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INVESTIGATION ON ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF SOME SELECTED MEDICINAL PLANTS IN ETHIOPIA

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This is to testify that the thesis prepared by Abiy Abebe: entitled "***Investigation on Antibacterial and Antifungal Activity of Some Selected Medicinal Plants in Ethiopia"*** and submitted in partial fulfillment of the requirements for the degree of Master in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology Specialty) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Table of Contents

Contents Page

List of Figures

List of Tables

List of Abbreviations and Acronym

AMR	Antimicrobial Resistance
ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
CFU/g	Colony Forming Unite/gram
CFU/ml	Colony Forming Unite/ milliliter
CLSI	Clinical Laboratory Standards Institute
DMSO	Dimethyl Sulfoxide
DRERC	Department of Research and Ethical Review Committee
EPHI	Ethiopian Public Health Institute
ESBL	Extended-Spectrum Beta-Lactamase
HAIs	Hospital-Acquired Infections
IC_{50}	50%Inhibitory Concentration
JMP	John Mackintosh program
MBC	Minimum Bactericidal Concentration
MDR	Multi-drug resistant
MeOH	Methanol
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum Inhibition Concentration
MRSA	Methicillin-Resistant *Staphylococcus Aureus*
OECD	Organization for Economic Co-operation and Development
SDA	Sabouraud Dextrose Agar
SOPs	Standard Operating Procedures
TMMRD	Traditional and Modern Medicine Research Directorate
WHO	World Health Organization

ABSTRACT

Background: Cognizant of the fact that, there has been an alarming increase in the incidences of infectious diseases, emerging and re-emerging antibiotic resistance organisms; there is an urgent need of novel antimicrobial compounds with new mode of action, effective and inexpensive. One potential source for the search of such agent is medicinal plants due to the presence of wide range secondary metabolites in their tissue.

Objectives: The aim of this study was to investigate the antimicrobial activity of 12 traditionally used Ethiopian medicinal plants for their potency, spectrum of antibacterial, antifungal activity and acute toxicity.

Methods: Ethnobotanical literature survey was conducted to select 12 medicinal plants. Antimicrobial activities of 80% Ethanol extracts of all plants were investigated by agar well diffusion techniques against 22 organisms. Agar dilution techniques were employed to determine the Minimum Inhibitory, Bactericidal and Fungicidal Concentrations. The Oral acute toxicity study was determined on the most active plant extract *B. abyssinica*.

Results: Among investigated medicinal plants, *B. abyssinica, X. strumarium, R. natalensis, Z. scabra*, *E. cymosa, C. abyssinica* and *S. abyssinica*, showed strong antibacterial activity with inhibition zone ranging from 9-27 mm; while, *B. abyssinica* (42 mm) and *X. strumarium* (48 mm) showed strong antifungal activity. The extracts of *B. abyssinica* showed most potent antimicrobial activity with MIC value ranging 0.250-16 mg/ml. *Staphylococcus aureus* was the most sensitive organism against *B. abyssinica* with MIC value of 0.25 mg/ml. Nevertheless, *K. pneumoniae* and *Citrobacter* species were the least sensitive bacteria with 16 mg/ml MIC and MBC value. *T. mentagrophytes* and *T. rubrum* were the most susceptible fungal organism to the treatment of *B. abyssinica, X. strumarium* while, *A. niger* was the most resistant fungus. The LD 50 of *B. abyssinica* was found to be 4103.175 mg/kg.

Conclusion: The majority of the plants tested showed promising antibacterial and antifungal potency, especially on gram positive bacteria and dermatophytes. Further studies are recommended to explore *in-vivo* efficacy, mechanism of action and toxicological studies of the most promising plant.

Key Words: Antibacterial, Antifungal, Medicinal Plant, *B. abyssinica*, MIC, Acute Toxicity

1. INTRODUCTION

1.1 Background

Marked landscape shift of patient care was achieved soon after the successful discovery of modern medicine such as penicillin. Millions of individuals' lives were saved by receiving necessary treatments and therapy than ever before [1]. Consequently, the average life expectancy of individuals within the twentieth century has been remarkably increased [2]. Unfortunately, widespread use for more than 75 years, the efficacy of currently available antibiotics are not as effective as they used to be [3]. Under use and overuse of antibiotics, alarmingly increases in the incidence of new and re-emerging drug resistant organisms, upcoming of undesirable side effects of certain antibiotics could be the factor for the downfall of the antibiotics success, which makes, infectious diseases to continue to be one of a major threat to humankind [4, 5]. Drug resistant microorganisms are the main contributors to the currently observed dramatic increments in morbidity and mortality of patients affected by infectious diseases [6]. The incidence of infections by antibiotic-resistant bacteria has increased significantly over the last decade [7]. Moreover, they are also responsible for significant increment in the costs of diagnostics, pharmaceutical treatments and hospital stay [6].

Approximately more than 4 million patients acquire healthcare-associated infection (HAIs) and almost 75,000 deaths occurs in the USA each year and 40,000 die in Europe as a direct consequence of it [8]. This infection results in up to $4.5 billion additional health care expenses annually [9]. HAIs infections are responsible for a quite large number of extra hospital stay. More than 70% of the bacteria that causes HAIs are resistant to at least one of the drugs most commonly used to treat them [10]. For instance, Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infections and very difficult to cure because these strains are resistance against almost all clinically available beta-lactam antibiotics [11]. According to CDC Estimates; MRSA was responsible for 80,461 morbidity and 11,285 mortality annually occurred in 2011 [12]. Up to 53 million people are carriers of MRSA in the developing world [13]. Similarly, Shigella causes an estimated 164.7 million infections globally each year, 99% of infections occur in developing countries and the majority of cases and deaths occur amongst children less than five years of age [14].

On the other hand, *Pseudomonas aeruginosa* is the other cause of severe nosocomial infections these days. While, multidrug-resistant *P. aeruginosa* strains are familiar and becoming a global challenge. This is due to its ubiquitous nature, ability to survive in moist environments and resistance to many antibiotics and antiseptics [5].

Similarly, *Escherichia coli* is the other most frequent cause of community and hospital acquired urinary tract infections and resistance to a third-generation cephalosporin, which are widely used for intravenous treatment. A recently emerging threat carbapenem resistance in *E. coli*, which confers resistance to virtually all available beta-lactam antibacterial drugs were observed [15]. Moreover, *Klebsiella pneumoniae* is the most common causes of urinary, respiratory tract, neonates and bloodstream infections. This bacteria can spread readily between patients, leading to nosocomial outbreaks. Now a day's carbapenem-resistant *K. pneumoniae* was reported worldwide, which means patients infected with these bacteria, there are no clinically effective treatments, only the last-resort drugs tigecycline or colistin which are less effective and unavailable [16].

One of the possible ways to overcome the challenges of widespread infection and multidrug resistance is a continual search for novel antimicrobial substance. A possible source of new antimicrobial agent is natural product, particularly medicinal plants. Since they are a rich sources of secondary metabolites that could be selectively toxic against pathogenic microbes, effective against resistant strains, cheap and easily available for use [17]. Many studies have revealed that, these secondary metabolites are Alkaloids, Flavonoids, Glycosides, Tannins, Saponins, Steroids, Terpenoids and Phenolic compounds and the like which are extracted from different parts of the plant and they have been associated with different antimicrobial activity and other function [18, 19]. Though, the use of medicinal plants for the treatment of infectious diseases traced back over five millennia (early civilization in India, Eastern countries and China), it is still the main pillar of traditional medicine that are currently estimated to be utilized for primary health care for 80% of the world population [20, 21]. Even with the currently used modern medicine, medicinal plants have huge share, more than 130-140 drugs or 80% of antimicrobial, cardiovascular, immunosuppressive and anticancer drugs were extracted from plants or further modified synthetically; their sales exceeded US$ 65 billion in 2003 [22].

