol in the extracts, the dissolved extracts were treated with few drops of 5% glacial acetic acid and 5% $NaNO_2$ solution. The brown precipitate occurs indicates the presences of phenol in the extracts (Joseph *et al*., 2013).

Saponin test

To do this test, extracts solution were mixed with 20ml of distilled water and continuously shakes for 15 minutes. The formation of foam indicates the presences of saponin (Joseph *et al*., 2013).

Glycoside test

Two milliliters of glacial acetic acid was added to 5ml of plants extracts. Then 1 drop of 5% $FeCl_3$ and 5ml of conc. H_2SO_4 were added along the side of the test tube respectively. Brown color was formed at the junction of two layers which indicates the presence of glycosides (Joseph *et al*., 2013).

Flavonoid test

For this test, few drops of 10% NaOH were added to 1ml of the extracts then an intense yellow color formed. Few drops of 2% HCL were again added to yellow color mixture and allowed for a colorless change that indicated the presence of flavonoids (Hossain *et al*., 2013).

Tannin test

This test is determined by adding few drop of 1% lead acetate in 2ml of stock extracts solution. The formation of yellowish precipitate indicates the presences of tannins in the extracts (Nani *et al*., 2013).

Steroid test

To identify the presence of steroids in the extracts, 1ml of extracts were dissolved in 10ml of chloroform, and then 10ml of concentrated H_2SO_4 was added by the side of the test tube. The red color was formed in the upper layer and sulpheric acid layer showed yellow color with green fluorescence which shows the existence of steroids (Gibbs, 1974).

Terpenoid test

For this test mixing extracts solution with 2 ml of chloroform with the careful addition of concentrated H_2SO_4. The formation of a reddish brown colour at the interface confirmed the presence of terpenoids (Chandra *et al*., 2015).

Steroidal Glycosides test

This test done by dissolving the 2ml of the tested extracts with glacial acetic acid and makes it cool. Add 2 drop of 5% $FeCl_2$ and transfer it into a test tube which contains 2ml of concentrated H_2SO_4. The reddish brown color was observed at the junction of two layers (Nani *et al*., 2013).

Resin test

For this test, 2ml of distilled water was poured into 1ml of extract dissolved in acetone. Turbidity of the solution was observed to determine the presence of resin (Keo *et al*., 2017).

3.4. Test organisms

3.4.1. Bacterial strains

Both standard American Type Culture Collection (ATCC) and clinical isolates of different gram-negative and gram-positive bacterial strains were used. The standard strains used were *E. coli* (ATCC 25922), *P. aeruginosa (*ATCC 2785315), *S. aureus (*ATCC 25923) and *S. typhi (*ATCC 13311*)* and clinical isolates of the same species. Both the standard strains and clinical bacterial isolates were obtained from the Ethiopian Public Health Institute (EPHI) Microbiology Department. Those bacterial strains were cultivated and maintained in appropriate culture media.

3.4.2. Bacterial inoculum preparation

All standard and clinical bacterial strains were streaked on selective or differential media to avoid contamination and to obtain pure culture. The standard and clinical strains of *S. aureus* streaked on mannitol salt agar, *S. typhi* on S.S agar, *E. coli* on ethyl methyl blue (EMB) agar and *P. aeruginosa* on pseudomonas-isolator agar those were prepared according to the instruction of the manufacturer and the operating standard procedures.

Three to five well-isolated colonies selected from an overnight culture at 37^{o}C of selective media were touched with sterilized loop of inoculator and transferred into a tube containing 5ml of sterile nutrient broth and cultured for 24 hours at 37^{o}C. Then the bacterial isolates were made to grow on nutrient agar (Oxoid LTD., Basingstoke, and Hampshire, England) for 24 hours at 37^{o}C. At least three to five well-isolated colonies of the same morphological type were selected from an overnight culture at 37^{o}C on nutrient agar plate medium and transferred into a pre-labeled tube containing 99.9% sterile physiological saline to dilute the suspension.

The turbidity of the actively growing bacterial culture was adjusted optically comparable to that of the 0.5 McFarland standards (that was prepared by adding a 0.5ml aliquot of 0.48 mol/L $BaCl_2$ to 99.5 ml of 0.18 mol/L H_2SO_4 (1%v/v) (McFarland, 1979) through visually comparing the physiological saline inoculum tube and the 0.5 McFarland standards against a card with a white background and contrasting black lines which is resulted in a suspension containing approximately 1.5 x 108 CFU/ml for microorganism. The prepared saline culture (suspension) was used to inoculate the Mueller Hinton agar (MHA) (Oxoid LTD., Basingstoke, Hampshire, England) plates for antibacterial susceptibility testing.

3.5. Standard Antibiotics

Chloramphenicol was used as positive control; whereas sterilized distilled water was used as a negative control for the antibacterial susceptibility test.