The development of modern standardized products were started in 1805 when the young German pharmacist, Friedrich Sertürner, isolated the first pharmacologically-active compound morphine from the opium plant which is considered as the beginning era of “modern” drugs [23]. The subsequent discovery of penicillin from mold in 1928 was another natural product based modern medicine, which began the era of antibiotics, has been recognized as one of the greatest advances in therapeutic medicine. Due to this a dramatic reduction of illness and mortality from infectious diseases onwards [24]. Now antibiotics save millions of lives each year around the world. Similarly, the discovery of artemisinin from medicinal plant *Artemisia annua* was another success of drug development from natural products which can be traced back to the 1972, presently used widely for the treatment anti-malaria [23]. Likewise, among anticancer drugs approximately 54% of them were derived from natural products. Vinca alkaloids from *Catharanthus roseus* and terpene paclitaxel from *Taxus baccata,* Taxol from *Taxus brevifolia.* Aspirin, from the leaves of willow tree *Salix spp*., digitoxin from the *Digitalis purpurea* were just to mention a few plant origin medicine [23, 25].

Ethiopia is believed to be a home for about 6,500 species of plants with approximately 12% endemic, hence making it one of the six plant biodiversity-rich countries of Africa [26]. The country has a long history of using medicinal plants to treat a variety of human ailments. That is why there are a considerable number of research works of the various aspects of medicinal plants, like *Phytolacca dodecandra* commonly known as "Endod", used as an effective molluscicide to control schistosomiasis [26, 27].

Cognizant of the highly infectious disease burden, the shortage and the drawback of currently existing antimicrobial agents and the availability of highly unexplored natural resources in Ethiopia, there are great interest of many academic institutions and pharmaceutical industries working on it. Focusing on searching for a novel, highly effective, better active, safe and affordable antimicrobial agents from natural product. Therefore, the current study is aimed at evaluating the antimicrobial potential of selected medicinal plant extracts against major human pathogenic bacteria and fungus.

1.2 Statement of the Problem

Since the discovery of penicillin, antimicrobials have saved million's life and also eased the pain and suffering of human illness. However, after more than 75 years of widespread use, many antimicrobial agents are not as effective as they used to be. Consequently, the number of deaths caused by infectious diseases is falling slowly worldwide [28]. In 1990, approximately 16 million people died from infections. In 2010, the number of deaths had fallen only to 15 million. The World Health Organization (WHO) forecasts 13 million deaths will be attributed infectious disease in 2050 [29]. Even though, there is a gradual reduction of death due to infectious disease, the rate of drug resistance is alarmingly increasing which, if not controlled, will put us back to pre-antibiotic era/post-antibiotic era. In low and middle income countries, the problem of infectious disease and drug resistance is set to remain a double burden for decades to come [16, 29].

The most recent estimates, a total of 700,000, people die every year from drug resistance strains of common infections organisms [30]. Currently, more than one million people have lost their lives due to drug-resistant organisms. This number is likely to be underestimated due to poor reporting and surveillance in developing countries [31]. According to CDC estimates in the US more than 2 million people acquire serious infections with resistance bacteria and an estimated 140,000 Healthcare-Associated Enterobacteriaceae 26,000 drug-resistant and 1,700 deaths are attributable to Extended-Spectrum Beta-Lactamase (ESBLs) occur each year [12]. Overall, 20,000 (30%) of hospital-acquired enterococcal infections per year are vancomycin-resistant, leading to 1,300 deaths. Candida is responsible for about 46,000 HAIs in the US annually. Roughly 30% of candidemia patients die due to drug-resistance Candida during their hospitalization. Consequently 23,000 and 25,000 deaths attributable to selected antibiotic-resistant annually in the US and European Union countries, respectively [12, 28].

In low and middle income countries, the magnitude and burden of HAI remains underestimated or mostly even unknown, since it needs complex diagnosis and surveillance activities to guide interventions require expertise and resources [32]. In Thailand, an estimated 19,000 deaths occur each year in patients with HAI due to Multi-drug resistant bacteria (MDR). *E. coli* and *K. pneumoniae* were the most common causes. Which is about 3 to 5 times larger than those of the US and EU countries [33]. In India an estimated, 58, 319 deaths could be attributed to ESBL and

MRSA. Whereas, 106, 514 neonatal mortality due to Gram-negative and *S. aureus* [34]. In Tanzania, an estimated 27% and 34% mortality attributable to ESBL and MRSA in neonatal sepsis respectively [35]. Similarly, in Mwanza, Tanzania, the overall prevalence of ESBLs at a tertiary hospital out of 377 clinical isolates, the ESBL prevalence was 64% *K. pneumoniae*, whereas 24% was *E. coli* [36].

In Algeria, Burkina Faso and Senegal hospital-wide HAI prevalence varied between 2.5% and 14.8%. In Ethiopia, there is no comprehensive research that presents the whole picture regarding HAI, a cumulative incidence in hospitals, surgical wards ranges from 5.7% to 45.8% in studies conducted in Ethiopia and Nigeria [32]. In similar other study conducted in Ethiopia shows that, more than 75% gram negative isolates were multiple antibiotic resistance. 95.7% *Acinetobacter,* 83.3% *E. coli*, 66.7% of *P. aeruginosa* and 60% of *K. pneumoniae* species were resistant to more than five antibiotics tested. 34.8% *Acinetobacter* species and 12.5% *E. coli* are Pan-antibiotic resistance. 50% of gram positive isolates were resistant to at least one of the antibiotics tested. 5.3% of the *S. aureus* isolates were found to be resistant, at least five of the antibiotics tested [37].

Controls of infectious diseases were not more successful due to the frequent emergence of drug resistance organisms, failures to develop and deliver effective technology and the dearth of new antibiotics [29]. By the early 1970s, there were eleven distinct classes of antibacterials and over 270 different drugs in clinical use. Unfortunately, in the past three decades, only two new classes of anti-bacterial and antiviral drugs have been developed and introduced into clinical practice such as Oxazolidinones in 2000, cyclic Lipopeptides in 2003 and ART [13]. Consequently, the emergence of AMR has prompted the (CDC) and the WHO spearhead efforts aimed at combating this problem through public education about the misuse of antibiotics, increased surveillance and research focused on research and developing new, more effective therapies [38].

To address this major challenge of drug resistance, it is extremely important to undergo urgent and intensive research to come up with best quality, efficacious, safe and affordable antimicrobial from natural product particularly medicinal plants. Since medicinal plants remain the main focus of scientists and researcher globally as a novel source of principal compounds in the development of new antimicrobial agent [21, 39].

Ethiopia with a wealth of unexplored natural products, it is an ideal place to search for new medicine for the treatment of infectious diseases. Even though, some studies have been conducted to explore the antimicrobial potential of various medicinal plants, these studies are insufficient as compared to the current widespread problem and prevalence of drug resistance microbe as well as the urgent needs of new medicine. Furthermore, availability and biodiversity of medicinal plants in the country are very high, the studies done so far are not sufficient to address the existing challenges. In addition, some studies done previously focused mainly on antibacterial activities of medicinal plants without considering antifungal activities and their safety [40-42].

Therefore, the present studies employ efforts on focusing efficacy, preliminary assessment of acute toxicity on one of the best selected medicinal plants. Assessing these factors are extremely significant in order to achieve a safe treatment with medicinal plant product. Furthermore, the current study fill the indicated gaps and it provides a scientific basis for their traditional use of local culture.

1.3 Significance of the Study

As studies indicated, the high burden of infectious diseases; elevated treatment failure of almost all available antibiotics; expensiveness and unwanted side effects of conventional antibiotics; and the availability of unexplored natural product in Ethiopia, these studies initiated to assess safe and effective medicinal plant against selected human pathogenic bacteria and fungus. This study focuses on evaluating antimicrobial potentials of maximally unexplored medicinal plants of the country. The finding of this experimental study primarily helps to identify plants with better antibacterial and antifungal efficacy. This ultimately can help the scientific communities to undergo further investigation on identified plants to develop novel medicinal products with sufficient supplies to resolve drug resistance problem.

2 LITERATURE REVIEW

2.1 Infectious Diseases and Associated Burden

According to WHO estimates, there are approximately 56 million peoples' deaths annually worldwide, predominately die of infectious diseases account for almost one third of all deaths. Approximately 6.6 million of those deaths occur among children less than five. The most encountered infections are caused by microorganism particularly caused by drug resistant bacteria and/or fungi, HIV/AIDS, malaria and others [43,44]. Several studies conducted in European countries revealed that, severe infections caused by Gram-negative bacteria, including those strains resistant to multiple classes of antibiotics (MDR) are significantly increasing over the last years. Especially those third-generation cephalosporins-resistant *Enterobacteriaceae*, MDR *Acinetobacter baumannii* and *P. aeruginosa* are increasing [45]. In South Asian countries in the other hand, neonatal sepsis-causing organisms, that are associated with antimicrobial drug resistance, such as *S. aureus*, *E. coli*, *Enterobacter* and *Acinetobacter species* and *Pseudomonas* caused estimated deaths of 300,000 infants annually [46].