3.6. Antibacterial susceptibility test

3.6.1. Agar well diffusion

The antibacterial activity of both plants extracts were done by using agar well-diffusion method (Cheruiyot *et al*., 2009). Sterilized MHA plates were prepared by pouring 25ml of molten media into 90mm diameter sterile petri dish and plates were allowed to solidify for 5 minutes for any surface moisture to be absorbed. Fresh inoculums of each bacterium were swabbed uniformly on the media using a sterile swab and allowed to dry for 5 minutes. Then, five equidistant wells were bored into the medium using a sterile 6mm diameter cork borer. The three corresponding wells were filled with 500mg/ml crude plant extracts of different solvents. Chloramphenicol 30µg/80µl and distilled water were loaded on two additional wells as positive and negative controls respectively. After filling the extracts in the wells, the plates were left undisturbed for about 2 hours at room temperature for pre-diffusion (Dahiya and Purkayastha, 2012) and then, incubate at 37°C for 24 hours. 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 triplicate.

3.6.2. Determination of minimum inhibitory concentration

The minimum inhibitory concentrations (MIC) of the crude extracts were determined as the lowest concentration of the aqueous and methanol extracts inhibiting visible growth of the tested bacteria (Andrews, 2001). The MIC was determined by two fold serial dilution technique using nutrient broth media (CLSI, 2015). Accordingly, a stock solution with the concentration of the plant extracts of 250mg in 1ml of distilled water was diluted using a two-fold serial dilution. This provides the series of test concentrations of 125, 62.5, 31.25, 15.25, 7.8, 3.9 and 1.9mg/ml respectively. The plant's antibacterial activity testing was done by incubating each concentration of the extract with inoculum.

The inoculum was prepared by taking 3-5 colonies of overnight fresh cultures of bacterial strains in physiological saline solution and comparing the turbidity with that of the standard 0.5 McFarland solution. Then, 1ml of inoculum was taken from standardized saline solution inoculum prepared

according to 0.5 McFarland solutions and added to 150ml of sterilized Muller Hinton broth (MHB) without any plant extract or any bacterial suspension to dilute the concentration of the inoculum. Next, 1ml of inoculum was taken from diluted bacterial suspension added to each concentration of both plant extracts. The test tube containing broth without inoculum was the negative control and the one containing broth plus inoculum was considered as positive control and all tubes were incubated at 37 °C for 24 hours. Lastly, the presence and absence of growth was recognized by turbidity and clear solution respectively and the average value of triplicates was taken as MIC.

3.6.3. Determination of minimum bactericidal concentration

After MIC determination, minimum bactericidal concentration (MBC) was determined by sub culturing the samples having values of greater or equal to MIC values. The highest dilution (lesser concentration) that yielded no single bacterial colony was taken as MBC (Andrews, 2001).

3.7. Oral acute toxicity determination

The test was performed according to Organization of Economic Cooperation and Development (OECD) guideline No. 420 (OECD. 2001). Twenty female Swiss albino mice weighing 20-25g were used for the toxicity test. Those mice were obtained from the animal house of the DMCMB. The mice were randomly selected, marked and distributed to permit individual identification and kept in their cages for at least 5 days prior to dosing. Then they were housed in clean cages to allow for acclimatization to the laboratory conditions. They were fed with standard mice pellet diet and water *ad libitum*.

The mice were left fasting for 3hours prior to dosing. Following the period of fasting, the mice were weighed by sensitive digital electronics beam balance (A & D Company, Japan) and distributed in to four groups of five mice in each group. Then mouse was administered orally with the dose of 1000, 1500 and 2000mg/kg bodyweight (BW) of methanol crude extracts of both plants for group one, two and three respectively and water was administered for the negative control mice group by using specially designed mice oral needle (Cadence Science Ltd., America). The extracts were delivered orally based on the average BW of the mice in each group. After the extract administration, the animals were observed within 30 minutes, 4 hours and after 24 hours checking for signs of behavioral manifestations and the mice were further checked for 14 days for any signs of acute toxicity. BW of the mice were further measured on days 7 (D7) and 14 (D14) to notice any change.

The packed cell volumes (PCV) of each group mice were measured before extract administration and at D14 after administration. Blood samples were drawn from tail of each mouse in heparinized micro-hematocrit capillary tubes up to 3/4th length to measure PCV. The tubes sealed by sealer and placed in a micro-hematocrit centrifuge (HAWKSLEY, England). The samples centrifuged at 12,000 rpm for 4 minutes. PCV of experimental mice was calculated as follows (Bull *et al.,* 2000).

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

Finally, acute toxicity test was determined by gross physical, BW and PCV changes for 14 days.

3.8. Data analysis

Data were analyzed by using SPSS software, version 22 (IBM SPSS, United States). The results were presented as the mean ± standard deviation (SD) and statistical significance was considered at 95% confidence interval ($P<0.05$).

3.9. Ethical clearance

Ethical clearance for this study was obtained from the College of Natural Sciences Institutional Review Board (CNS-IRB), AAU.

4. Results and Discussion

4.1. Yield of the Crude Extracts

In this study, the method used for the extraction achieved different extract yields 4.69, 12.63 and 15.35% of ethyl acetate, aqueous and methanol, respectively, for *A. abyssinicus* (Hochst.) Radlk.and 3.84, 11.35 and 13.782% of ethyl acetate, aqueous and methanol for *L. vulgare* respectively. The highest percent extract yield was obtained from *A. abyssinicus* (Hochst.) Radlk.leaf (15.35%) and the lowest (3.84%) was from *L. vulgare* leaf (Table 1). The percentage yields of the extract were calculated according to Tura *et al.*, 2017.