The burden is high among disadvantaged world regions and vulnerable groups. More than 3 million under five children die each year due to acute respiratory infections in developing countries. Nearly 70% of these infections is assumed caused by antibiotic resistant *Streptococcus pneumoniae* and it is also responsible for the causes of otitis media, bacteremia and bacterial meningitis [13]. Similarly the other deadly diarrheal causing and very contagious pathogen is Shigella, having four species (*dysenteriae, flexneri, boydii, and sonnei*) all have shown resistance to antibacterials. There is also evidence of increasing resistance to ciprofloxacin, which is the WHO recommended treatment for Shigellosis [13]. Bloodstream associated bacteriemia such as, isolates of *Enterococcus faecalis, S. aureus*, *K. pneumoniae, A. baumannii, E. coli*, *P. aeruginosa* and *Proteus* spp., causes the majority of HAI and successfully “escape” the effects of antibacterial drugs [47].

Similarly, the other neglected topic by public health authorities were fungal diseases. As a review of 2017, nearly one billion people are estimated to have fungal infections, 150 million people have serious fungal diseases, >1.6 million associated mortality, which is similar to that of tuberculosis and >3-fold more than malaria [48]. *Candida* spp, are opportunistic pathogens in

susceptible or immunocompromised individuals. *Candida albicans* is most commonly isolated, followed by *Candida glabrata* and *Candida krusei* [49]. It is the fourth most common cause of nosocomial bloodstream infection [50]. They are commensal members of the gastrointestinal micro-flora. However, when this homeostasis are disrupted candidemia and invasive candidiasis arise from the intestinal tract and increased its incidence over the past two decades, especially in the internal medicine wards with prevalence ranging from 24% to 57% and associated mortality range from 30-50% soon after hospital admission [51].

Filamentous fungi such as *Aspergillus* species (*A. fumigatus*, *A. flavus* and *A. Terreus*) are the other life-threatening invasive fungal infection, caused by spore inhalation resulted from contaminated environment [52]. A recent estimates of greater than 10 million, cases of fungal asthma and 3 million, cases of chronic pulmonary aspergillosis found globally and 1 million, cases of fungal keratitis occur annually [48]. Particularly immunocompromised patients with prolonged neutropenia, allogeneic hematopoietic stem cell transplant, solid organ transplant, inherited or acquired immunodeficiencies and individual on corticosteroid treatment are the most affected one [53]. Moreover, this fungus also causes local disease in immunocompetent individual [54]. On the other hand, Dermatophytes known to cause superficial fungal infection (dermatophytosis) and the major causative agents are *Trichophyton rubrum* and *Trichophyton mentagrophytes* are known to cause deep-seated infections in immuno compromised individuals [55].

2.2 Antibiotic Resistance and Dearth of New Antibiotics

2.2.1 Antibiotic Resistance

Even though, antimicrobial resistance is a natural phenomenon that occurs spontaneously as microbes evolve, human activities have accelerated the pace at which microorganisms develop and disseminate resistance. The indiscriminate and excessive usage of antimicrobial drugs without treatment indication appears to be the most significant factor associated with the emergence and fast spread of resistant microorganisms in recent years [47]. Moreover, antibiotic consumption has increased by 36% in the past decade, no new classes of these drugs have been discovered since the 1980s to solve the currently prevailing problems. That is why antibiotic resistance described as "*a silent tsunami, crumbling down the pillars upon which modern medicine is built*." [56, 57].

For instance, when we see respiratory tract infections, it is mostly considered as self-limiting viral origin, such as common colds, viral sore throats and bronchitis which, antibiotic treatment has limited effect on symptoms. However, it accounts the most commonly treated acute problems in the primary care in Europe. Both the upper and lower respiratory tract infections account for 57% and 30% of antibiotics used respectively [58]. As CDC estimated that, 47 million unnecessary antibiotic were prescribed by U.S. doctors' more than 100 million worldwide with offices and emergency departments each year. Around 50% of these unnecessary antibiotics are prescribed for acute respiratory infections. Likewise, 75% of antibiotics prescribed incorrectly in nursing homes. The two extremes ages, children under 2 and 65 and above older adults receives most of the antibiotic prescriptions [59]. The most common associated problems are wrong antibiotic usage, incorrect dose or duration, patient non adherence to the treatment regime. Which may lead harmful side effects, allergic reactions, *C. difficile,* fungal and antibiotic-resistant organism infections [13, 59].

The issue has received high attention from various organizations, including WHO, Infectious Diseases Society of America, who recognized AMR as one of the greatest threats to human health worldwide [16, 58]. MRSA alone is the significant cause of death each year in Americans than combinations of emphysema, HIV/AIDS, Parkinson's disease and homicide [58]. In 2012 the European population-weighted mean percentage of carbapenem resistance was 6.2% [51]. The highest burden of carbapenem resistance *K. pneumoniae* (28.8%) was seen in Greece, followed by Italy nearly 19%, this might be associated with the highest rate of antibiotic consumption, which is much higher than other European countries like Sweden around 1% [51,58].

Apart from spreading resistance, an alarming pattern of multi and pan-drug resistant Gram-negative bacteria are currently emerging; multi-resistant Enterobacteriaceae is an increasing major concern worldwide [60]. Consequently, on the bases of their ability to escape the effect of available antimicrobial drugs and posing most the hazards, these organisms have been clubbed together under the term "ESKAPE," pathogens i.e., *Enterococcus faecium, S. aureus, K. pneumoniae, A. baumanii, P. aeruginosa* and *Enterobacter* species [61].

2.2.2 The Possible Reason for Dearth of Novel Antimicrobial

Researching to develop novel antimicrobial drug is extremely difficult science, since it needs diversified expertise and very expensive activities. Hence, it must be supported by adequate funding. The area requires an estimated developmental capitalized cost of $800 million to over $2 billion needed depending on the class of pharmaceutical. As well as it requires larger economic incentives for the participated scientists [62]. In addition to successfully discovering technical challenges, in undergoing antibiotic clinical trials in hospital settings is very challenging and costly demanding. At the same time it requires different trials for each new indication in varying organ systems. Another hurdle is making this research economically attractive to companies [61].

Unfortunately, many antibiotics are prescribed for a relatively short course, from 3 days to 2 weeks, as compared to a course of years or decades for chronic diseases, antihypertensive or cholesterol medications and relative expensiveness of neurological musculoskeletal drugs. Therefore, pharmaceutical industries are generally less attracted to a limited commercial return corporate investment like research and developments on antimicrobial drugs [13, 61- 63]. Furthermore, the number of pharmaceutical industries is also significantly diminished through time. In 1990s, there were estimated 18 large pharmaceutical companies, by 2005 they had dropped to 8, in the last seven years only 5 major companies are still actively involved in antibacterial research and developmental activities [13].

2.2.3 Natural Products as Antimicrobial Source

Even though, there is a striking lack of new antimicrobial drug which are very potent against the currently prevailing AMR, there are many research undergoing here and there to resolve the current urgent needs of new antimicrobial agents to fill pipeline [63]. Natural products have played an enormous role throughout the world in treating and preventing different kinds of human and animal's ailments for thousands of years. Since, natural product based medicines have come from various source materials, including terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates [64].

The discovery of antibiotics is considered as one of the most valuable findings related to human health [24]. Antibiotics are natural products of small organic molecules, usually less than 1000 Daltons in size. The majority of them are originated from the genus *Streptomyces* the most valuable microorganisms which are within the filamentous bacterial group (Actinomycetes) and by filamentous fungi. Generally, these molecules are produced in their stationary phase of growth, parallel to spore formulation and are not essential for growth or sporulation [62]. To mention some aminoglycoside, streptomycin, penicillin, ß-lactam antibiotic, polymyxines from soil bacteria, erythromycin, azithromycin, clarithromycin and the ketolides, chloramphenicol, vancomycin from soil organism, rifamycin and metronidazole were to mention some of them [65]. Whereas, some antibiotics are synthesized partially from natural product as a template [62]. These natural compounds often serve as lead molecules whose activities can be enhanced by manipulation through combinations with chemicals and by synthetic chemistry [66].

2.2.4 Plants as a Source of Medicine in General

On our planet earth an estimated 250,000- 500,000 species of plants can exist, among which 287,655 species have been identified as of 2004 [67]. Plants have provided man with all his needs in terms of shelter, clothing, food, medicines, flavors and fragrances. Plants have formed the basis of sophisticated traditional medical systems that have been in existence for many centuries as a source life and continue to provide mankind with new remedies. Human being starts to use medicinal plants instinctively, as is the case with animals [67, 21]. For instance, before the discovery of aspirin from *Spiraea ulmaria* the ancient Greeks and Egyptian papyri used the bark of the willow tree, as a source of a medicine for relieving of pain, fever and swelling [25, 67].

The therapeutic effect of medicinal plant is related to the presence of secondary metabolites which is synthesized in all parts of the plants, like alkaloids, terpenoids, glycosides, phenolic and others organic compounds [68]. Consequently, scientists throughout the world have discovered thousands of phytochemicals synthesized by primary or secondary metabolism of the plants which have a potential inhibitory effect on all types of microorganisms in vitro [69, 70]. These secondary metabolites apart from their antimicrobial and antineoplastic activities, plants possess antioxidant compounds like flavonoids, which is responsible for scavenging ‘reactive oxygen

species' (ROS) to control of heart diseases, stroke, arteriosclerosis, antidiabetic, hepatoprotective, neuroprotective and cancer [69].

2.2.5 Plants as Antimicrobial Sources

Herbal medicine is a kind of medicine originated from all parts the plant materials (leaves, flowers, fruits, seeds, barks, stem, root etc.). Herbal medicine, also called botanical medicine, phytomedicine or physiotherapy, which has a huge public acceptance of, its potency in preventing and treatments of various human and animal ailments [18, 20, 67]. Worldwide medicinal plants have been evaluated not only for their precious sources of pharmaceutical products, but also for their action as a resistance-modifying agent, the main reason why drawn the attentions of so many researchers found globally [66].

Currently, more than 130 drugs are developed from plants or further modified synthetically [20, 22]. Even though, herbal medicine is inseparable with the survival, human beings in this universe, over the past century, it has been challenged with the introduction of western medicine (or "conventional" medicine). Despite its long history of effective use, due to lack of scientific evidence in the context of contemporary medicine, medicinal plants are challenged by modern medicine practitioner [20, 67].

However, in recent years, there has been a renaissance in the consumption and demands of herbal products in both developed and developing countries [71]. In developed countries this may be partly due to the dissatisfaction and lack of a complete cure with conventional medicines. Whereas, with the developing countries due to lack of medical doctors, shortage, and unaffordable prices of pharmaceutical products, widely acceptance, trust and respect of traditional practitioners might be the possible causes for the revival of interest in the use of medicinal plants [72, 73]. The other factor might be the side effects of chemical drugs, lack of curative modern therapies for several chronic diseases and the prevalence of microbial resistance to the currently available conventional medicine [18, 67, 71]. Although wherever, modern health facilities exist, traditional medicine is incomparable [74].

According to the WHO, almost 65% of the world's and 80% populations of developing countries have relied on plants integrated TM into their primary health care systems of both humans and animals [71, 72]. WHO has set precise guidelines for the evaluation of the safety, efficacy and

quality of herbal medicines [71]. Consequently, many countries in the world such as China, Ghana, Korea, India, Thailand, Indonesia, Sri Lanka, Viet Nam, Madagascar, Mali, Nigeria and Ethiopia has included traditional medicine research and development in the national health research agenda and produce scientific evidence on the safety, efficacy and quality of traditional medicines and link with health services and policy-makers to facilitate the utilization of research results [72, 75].

Therefore, through time, the global pharmaceutical companies armed with modern science/technology to isolate, purify and structurally characterize active constituents. Hence, they have begun to rediscover biologically active herbal products as a potential source of new drug candidates and renewed their strategies in favor of natural product drug development and discovery [67]. Currently, many practitioners of “conventional” medicine do not hesitate to recommend herbs, herbal products or complementary and alternative medicine therapy to their patients for the effective treatment of certain diseases [67].

The fundamental contribution of medicinal plant in the development of official modern medicines, such as anticancer, antihypertensive and antimigraine medication benefited greatly from plant based natural products [23]. Drugs like Digoxin and Digitoxin an indispensable cardiac drug from the *Digitalis purpurea* leaves; Artemisinin from *Artemisia annua,* quinine from the bark of *Cinchona officinalis* for the anti-malaria treatment; morphine from *Papaver somniferum*, ergometrine, atropine, antihypertensive reserpine from *Rauwolfia vomitoria* [22, 23, 74]. The anti-cancer Vinca alkaloids, Vincristine and Vinblastine from *Catharanthus roseus* of Madagascar used in the management of leukaemia and again anti-cancer compound bruceantin, from the Ethiopian plant, "yedega abalo" named *Brucea antidysentrica*, which to commemorate Scottish travelers James Bruce were just to name a few examples of the contributions of traditional pharmacopoeia and how research on natural products has made a significant contribution in drug development [73, 74].

Ethiopian is well known for its significant geographical diversity which favored the formation of different habitat and vegetation zones. The county, is also a home of many languages, cultures and beliefs which in turn have contributed to the high diversity of traditional knowledge and practices of the people, which includes the use of medicinal plants [76]. The country also believed as a home for an estimated 6,500 species of higher plants with approximately 12%

endemic, hence making the country one of the six plant biodiversity-rich countries of Africa and one of the most diverse floristic regions in the world as well. Therefore, the availability of a huge floral diversity offer the country great possibilities for the discovery of new compounds with antimicrobial activities [21, 26, 73].

Several previous studies conducted in Ethiopia on indigenous plants used in traditional medicine have shown the anti-microbial activities, even many of them were also found to be effective against resistant microbial strains [77]. Among the traditionally used medicinal plant of Ethiopian which have shown very effective contributions for some ailments of human and domestic animals. Such plants include *Phytolacca dodecandra* commonly known as "Endod", used as an effective molluscicide to control schistosomiasis infection [27, 73].

2.2.6 Review of Some Medicinal Plants Included in This Study

All of the medicinal plant materials used in this study have been reported to be used traditionally by local Ethiopian people in treating different kinds of human and animal's ailments [78-89]. Thus, in the present study, the selection of 12 plant species was based on the ethnobotanical literature survey, gathered information from local healer and knowledgeable individuals about their anti-microbial properties which are traditionally used for treating various ailments.

***Bersama abyssinica* Fresen.** *Bersama abyssinica* Fresen, belongs to the family Melianthaceae; the genus Bersama, have estimated sixty four species and two subspecies in East Africa; *Bersama abyssinica* sub-spp. Paullinioides and the only subspecies that occur in Ethiopia *Bersama abyssinica* sub-spp. abyssinica [90, 91]. With vernacular name “Azamir” (Amharic), "Lolchissa (Oromifa), Tintala-shoa "Welaita", Winged bersama "English" It is a very common sub-Saharan African medicinal plant which is found in the Horn of Africa, West and southern part of the continent (Ethiopia, Kenya, Sudan, Congo, Tanzania, Uganda, Angola, Nigeria, Zambia, Zimbabwe and Mozambique) [92, 93].

Bersama abyssinica is a medium sized a shrub or small evergreen tree, usually 3–7 m but to 15 m in forest and it is often with twisted trunk, Dark green leaves, about 10 cm long and compound with 5–10 pairs, plus one at the tip. The leaf stalk may reach 60 cm and winged, green-cream flowers, slightly pink, each 2 cm across with a bright orange-red seed [93].

Figure 1a. *Bersama abyssinica* showing it's winged leaf arrangements.

This medicinal plant traditionally used for treatment of different human and animal ailments such as intestinal parasites, skin infection, horse scabies, treatment of febrile illness, bronchitis, stomach ache, diarrhea, constipation, pest control, malaria, snakebite were to mention a few of its usage. For instance, traditionally stems and roots of this plant are chewed or powders drunk against intestinal parasites. Dry leaves powder mixed with butter and applied for treating skin infection [79, 82, 89]. Orally administered leaf-juice for treating dysentery and roundworm infestation [93]. Furthermore; when used for the treatments of febrile illness to potentiate its effectiveness they mix, it's root powder with a leaf of *Ruta chalepensis* and fruit of *Zingiber officinale* [88].