Percentage extract yield (%) $= \frac{Weigh\ of\ dreid\ extract}{Weight\ of\ dried\ powder} 100\%$

In both plants the highest yield was obtained from the extracts of methanol and the least yield was obtained from ethyl acetate. From this data, it is possible to conclude that methanol dissolves more active components compared to ethyl acetate and water. Since, the extraction yields indicate a measure of the solvent efficiency to extract specific components from the original material.

Table 1: The percentage yields of crude extracts of the leaves of *A. abyssinicus* (Hochst.) Radlk. and *L. vulgare* at concentration ratio of 100 mg powder dissolved in 1000 ml of solvents.

Parts of plants	Solvents	Powder weight (g)	Extract weight (g)	Yield (%)
A.abyssnicus	H_2O	100	12.63	12.63
	MeOH	100	15.35	15.35
	EtOAc	100	4.69	4.69
L.vulgare	H_2O	100	11.39	11.39
	MeOH	100	13.78	13.78
	EtOAc	100	3.84	3.84

Note: H_2O = water, MeOH = methanol, EtOAc = Ethyl acetate.

4.2. Phytochemical detection test of *A.abyssinicus* (Hochst.) Radlk. and *L. vulgare* leaves

Phytochemical constituents are bioactive compounds with many antibacterial activities. In this study, phytochemical detection test of leaves of *A. abyssinicus* (Hochst.) Radlk.and *L. vulgare*

indicates the presence of various secondary metabolites or active compounds as shown in Table 2. Variations were also observed in the concentration of the phytochemical constituents of the plant species and the solvents used. Therefore, the phytochemicals including phenol, flavonoid, tannin, steroid, alkaloid, saponin and glycoside present in water extracts of *A. abyssinicus* (Hochst.) Radlk.leaf**,** but terpenoid, steroidal glycoside and resin were absent in water extracts and only resin was absent methanol extracts of *A***.** *abyssinicus* (Hochst.) Radlk.

Similarly, the phytochemicals including phenol, flavonoid, tannin, steroid, terpenoid, alkaloid, saponin and glycoside present in water and methanol extracts of *L. vulgare* leaves**,** but steroidal glycoside were absent in water extracts and resin was absent in water and methanol extracts of *L. vulgare*.

Table 2: Phytochemical analysis of the crude extracts of the leaves of *A. abyssinicus* and *L. vulgare*.

	A. abyssinicus		***L. vulgare***	
Phytochemicals	MeOH	H_2O	MeOH	H_2O
Phenol	+	+	+	+
Flavonoid	+	+	+	+
Tannin	+	+	+	+
Steroids	+	+	+	+
Terpenoids	+	-	+	+
Steroidal glycoside	+	-	+	-
Alkaloids	+	+	+	+
Saponin	+	+	+	+
Resin	-	-	-	-
Glycoside	+	+	+	+

Note: + = presence of phytochemicals, - = absence phytochemicals, MeOH = methanol, H2O= water.

4.3. Antibacterial susceptibility tests

In this study, the antibacterial activity of the methanol, ethyl acetate and water extracts of *A. abyssinicus* (Hochst.) Radlk and *L. vulgare* leaves were evaluated against four clinical and four standard selected human pathogenic bacteria species using agar-well diffusion method. Tables 3 show the antibacterial activity of the two medicinal plants. The two plant extracts were shown varying degree of inhibition against the test organisms at a concentration of 500mg/ml.

Table 3: Antibacterial activity of *A.abyssinicus*and *L. vulgare* leaves extracts of different solvents against the test bacterial strains at the concentration of 500mg/ml.

Types of Plants and control	types of extracts	Zone of inhibition (mm) (mean ± SD)							
		*E. coli**	*E. coli***	*S. aureus **	*S. aureus ***	*S. typhi **	*S. typhi ***	*P. aeruginosa**	*P. aeruginosa***
A. Abyssinicus	MeO	19±1.0^{b}	17±1.0^{b}	22±1.0^{b}	20±1.0^{b}	21±0.577^{b}	20.66±1.15^{b}	-	-
	EtOAs	-	-	-	-	-	-	-	-
	H_2O	17±1.0c	15.33±0.577^{b}	20.66±0.577^{b}	19±1.0^{b}	20.33±0.577^{b}	19.33±0.577^{b}	-	-
L. vulgare	MeO	16.66±0.57^{b}	15±1.0^{b}	22±0^{b}	21±0^{b}	20.66±0.577^{b}	19.66±1.52^{b}	-	-
	EtOAs	-	-	-	-	-	-	-	-
	H_2O	16.33±0.57^{b}	14.66±0.577^{b}	21.33±0.577^{b}	20.66±0.577^{b}	20.33±0.577^{b}	19.33±0.577^{b}	-	-
Positive control	Chl	28.33±0.577^{a}	27.33±0.577^{a}	31.33±0.577^{a}	30.0±0^{a}	30.33±0.577^{a}	29.33±0.577^{a}	19. 33±0.577^{a}	18.0±1.0^{a}

Note: MeOH=methanol; EtOAc=ethyl acetate; H_2O=water; Chl=chloramphenicol; (mm) =millimeter; (-) =no inhibitory activity;SD=standard deviation; (*) =standard strain; (**) = clinical strain. Treatment mean in the same column having the same superscript have no significant difference.