Other scientifically proven results have shown that, methanol root-bark extracts of *B. abyssinica* has displayed antiretroviral activity by inhibiting replication of HIV-1 at 50% effective concentrations of 5.2 µg/ml that were non toxic to MT-4 cells [94]. This plant extract significantly inhibited malarial parasitemia dose-dependently [95]. There is also a reports of leaves, stem bark and root bark extracts of *B. abyssinica* possess antimycobacterial activity against the two fast growing bacteria *M. madagascariense* and *M. indicus pranii* [96]. The root bark of *B. abyssinica* was reported to exhibit antitumour activity and also it showed antibacterial activity against *Bacillus subtilis* as well as the strong antifeedant effect on the cotton pest insect observed [93, 95]. In addition, the extract of *B. abyssinica*, showed a significantly insecticidal effect on *Melophagus ovinus* (sheep ked) [97]. The methanol leaf extract of this plant also displayed significant free radical scavenging activity with an IC_{50} value of 7.5 µg/ml [93].

According to the study conducted in Côte D'Ivoire, with stem-bark aqueous extract of *B. abyssinica*, on multi-resistant strains and ATCC of *Staphylococcus aureus* test organisms. The result showed a diameter of inhibition zone ranging from 11.1±0.1 to 18,0±0.3 mm. The MICs were found between 1.563±0.0 to 0.195±0.0 mg/ml and MBC varies from 1.563±0.0 to 0.098±0.0 mg/ml [90].

In another similar study conducted in Côte D'Ivoire, on Root ethanol and aqueous extracts of *B. abyssinica* on 3 gram negative test organisms. This plant exhibited the best test results among other 13 plants. Ranges of 2.5 to 80 mg/ml MIC and MBC values were obtained against *S. typhi, S. typhimurium* and *P. aeruginosa* using tube dilution method [98].

A study conducted, in Cameroon; on *Bersama engleriana* methanol extract roots, stem bark, leaves and wood. Antimicrobial sensitivity test were also done using an agar disc diffusion assay showed that all the tested extracts were active on all tested microorganisms. The MIC results ranging from 9.76 to 156.25 µg/ml for those of the leaves and wood. The lowest MIC value of 9.76 was obtained by root extracts on *Shigella dysenteriae.* This plant also possesses the major secondary metabolites such as alkaloids, anthraquinones, triterpenes, saponins, phenols and flavonoids [99].

A study conducted in Tanzania to confirm the phytochemical presence of therapeutically effective bioactive compounds on the methanolic fraction of *Bersama abyssinica* leaf, stem bark and root bark using Gas chromatography coupled to mass spectrometer (GC-MS) analysis. A total of 24 phytocompounds from the leaves, 21 compounds from stem bark and 19 from root bark; were identified. Most of the identified compounds terpenes, vitamin, carotenoid (rhodopin), flavonoids, steroid, unsaturated and saturated fatty acids. Previously were reported to possess antimicrobial, antitumor, antiseptic, preservative, insecticidal and antioxidant activities. (3,7,11,15-Tetramethyl-2-hexadecen-ol), (1,2,3-Benzenetriol), Capric ether, (2,3-Dimethylfumaric acid), (2-furancarboxaldehyde, 5-methyl), (Ethyl iso-allocholate) these compounds were to name a few which are responsible for Antimicrobial, Fungicide, Candidicide, anticancer activities [92].

A preliminary antimicrobial screening test was conducted in Ethiopia on 67 plant species such as *B. abyssinica* (root bark), *Artemisia abyssinica*, *Stephania abyssinica* crude methanol, petroleum

ether and aqueous extracts were subjected to against 10 strains of bacterial species and 6 fungal strains using the agar dilution method on a range of concentration of 250–2000 µg/ml for the bacterial and 500–4000µg/ml was used for fungal pathogens. *B. abyssinica* was effective for five organisms *S. flexineri, S. dysenteriae, B. cereus, N. gonorrhoeae, S. aureus. S. abyssinica* was also effective to *N. gonorrhoea were as, A. abyssinica* was effective *B. cereus, N. gonorrhoeae, S. aureus.* However, all the above mentioned plant species did not show any activity on selected fungal pathogens [100].

A similar antimicrobial study was conducted in Ethiopia; using in-vitro broth microdilution method against one yeast and 11 bacterial strains on 23 ethanol extracts of medicinal plant such as, *Clutia abyssinica, Clematis hirsuta, B. abyssinica, Croton macrostachyus* are to mention some against the 12 microbes. Range of MIC results 64 to 512 µg/ml were observed. Ethanol root extracts of *B. abyssinica* were found effective against *Listeria monocytogenes, S. epidermidis, C. albicans* with MICs 512 µg/ml. Ethanol root extracts *C. abyssinica* and *C. macrostachyus* were effective against *S. pyogenes* at 256 mg/ml [101].

***Xanthium strumarium* L**: *Xanthium strumarium* L. English Cocklebur, local name in Amharic "Yemogne Fikir, Gebere nikel, or Bandaa" is belonging to the family Asteraceae, genus Xanthium an estimated 25 species annual short-day herbaceous plant of worldwide distribution. Especially, found tropical and sub-tropical regions of the world. This herb has erected stem up to 30-120 cm in height with frequently branched resulting somewhat bushy plants character. It has dark green leaves on the upper surface, broadly triangular-ovate in shape similar with grape leaves, 15 cm in diameter and roughly textured with minute bristles. The flowers are white or green; monoecious numerous male uppermost, female ovoid, covered with hooked bristles and are pollinated by insects. The seeds, 1.2 cm to 2 cm long, are covered by a hard green husk with numerous hooked spines which facilitates its spread [102,103,104]. It can be propagated through seeds. This weed has been considered one of the worst weeds since, it is easily dispersed through animals as the fruits have hooked bristles and two strong hooked beaks [105].

Figure 1b. *Xanthium strumarium* L.

This medicinal plant as different literature data showed that it possesses several efficacy as antiviral, antibacterial, fungicidal, antimalarial, antitrypanosomal, anti-inflammatory, high antioxidant potential, insecticidal curing nasal sinusitis, headache, urticaria, arthritis and cytotoxic activities against cancer cell lines were to mention a few [105, 106]. This property is attributed largely due to the presence of xanthanolides (a class of sesquiterpene lactones), rich in phenolic as well as flavonoids compounds [102]. This weed found throughout Ethiopia and used traditionally for the treatment of fungal infections of human skin leaf powder mixed with lime is usually applied on the skin against *Tinea versicolor* [107]. These have to be examined to assure medicinal plant's efficacy in treating different aliments traditionally by conducting in-vitro investigation.

For instance antimicrobial study was conducted in Brasil, on *X. strumarium* leaf extracted using 80% ethanol, methanol and ethyl acetate against standard test organism *S. aureus*, *E. coli, S. typhimurium, P. aeruginosa* and *C. perfringens.* MIC were carried out using 96 wells, test plate. The results indicate that *S. aureus, P. aeruginosa and E. coli* were the most susceptible with MIC values between 0.4 to 0.6 mg/ml, were as *S. typhimurium* and *C. perfringens* 0.6 to 0.8 mg/ml [104].

On another antimicrobial activity conducted in Bangladesh, using *X. strumarium* crude leaves and stem bark chloroform extracts were tested by the standard disc diffusion method against clinical isolates of *B. megaterium*, *B. subtilis*, *B. cereus*, *S. aureus, Sarcina lutea*, *V. mimicus, V. parahemolyticus, P. aeruginosa*, *E. coli, S. boydii*, *S. dysenteriae*, *S. typhi, S. cerevaceae, C. albicans* and *A. niger* were clinical isolated test organisms tested using 500 µg/disc impregnated test substances following subsequent incubation time average, ranging 10-24 mm inhibition zone exhibited [108].

An *in vitro* antimicrobial investigation was conducted in India, on *X. strumarium* leaf methanolic extract against eight standard pathogenic bacteria such as, *K. pneumoniae*, *P. vulgaris*, *P. aeruginosa*, *P. putida*, *B. cereus*, *B. subtilis*, *S. epidermidis*. By well diffusion method at a concentration of 50 mg/ml and 100 mg/ml their activities were recorded by zones of inhibition. *K. pneumoniae* were shown highest activity of (15 mm) at both concentrations. Followed by *P. aeruginosa*, S. *typhimurium* exhibited 13 mm at 100 mg/ml and 11 mm at 50 mg/ml were seen. The remaining organism shown promising activity of an average of 12 mm zone of inhibition [109].

Antimicrobial activities leaf crude extracts of *X. strumarium* were evaluated in Ontario Canada, against different bacteria; *E. coli*, *Aeromonas caviae, Paenibacillus alvei*, *Micrococcus luteus*, *Mycobacterium avium* and *Bacillus cereus* were using hole plate diffusion methods. A range of 9.3-35.3 mm zone of inhibitory activity exhibited. *B. cereus* 26.70 mm, *M. luteus* 35.3 mm, *M. avium* 30.0 mm, *P. alvei*. 25.0 mm, whereas no effects were seen on *E. coli and A. caviae.* Greater than 5 mg/ml MIC and MBC were observed in all test organisms [110].

A study conducted in Ethiopia, using two pathogenic gram positive bacteria *S. aureus* and *S. agalactiae* isolated from bovine mastitis. The test was performed using the agar disc diffusion method on *X. strumarium* leaf ethanol extract at 10%, 5%, 2.5%, 1.25% and 0.625% concentrations. Encouraging results were found against the test bacteria. Mean Zone of inhibition *S. aureus* 20.5, 18.5, 16, 15.25, and 13 mm and *S. agalactiae* MZI 21.5, 19.25, 17.25, 15.75, 13.25 mm, respectively [111].

Another similar *in-vitro* antibacterial study conducted in Ethiopia 2017; against *S. aureus* and *S. Agalactiae* isolated from bovine mastitis using ethanol leaf extract of *X. strumarium*. Discs diffusion method was used to study their efficacy with different concentrations (10%, 5%, 2.5%, 1.25% and 0.625%). The crude extract of both plants inhibits the growth of *S. aureus* and *S. agalactiae* at all concentration (10% to 0.63%) except *C. molle* leaf at 0.63% had no antibacterial effect on *S. agalactiae.* Leaves extract of *X. strumarium* resulted in 20.67 mm growth inhibition zone against *S. aureus* and 21.33mm on *S. agalactiae*, were observed, respectively [112].

3 OBJECTIVES

3.1 General Objective

- To Investigate Antibacterial, Antifungal Activity and Acute Toxicity Study of Traditionally used Ethiopian Medicinal Plants

3.2 Specific Objectives

- To conduct ethnobotanical literature survey to select claimed medicinal plants
- To evaluate antibacterial and antifungal potency and spectrum of selected medicinal plants using qualitative methods
- To determine MIC, MBC and MFC on the most active crude plant extract against the selected test organisms
- To evaluate the acute toxicity of the most active crude extract of the selected medicinal plant

3.3 Hypothesis

Ho: The Selected Ethiopian medicinal plants have antimicrobial activity against selected bacterial and fungal pathogenic organisms.

4 MATERIALS AND METHODS

4.1 Study Area and Period

The study was conducted from January 2017 to April 2018. Medicinal plants which claimed of having antibacterial and antifungal properties, were collected from different parts of Ethiopia such as, Oromia region Bale Zone around 430 km southeastern part of Ethiopia and from Shashamane, 240 km from the capital of Addis Ababa. Amhara region (North Shewa Zone Ankober district) which is 172 km northeast of the capital Addis Ababa and 46 km away from Debre Brihan town. Southern Ethiopia Wondo Genet, around 265 km, from Arba Minch 500 km away from Addis Ababa. And from botanical garden of Ethiopian Public Health Institute (EPHI) Traditional and Modern Medicine Research Directorate (TMMRD) Addis Ababa. The laboratory work was carried out in (EPHI, TMMRD) laboratories.

4.2 Medicinal Plant Collection and Identification

The Ethnobotanical literature survey was performed to gather the necessary information from published articles, books, student theses (dissertation) and from traditional healers/ knowledgeable individuals regarding potentially claimed medicinal plants. Adequate fresh herbal materials such as (leaves and root) were collected from their natural habitat based on local/ vernacular name. The collection was performed with the assistance of local traditional healers, and field observations of the plants from four botanical gardens. The selected plants were collected according to traditional usages. Voucher specimens were collected, pressed for taxonomical identification and verification were also performed by Botanist Dr. Getachew Addis (a Lead researcher and botanist of EPHI, Traditional and Modern Medicine Research Directorate). Finally the voucher specimens were deposited in the herbarium of TMMRD of EPHI for future references.

Figure 1: Picture of Some Selected Medicinal Plants for Screening; (a) *Bersama abyssinica* Fresen, (b) *Rhus natalensis* Bernh, (c) *Clutia abyssinica* and (d) *Ehretia cymosa*

4.2.1 Herbal Material Preparation and Extraction

Collected plant materials were washed 2-3 times with running tap water and rinsed with distilled water to remove dust, external pollutants, sediments, insecticides and parasites. Then air dried at room temperature in the shade, pulverized with a mortar and pestle finely ground into powder using an electric mill. This was done in order to lowering particle size and allowing for maximum penetration of extracting solvents (like ethanol, methanol) in to finely ground plant materials so as to maximize the releases of its' bio-active components. Powdered samples were packed and labeled in amber colored bottle/ polyethylene bags to avoid the entrance of air and any other contaminant and stored in closed containers for further extraction [4, 113, 114].

4.2.2 Extraction of Herbal Material

The extraction of plant materials was performed according to standards and their traditional use and preparations of the plant. Each powdered plant material (50g) was weighted, macerated in 500 ml, 80% ethanol organic solvent (1:10 solute: solvents) ratio in an Erlenmeyer flask with a stopper for 72 h kept in rotary-shaker (VWR DS-500; The Lab World Group, Boston, MA, USA) at 100 RPM room temperature. Every 24 hours, the macerate was filtered using funnel plunged with Whatman No.1 filter paper (Whatman Ltd. International, Maidstone, UK cat No 1001185) into clean and dry Erlenmeyer flask. The marc/residue obtained was re-suspended and macerated with new batches of solvent (80% Ethanol) (Sigma-Aldrich) three times within 3days. Each filtrate was evaporated (concentrated) to remove the solvent (ethanol) at 40°C to dryness through the rotary evaporator (R-200 Buchi, Switzerland) under vacuum to obtain the crude extract. Each crude extract obtained were then kept in hot air dry-oven at 37^0C for 72 hours to change to complete drying form weighed, labeled and sterilized overnight in UV-irradiation and stored in a desiccator until used, sterility was checked on nutrient agar plates during the experimentation [114, 115]. Finally, percentage yields (% extract yield) of each plant extract were calculated.

Figure 2: Rota Vapor used for Concentrating the ethanolic Plant extracts

4.3 Microbiological Assay

4.3.1 Test Organisms

A total of 22 organisms, 17 bacterial (6 gram positive, 11 gram negative) and 5 fungal species were used in the study. American Type Culture Collection (ATCC) or reference strains:

Staphylococcus aureus (ATCC 25923), *Escherichia Coli* (ATCC 25922), *Proteus mirabilis* (ATCC 35659), *Klebsiella pneumoniae* (ATCC 700603), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Streptococcus pyogenes* (ATCC 19615), *Streptococcus agalactiae* (ATCC 12386), *Streptococcus pneumoniae* (ATCC 49619) and *Shigella flexneri* (ATCC 12022). Clinical isolates; *Shigella dysentery, Proteus vulgaris, Acinetobacter, Citrobacter, Enterobacter* species and MRSA and Fungi: *Trichophyton mentagrophytes* (ATCC 18748), *Trichophyton rubrum* (ATCC 28188), *Aspergillus flavus* (ATCC 13697), *Aspergillus niger* (ATCC 10535) and *Candida albican* clinical sample. All the ATCC standard, clinical isolates organisms and working standard antibiotic drugs were generously obtained from EPHI Clinical Bacteriology and Mycology Reference Laboratory and TMMRD and Tikur Anbessa Specialized Hospital (TASH).