The methanol and aqueous extracts of *A. abyssinicus* leaves indicated considerable antibacterial activity against both standard and clinical bacterial strains. The methanol crude extract had relatively higher antibacterial activity than aqueous extract but this variation is statistically not significant ($p<0.05$) with exception of standard *E. coli*. Although there is no published data available till now about antimicrobial activity of *A. abyssinicus* leaf in particular and other parts in general, the result is comparable to other similar studies conducted on *A. cobbe (L.) Raeusch* of same genus in which aqueous extracts were found less effective as compared to methanol extracts (Chavan and Gaikwad, 2016). Both methanol and aqueous extracts were ineffective against both clinical and standard strains of *P. aeruginosa*. The data is in line with a study conducted by (Mishra and Pandhy, 2013) in which the methanol extract of the same family *Schleichera oleo (lour).oken* seed had antibacterial effects against S. *aureus, E. coli* and *P. aeruginosa*.This finding was not similar with this study. The difference may be due to the procedure followed, the difference in plant and the age of plant and climate condition of plant collected area. Ethyl acetate extract of *A. abyssinicus* leaf was ineffective against all test organisms, the reason may be due to inefficacy of the solvent to dissolve secondary metabolites but, it does not mean it is not effective to other test organisms that were not included in this study.

The test organisms were susceptible to aqueous and methanol extracts of *A. abyssinicus* leaf but *P. aeruginosa* was resistant to all extracts. Standard *S. aureus* was highly susceptible bacterial strain for methanol and aqueous extracts respectively. In line with this study, *S. aureus* and *E. coli* were susceptible to the methanol extracts of *Schleichera oleo (lour).oken* seed of the same family (Mishra and Pandhy, 2013). Antibacterial activity of the same family *Schleichera oleo* and the same genus *A. cobbe (L.)Raeusch* on test organisms is due to the availability of different phytochemicals including alkaloid, saponin, flavonoid,terpenoid and tannin which had incredible antimicrobial effects of the same genus (Chavan and Gaikwad, 2016; Mishra and Pandhy, 2013).

There were significant differences among the crude extracts and the positive control chloramphenicol ($p<0.05$). All test bacterial organisms were highly susceptible to chloramphenicol except *P. aeruginosa* strains. In line with this study, *P. aeruginosa* exhibited a considerable level of resistance to chloramphenicol (Mulu *et al*., 2017).

Likewise of *A. abyssinicus,* methanol and aqueous leaf extracts *L. vulgare* indicated considerable antibacterial activity against both standard and clinical bacterial strains. The methanol crude extract had comparatively higher antibacterial activity than aqueous extract but this variation is statistically not significant ($P<0.05$). Similar to *A. abyssinicus,* standard *S. aureus* was highly susceptible

bacterial strain for methanol and aqueous extracts *L. vulgare*.A study conducted by Qusti *et al*., (2018) in which the methanol extract of the same family *Olea europaea* had inhibitory activity against *S. aureus* (12±1.4) and *E. coli* (39±0.5). This finding was not in agreement with the current study. The differences may be due to the procedure followed, the difference in plant and the age of plant and climate condition of plant collected area. Both methanol and aqueous extracts were also ineffective against both clinical and standard strains of *P. aeruginosa*.

Ethyl acetate extract of *L. vulgare* leaf was ineffective against all test organisms the reason may be due to inefficacy of the solvent to dissolve the secondary metabolites of the plant. But, it does not mean it is not effective to other test organisms that were not comprised in this study.

All test organisms were susceptible to both methanol and water extracts of *L. vulgare* leaf but both *P. aeruginosa* strains were resistant to all crude extracts. Standard *S. aureus* was the most susceptible for methanol crude extract among all test organisms. But, clinical *E. coli* was least susceptible for aqueous extract.

The positive control chloramphenicol showed significantly higher inhibitory activity than that of all solvents crude extracts of *L. vulgare* ($P<0.05$). But both strains of *P. aeruginosa* were least susceptible to chloramphenicol. The same with this study, *P. aeruginosa* exhibited a considerable level of resistance to chloramphenicol (Mulu *et al*., 2017).

4.4. Determination of minimum inhibitory and bactericidal concentrations

The MIC of the two plants extracts were evaluated by using the two folds broth dilution method. MBC of the extracts was evaluated on agar plate. The results shown in (Fig. 3) the MIC and MBC values obtained from the crude extracts of *A. abyssinicus* (Hochst.) Radlk and *L. vulgare* were variable against the tested bacterial isolates. Among the extracts of *A. abyssinicus* (Hochst.) Radlk leaf, methanol exhibited the lowest MIC against both standard and clinical *S. aureus* strains which indicate that the methanol extract could suppress the growth of *S. aureus* strains in much lower dose. A similar study on the *Schleichera oleo (lour).oken* seed of the same family, methanol extract reported lower MIC value against *E. coli* and *S. aureus* (Mishra and Pandhy, 2013). The discrepancy may be due to difference in plant or the specific protocol followed. The same value was recorded for the methanol extract against both *S. typhi* strains. Both methanol and aqueous extracts were relatively less effective against *E. coli* strains in lower doses with exception of methanol extract against standard *E. coli* (3.9mg/ml). The MIC value (7.8mg/ml) was obtained