4.3.2 Inoculum Preparation

From stock cultures which was maintained at 4°C on slopes of nutrient agar. Active cultures for the experiments was prepared by transferring a loop full sample of the stock cultures to subculture on nutrient and sabouraud dextrose and blood agar incubated overnight at 37°C for bacteria and 25°C for fungal test organisms. The inoculum suspension was prepared by picking four to five colonies and diluted with 5 ml fresh Mueller-Hinton and Sabouraud dextrose broth (Oxoid Ltd, UK) to achieve the required standardized turbidity of the cell suspension (Optical Densities OD) by measuring using UV-Visible Spectrophotometer (Thermo Scientific Evolution 60S CAT 840210100) at 625 nm, (OD values range from 0.08 to 0.1) which is equivalently match with the turbidity of 0.5 McFarland barium sulfate standard corresponding to 1.0×10^8 colony forming units (CFU/ml) and 1.0×10^7 bacterial and fungal species, respectively. The suspensions were then diluted to 1:10 in respective broth to obtain 1.0×10^7 (CFU/ml) for bacteria and 1.0×10^6 cell/spores, for fungal strains as recommended by the Clinical and Laboratory Standards Institute (CLSI) [68, 116, 117].

4.3.3 Antimicrobial Sensitivity Tests

The antibacterial activity was primarily screened qualitatively using well diffusion methods at a testing concentration of (100 mg/ml and 200 mg/ml) crude extracts. Quantitative techniques were employed to determine MIC, MBC/MFC on the best efficacious medicinal plant during the

qualitative study. The solution of all the plant extracts was prepared by dissolving in a diluted dimethyl sulfoxide (5% v/v DMSO). All the experiments were conducted in triplicates.

4.3.4 Antimicrobial Agar Well Diffusion Assay

The assay was conducted as described by the Clinical and Laboratory Standards Institute (CLSI M07-A10) guideline with slight modification to meet the present experimental conditions. MHA and SDA were prepared according to the manufacturer's instructions and the medium was sterilized by autoclave (all American 75x-120v Electric) at 121^{o}C for 15 minutes. From the sterilized and cooled to 45^{o}C molten agar medium, 20 ml was poured aseptically into sterile 90 mm diameter petri dish and allowed to solidify at room temperature in sterile condition in the Biological safety cabinet (TELSTAR T Nr 16325). Bacterial and fungal strains sub cultured in nutrient agar (for bacteria) and Sabouraud Dextrose Agar (for fungi) after 16-18 hours of incubation at 37^{o}C and 25^{0}C were suspended in sterile nutrient broth (bacteria) and SDB (fungi) adjusted Spectrophotometrically as described above in the inoculum preparation.

A sterile cotton swab was briefly dipped into the adjusted suspension by rotating several times and pressing firmly on the inside wall of the tube above the fluid level so as to remove excess inoculums from the swab. This was done to maximize uniform distribution of test microorganisms on the medium. The entire dried agar surface was evenly streaked in three different directions by rotating at 60^{0}, swabbing the rim of the plate as the final step. Wells with 8-mm diameter were punched on the inoculated agar media with the help of sterile cork borer, 30 mm apart from center to center to one another of each wells. Within 15 minutes of inoculation the pre-prepared stock solution having 100 mg/ml and 200 mg/ml concentration by re-suspending the dried extracts with 5% v/v DMSO (Sigma-Aldrich) in distilled water were aseptically pipetted to well with a volume of 100 µL.

Likewise, the same volume of 5% v/v DMSO in distilled water was pipetted for negative control well at the center of each petri dish. Reference broad spectrum antibiotic discs were used as a positive control. All dishes were pre-incubated at room temperature for 2 h to allow uniform diffusion of extract solution into the agar medium, and then incubated at 37°C for 24 h for bacteria and at 25°C for 48 h to 72 h or more for yeast and other fungal test species. The antibacterial activity was evaluated by measuring the bacterial growth inhibition zone diameter

in mm. The experiments were performed in triplicate [116-120]. Both growth and sterility quality controls were conducted in every experiment in parallel. Results were expressed as mean value ± standard error of the mean (SEM), P values less than 0.05 ($p < 0.05$) were considered significant.

4.3.5 Determination of Minimum Inhibitory Concentration MIC

The determination of MIC was performed using agar dilution methods in which two-fold dilution of plant extracts in a medium were conducted. The assay was performed according to CLSI antimicrobial susceptibility-testing standards. A tenfold stock solution of each desired concentration (16 mg/ml, 8 mg/ml, 4 mg/ml, 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25mg/ml to 0.0625 mg/ml) was prepared separately in a bottle. Extract of each 2 ml stock solution and diluted standard drugs were added into separate test tubes containing 18 ml of molten agar medium separately, then thoroughly mixed with Vortex Mixer (SN 40419022) and poured aseptically into 90 mm sterile petri dish.

The plates were then allowed to solidify at room temperature. The agar surface of the plates containing the plant extracts and the controls were inoculated with standardized and diluted 1:10 in sterile broth to a respective organism of (1.0×10^7 CFU/ml). Inoculation using standard lope which delivers 2µl (10^4 CFU/spot) 5 to 8 mm in diameter of final inoculum size.

The inoculated plates were allowed to dry for 30 minutes, then incubated at 37^0C for 16-20 h for bacteria and at 25 ± 2°C up to 7 days for yeast and other fungi. Endpoints for each experiment were determined by placing plates on a dark background and observing for the lowest concentration or highest dilution which inhibited visible growth of the respective organism that was recorded as the MIC. Dilutions of Erythromycin, Ciprofloxacin and Ketoconazole served as positive controls. All the experiments were performed in triplicate with quality controls [116, 120, 121].

4.3.6 Determination of Minimum Bactericidal/ Fungicidal Concentrations (MBCs/MFCs)

Minimum Bactericidal and Fungicidal Concentration (MBC, MFC) are the lowest extract concentration that completely exterminates the bacterial or fungal population. Microbial cells from the MIC test plates was sub-cultured on the fresh extract or drug-free solid nutrient agar

medium for bacteria and SDA for fungus by making streaks on the surface of the agar. The plates were incubated at 37ºC for 24 hour for bacteria and at 25ºC for up to 72 h for yeast and fungal test organisms. Plates that did not show growth's of the colony or 99.9 % reduction of CFU on the solid agar medium after sub culturing were considered to be the MBC/ MFC. The experiments were carried out in triplicates. [113, 121]

4.4 Acute Toxicity Study

The acute oral toxicity test was carried out to determine median lethal dose (LD50) of the plant extract. The LD50 is usually an initial screening step in the assessment and evaluation of the toxicity characteristic of a substance [122, 123]. It is the statistically derived single dose of a substance that causes death in 50% of the population of test animals which administered orally and expressed as mg/kg of substance per animal body weight. The larger the LD50 value, the lower the toxicity and the smaller the LD50 value, the more toxic, it is and the smaller the dose needed to cause death. Therefore, it is a way to measure the short-term toxic potential of a compound. In addition, human toxicity is estimated based on test results on animal models [124, 125].

The assay was performed in accordance with internationally accepted principles described by the Organization for Economic Co-operation and Development (OECD) Test Guideline TG 425, the Up-and-Down-Procedure (UDP) for testing of chemicals with slight modifications [126]. Since, all medicinal plants are may not be safe for consumption in the crude form. Thus, such plants should be investigated to better understand their properties, safety and efficiency [127].

4.4.1 Experimental Animal

The laboratory animals such as mice and rats are the primary mammalian species used for the evaluation of acute and chronic toxicity study. Therefore, 35 young adult female, Swiss albino Mice weighing (28 - 30g), 6–8 weeks of age, breed at the animal breeding units of EPHI, which are clinically healthy were employed for the experiment. The mice were housed in stainless steel cages with a semi powdered wooden feet resting. They were allowed to freely access a standard pellet diet and tap water *ad libitum* and maintained at a room temperature of (25 ± 2^{0}C) with a 12 h light/dark cycle [128,129].

All the mice were females nulliparous and non-pregnant, divided into seven groups, each consisting of five mice, based on weight and respective doses including one control group. Each of them was marked on their tail for easy identification. All the animals were acclimatized for a week in the laboratory environment and the gavage (feeding needle) prior to the experiment in order to minimize stress caused during the testing period. All the procedures were performed following internationally accepted principles (OECD) guideline for testing. Ethically reviewed and approved by the Addis Ababa University, College of Health Sciences Ethical Review Committee and conformed [124,126].