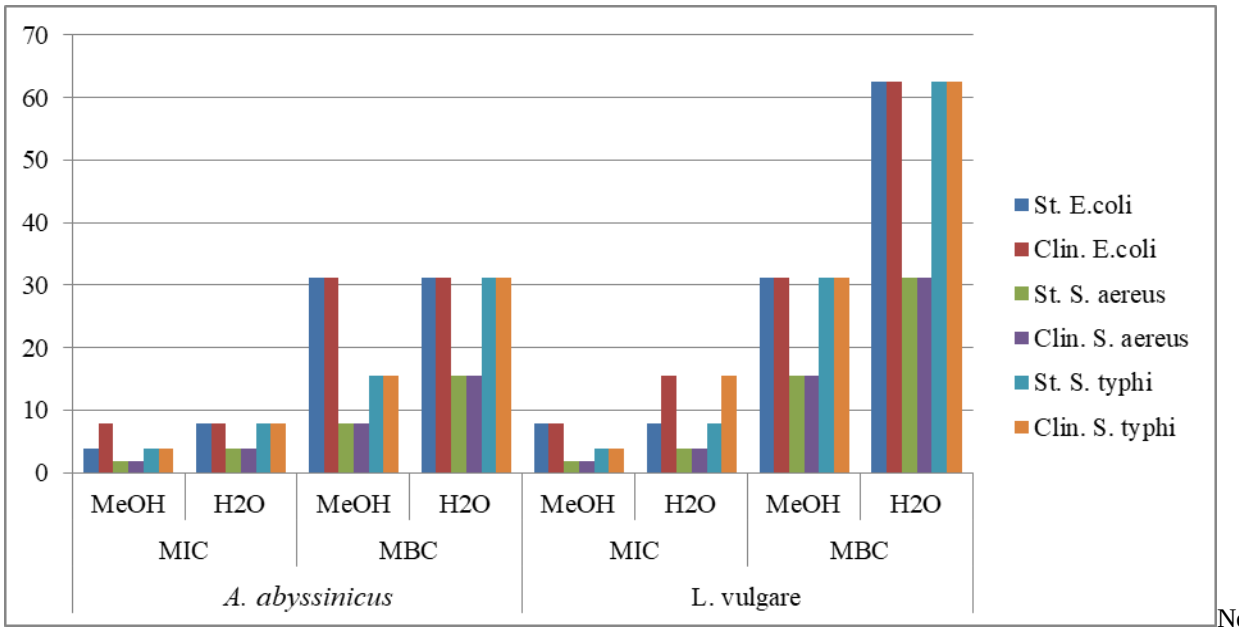
from methanol extract towards clinical *E. coli* and aqueous extract against both *E. coli* strains. Likewise, aqueous extract had the highest MIC value (7.8mg/ml) against both strains of *S. typhi.* Generally, *S. aureus* strains were the most susceptible pathogenic bacteria in this study.

MBC was standing for the lowest concentration of antimicrobial agent that completely killed the growth of culture. MBC was determined by sub culturing loop full tests from last MIC results for each bacterium on solid nutrient agar (Oxoid LTD., Basingstoke, Hampshire, England) and incubated at 37°C for 24 hours and the average of triplicates of the least concentration showing no visible growth on subculture was taken as the MBC. The highest MBC value of *A. abyssinicus* (Hochst.) Radlk. (31.25mg/ml) was recorded for methanol and aqueous extracts against both *E. coli* strains and aqueous extract against both *S. typhi* strains. Even if methanol extract could inhibit the growth of *E. coli* at low concentration (3.9mg/ml), it could not have a bactericidal effect until its concentration reached 31.25mg/ml. Comparatively lowest MBC value (7.8mg/ml) was recorded in methanol extracts against both strains of *S. aureus.* Mishra and Pandhy, 2013 noticed methanol extracts of *Schleichera oleo (lour).oken* seed of the same family exhibited lower MBC against *E. coli* and *S. aureus* which is different from this study. The discrepancy may be due to difference in plant genus and species.

Significant inhibitory activity was also noted for *L. vulgare* against the test organisms. Generally, the MIC values of both plants were relative against the test organism with exception of aqueous extract MIC value against clinical *E. coli* and *S. typhi*. The lowest MIC (1.9mg/ml) was recorded in methanol extract against clinical and standard *S. aureus* strains. The aqueous extract also exhibited the second lowest MIC value (3.9mg/ml) against the same test organism. The MIC value of methanol extract was 3.9mg/ml against both *S. typhi* strains. Supporting this study, water extracts of *Olea europaea L*. the same family exhibited MIC value of 31.25 mg/ml against *S. aureus* (Ali *et al.*, 2011) which is different from this study. The discrepancy may be due to difference in plant genus and species. On the other hand, study on the same family plant *O. europaea* methanol extract revealed MIC of 1.5 mg/ml and 6.25 mg/ml for *S. aureus* and *E. coli* respectively, which is related to the result of this study (Qust *et al.*, 2018).

Overall, bactericidal activity of *L. vulgare* was relatively weak compared to *A. abyssinicus* (Hochst.) Radlk. against some test bacterial strains with lowest MBC value (15.6mg/ml) for both *S. aureus* strains of methanol extract as high as 62.5mg/ml. Both plants methanol extracts exhibited a

better inhibitory activity against test bacterial strains in a very low MIC concentration (1.9mg/ml). It is possible to suggest that polar solvent methanol was most effective in extracting secondary metabolites responsible for the antibacterial activity than other solvents used in this study.



Note: MeOH=methanol; H_2O=water; (St.) =standard strain; (Clin.) = clinical strain; MIC=minimum inhibitory concentration; MBC=minimum bactericidal concentration.

Figure 3: MIC and MBC of *A. abyssinicus* and *L. vulgare* against standard and clinical bacterial strains

Both clinical and standard strains *S. aureus* were predominantly inhibited bacterium by the crude extracts of both plants compared with other test organisms. Since, *S. aureus* are gram-positive bacteria without outer membrane which makes it highly susceptible to the crude extract than that of the other gram-negative bacterial strains in this study.

4.5. Acute toxicity study of leaves of *A. abyssinicus* (Hochst.) Radlk. and *L. vulgare*

In this research, acute toxicity shows no mortality in the groups of mice that received water (negative control) and the groups that received 1000, 1500, and 2000mg/kg BW of the methanol extract after 24 hours. Uniformly, there was no acute toxicity with a dose level of 2000mg/kg BW of the methanol extract. Based on oral dose recommended by OECD guidelines 420 for testing acute toxicity. Even after 14 days, the mice did not show any BW reduction. Table 4 shows the mean bodyweights (MBW) of the mice in the three groups before and after 14 days of treatment. There were BW gains in all treatment and control groups. No behavioral changes were observed in any of the groups. When compared to control group, BW of the treatment groups did not

significantly changed. Throughout 14 day of monitoring, there was significant weight gain in both control and treatment groups ($p<0.05$). Since BW is among parameters of extract oral acute toxicity, the result of this study revealed that the leaf of *A. abyssnicus* and *L. vulgare* was non-toxic to the mice up to the dose of 2000 mg/kg.

Table 4: The effect of methanol crude extract of *A. abyssnicus* and *L. vulgare* on body weight ofexperimental mice

Test Plants	**Dose**	**MBW ±SD**			**% change**
	mg/kg	**D0**	**D7**	**D14**	**B/n D0&D14**
A. abyssnicus	1000	24.56±0.8	27.14±0.71$^{\#b}$	30.24±0.79*a	24.12
	1500	24.74±0.45	27.24±0.86$^{\#b}$	30.70±0.79*a	24.09
	2000	25.06±1.00	27.30±1.05$^{\#b}$	30.64±0.58*a	22.26
	Control	24.46±0.72	27.24±0.81$^{\#b}$	30.74±0.66*a	25.67
L. vulgare	1000	24.66±1.26	26.98±0.90$^{\#b}$	30.44±1.36*a	23.4
	1500	25.06±0.98	27.66±0.95$^{\#b}$	31.28±0.64*a	24.8
	2000	24.84±1.18	27.24±1.05$^{\#b}$	30.54±0.66*a	22.9
	Control	24.62±1.08	27.28±0.77$^{\#b}$	30.96±1.21*a	25.7

Note: BW =bodyweight; MBW = mean bodyweight in gram; D0= first day; D7 = day seven; D14 = day fourteen; SD = standard deviation ;(*) = significantly higher; (#) = not significantly higher.

The comparable increment of BW in both controls and treatment groups confirmed the absence of crude extracts effect on their BW change. Generally, the methanol extract of *A. abyssnicus* and *L. vulgare* leaves were not toxic on Swiss albino mice up to the dose 2000 mg/kg. Corresponding to this study Yesuf and Asres 2013 also reported the harmless effect of *A. abyssinicus* up to 2000mg/kg. There were reductions in the mean PCV of experimental mice after administration of *A. abyssnicus* and *L. vulgare* methanol extracts. Compared to the treatment groups, PCV reduction in the control groups were low (6.0% and 4.7%) for *A. abyssnicus* and *L. vulgare* respectively. As a result, the mean PCV end result showed that the crude extract of *A. abyssnicus* and *L. vulgare* effect on the PCV of the experimental mice was not statistically significant ($p<0.05$).

Table 5: The effect of methanol crude extract of *A. abyssnicus* and *L. vulgare* leaves on PCV of experimental mice

Test Plants	**Dose mg/kg**	**Mean PCV ±SD**		**% change**
		D0	D14	B/n D0&D14
A. abyssnicus	1000	51.66±1.17	48.56±1.12[#]	-6.07
	1500	49.74±1.46	46.38±1.14[#]	-6.75
	2000	48.68±2.30	44.12±2.6[#]	-9.36
	Control	50.23±1.30	47.18±1.6[#]	-6.00
L. vulgare	1000	49.24±1.28	46.8±1.8[#]	-4.95
	1500	48.12±1.21	45.58±0.65[#]	-5.27
	2000	49.26±1.72	46.3±0.58[#]	-6.00
	Control	48.04±1.36	45.76±1.36[#]	-4.7

Note: PCV=packed cell volume; D0= first day; D14=day fourteen; SD=standard deviation; (#) = not significantly higher

5. Conclusions and Recommendations

For both plants, the yield of methanol extract was higher than that of aqueous and ethyl acetate. This demonstrates the more of polar nature of important bioactive constituents of the plants and the effectiveness of methanol for their extraction. The preliminary qualitative phytochemical detection of the plants confirmed presence of major bioactive molecules and the presence of those secondary metabolites explain the considerable role of the plants against the test bacterial strains. The two medicinal plants have comparable antimicrobial activity against test bacterial strains. Among test bacterial strains *S. aureus* was highly susceptible bacterial strain and *P. aeruginosa* was resistant to both plants extracts. In both plants methanol extract was exhibited highest inhibitory activity and lowest MIC value than other solvents.

In oral acute toxicity test, the mice treated with crude methanol extracts of both plants had no any clinical signs, death or BW and PCV reduction within 14 days up to oral dose of 2000mg/kg.

Further research on the antibacterial activities of the plants is needed on other different strains of bacteria. This is important to establish whether the extracts have a broad spectrum activity or not. Bioactivity guided fractionation assay is therefore recommended for the possible isolation of novel chemotherapeutic agent(s).

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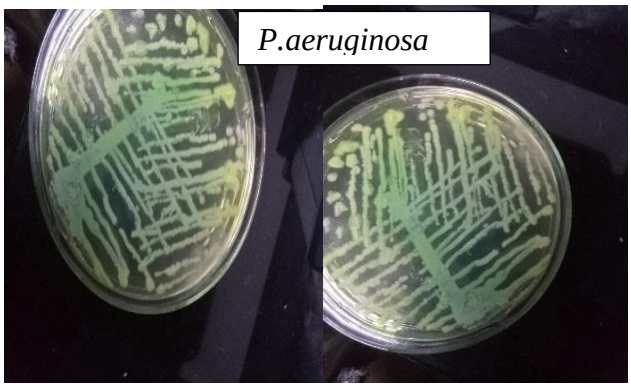
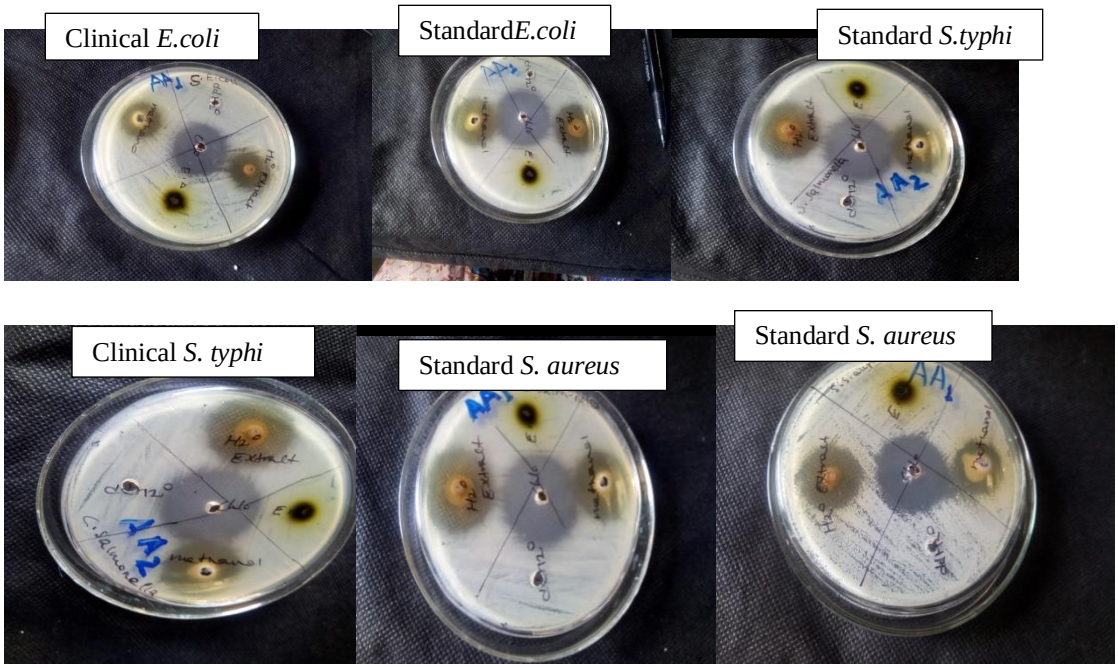
7. Appendixes

Appendix 1: Collection and preparation of plant material

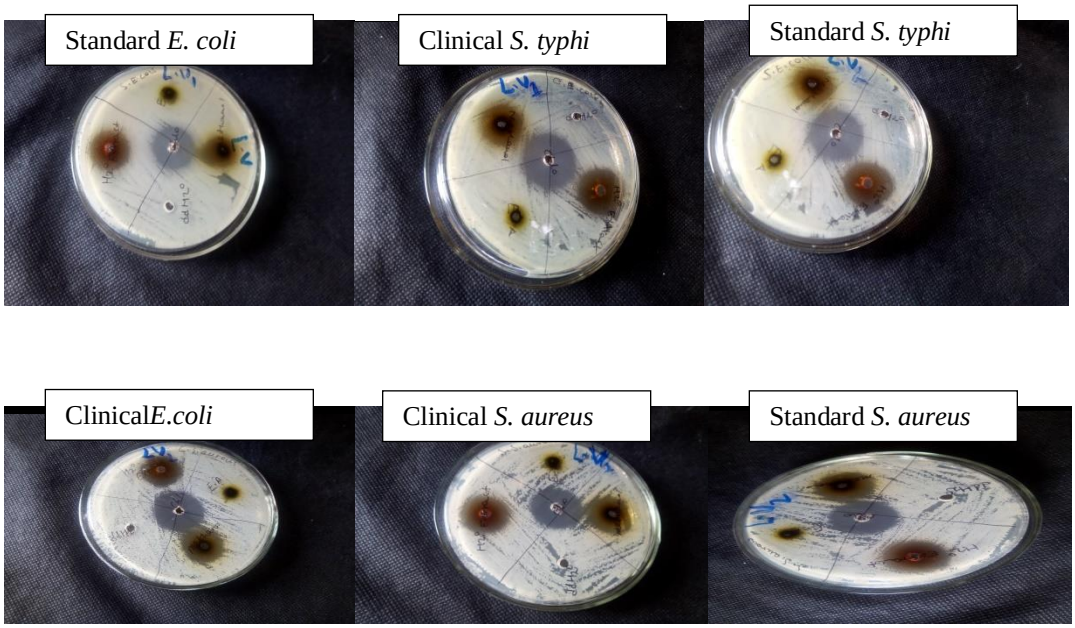
Appendix 2: Bacterial test organisms



Appendix 3: Antimicrobial result of *A. abyssinicus* leaf



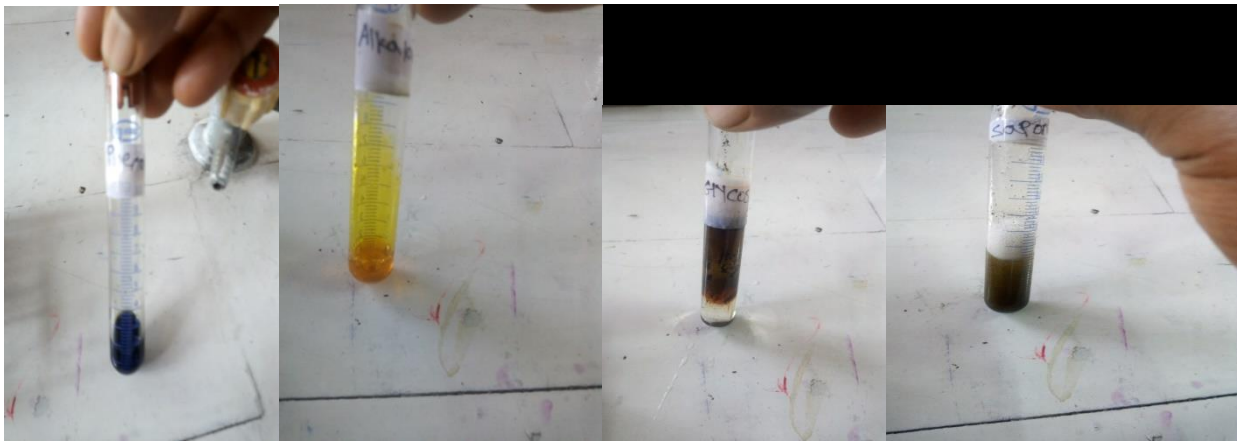
Appendix 4: Antimicrobial result of *L.vulgare* leaf



Appendix 5: Determination of MIC

Appendix 6: Determination of MBC

Appendix 7: Phytochemical test



Appendix 8: MIC and MBC of the aqueous and methanol crude extracts of the leaves of *A. abyssinicus.*

		Concentration in mg/ml	
Bacterial strain	**Solvent**	**MIC**	**MBC**
E. coli *	MeOH	3.9	31.25
	H_2O	7.8	31.25
E. coli **	MeOH	7.8	31.25
	H_2O	7.8	31.25
S. aureus *	MeOH	1.9	7.8
	H_2O	3.9	15.6
S. aureus **	MeOH	1.9	7.8
	H_2O	3.9	15.6
S. typhi*	MeOH	3.9	15.6
	H_2O	7.8	31.25
S. typhi **	MeOH	3.9	15.6
	H_2O	7.8	31.25

Appendix 9: MIC and MBC of the aqueous and methanol crude extracts of of *L.vulgare* leaf.

		Concentration in mg/ml	
Bacterial strain	**Solvent**	**MIC**	**MBC**
E. coli *	MeOH	7.8	31.25
	H_2O	7.8	62.5
E. coli **	MeOH	7.8	31.25
	H_2O	15.6	62.5
S. aureus *	MeOH	1.9	15.6
	H_2O	3.9	31.25
S. aureus **	MeOH	1.9	15.6
	H_2O	3.9	31.25
S. typhi *	MeOH	3.9	31.25
	H_2O	7.8	62.5
S. typhi **	MeOH	3.9	31.25
	H_2O	15.6	62.5