4.4.2 Dose Preparation, Administration and Observation

All the groups of mice were fasted for a period of 4hr ahead of performing the test in the laboratory, the fasted body weight of each mouse was determined and the dose is calculated before administration. Different doses of ethanol extract *B. abyssinica* leaf, with a doubling dose, 150, 300, 600, 1200, 2400, 4800 mg/kg was prepared based on their respective body weight, dissolved in distilled water, then administered a single dose orally using gavage No.22 fitted with a syringe. Equal volumes of diluent distilled water were administered to the control group and all are re-fasted for additional 2hr. The volume of the dose administered depends on the weight of the animals.

All the mice were observed frequently on the day of treatment for any signs of toxicity. Observations were made on the general behaviour and continuously monitored and recorded systematically after the first 30 minutes, 1hr, 2hr, 4hr, 6hr and 24hr of extract administration. All the surviving mice were monitored daily thereafter, for a total of 14 days, their weight changes calculated and recorded. The visual observations of any sign toxicity included changes in behavioral pattern, respiratory pattern, motility, sedation, writhing, salivation, diarrhea, food and water consumption finally the number of survivors was noted and the LD_{50} was estimated after the 14^{th} day [126, 122,]. The dose and respective mortalities were statistically analyzed employing probit analysis of SPSS software log dose/probit regression line method [130].

Figure 3: Female Swiss albino mice used for *in-vivo* acute oral toxicity study

4.5 Data Quality Assurance

All data quality control tools [pre-analytical, analytical and post analytical stages] of quality assurance that were incorporated into Standard Operating Procedures (SOPs) (Annex 1) and the CLSI guideline were strictly followed. All materials, equipment and procedure was also adequately controlled. Culture media tested for sterility and performance. The performance of equipments (Autoclave incubators) was monitored. All the antimicrobial experiment was performed in triplicate with quality controls such as positive, negative, growth and sterility controls to insuring quality and reproducibility results.

4.6 Data analysis and Interpretation

Data were entered into excel spreadsheet, exported to software and tested for normality and then subjected to analysis of variance using Minitab statistical software to identify possible differences between, biological activity followed by student t-test to compare significant difference between the control and treated groups. All the data obtained expressed as mean ± SEM. One way analysis of variance (ANOVA) followed by Tukey's test was used for statistical evaluation. P-values less than 0.05 ($p<0.05$) were considered as significant and the LD50 was determined by using probit analysis**.**

4.7 Ethical Considerations

The study was conducted after ethically reviewed and approved by the Department of Research and Ethical Review Committee (DRERC) of Department of Medical Laboratory Science College of Health Sciences, Addis Ababa University. Formal letter was also written by the DMLT and permission was also obtained from EPHI, TMMRD for the laboratory work.

5 RESULTS

5.1 Ethnobotanical Literature Review

Ethnobotanical data regarding the traditional uses of medicinal plants by the various indigenous communities for the treatments of different human and animal's ailments were collected from various studies and publications which were conducted in different parts of the county, and from knowledgeable individuals. Particularly, those plant species used for treating diseases of bacterial and fungal origins based on the sign of the diseases locally mentioned by the communities. The required information includes scientific and local names of medicinal plants, parts used for treatment, the kinds of diseases treated, route of application, areas where they available for collection, identification and other relevant information about the plants were searched and collected as summarized in Table 1. Extract percentage (% yield w/w) was calculated by dividing the weight of the dried extract with the weight of the plant powder used for extraction multiplied by 100 [77]. Yield (%) = (W1 ∗ 100) /W2.

Table 1: Ethno-medicinal information on the selected and tested medicinal plants, with their respective use

No	Scientific Name [Family]	Local Name	Parts used	Ethnomed-icinal use	Extract Yield	ROA	References
1	*Acmella uliginosa* Del. [Asteraceae]	Gutichit	Leaf powder	Tooth-ache	37.2 %	Local application	[80]
2	*Urtica simensis* Steudel. [Urticaceae]	Samma	Root powder,	Fibril illness, Gonorrhea,	28.8%	Oral, fumigate	[78,80,87]
3	*Rhus natalensis* Bernh. [Anacardiaceae]	Xaxessa OR	Leaf powder	Cough, Colds, headache, diarrhea	23.8 %	Oral	[78,79,80, 83, 85]
4	*Clutia abyssinica* Jaub. & Spach [Euphorbiaceae]	Feyele Fej AM, Ulee fooni OR	Fresh leaf	Bloody diarrhea Ophthalmic, Nasal infection, Toothache	34.6 %	Oral, hold in the teeth	[78,83,878 9]
5	*Xanthium strumarium* L.[	Yemogne Fikir	Leaf juice	Skin infection/	27.4	Topical	[80,88]

	Asteraceae]	/Gebere nqel/ Bandaa		animal para/ anthrax	%		
6	*Stephania abyssinica* [Dillon ex A. Rich.] Walp. [Menispermaceae]	Engochit AM, Etse Eyesus, Hidda K alaala/ OR	Either powdered or fresh root	Wound, Common cold, Inhalation, Malaise	23.4 %	Topical/ washing	[78,80,83, 84,87,88]
7	*Zehneria scabra* L.(linnf) Sand. [Cucurbitaceae]	Etsesabe k or Hareg-resa,	Leaf with root, stem	Wound, Malaise ,Cough, Common Cold, Fever, Diarrhoea, Conjunctivi tis	19%	Oral drink	[78,80,81,8 4,87,89]
8	*Bersama abyssinica* Fresen. [Melianthaceae]	Azamir Am Lolchiisa a OR	Leaf powder Seed	Wound, skin burn, dysentery, Dandruff, Constipatio n, Fever Bronchitis	22 %	Topical	[79,80,82, 87,88,89]
9	*Echinops kebericho* Mesfin [Asteraceae]	Kebercho	Leaves Fresh	Tonsillitis, common cold	11.6 %	Oral, Inhaling	[81,84,85]
10	*Ajuga integerifolia* [Lamiaceae]	Harmma guusa Or Akorarac h Am	Powder of root and leaf	Wound, Stomach trouble, skin disease	24%	Oral /Topical	[80,82,86]
11	*Artemisia abyssinica* L. [Asteraceae]	Chikugne /Tiroo	leaf powder	Stomach trouble, Whooping Cough, tonsillitis, Eye infection	26.6 %	Oral	[78,80,88, 89]
12	*Ehretia cymosa* [Boraginaceae]	Game Am *Checho*	Leaf powder	Toothache, wounds	25.2 %	Oral, Holding	[84,87,89],

Note: ROA: Root of Application; AM: Amharic; OR: Oromifa;

5.2 Determination of Antimicrobial Activity

In the present study, a total of 12 medicinal plants which are presented in Table 1, were collected from different parts of Ethiopia and subjected to extraction of their leaves and root of one plant using ethanol organic solvent. There antimicrobial potentials were evaluated *in vitro* against 22 pathogenic organisms (6 gram positive, 11 gram negative bacteria and 5 fungal test organisms). Agar well diffusion method was used for antimicrobial screening assay with a well bore size greater than 8 mm growth inhibition zone diameter was chosen as a break-point for microbial susceptibility at 100 mg/ml and 200 mg/ml test concentration. While, positive control antibiotics Ciprofloxacin, Erythromycin, Gentamicin, Vancomycin, Ceftriaxone, Ketoconazole and Negative control 5% DMSO were used.

The result obtained from the experiment showed that, all the plant extracts have inhibited *in vitro* growths of at list one or more microorganisms. Among these plants crude ethanolic leaves extracts obtained from 7 species of plants (*Bersama abyssinica, Xanthium strumarium, Rhus natalensis* and *Zehneria scabra, Ehretia cymosa, Clutia abyssinica, Stephania abyssinica*) were the most potent of all plant extracts which inhibited the growth of most gram positive, gram negative and fungal tested organisms as shown in Tables 3 to 6. However the results of the rest 5 plants with relatively minimum effects were presented in the annexed tables (**Annexes IV).**

5.2.1 Activities on Gram Positives Bacteria

In the agar well diffusion assay to determine antibacterial activity of hydro-alcoholic extracts of the 12 plants against gram positive bacteria, four plant extracts have shown remarkable efficacy Table 3. As result showed, MRSA clinical isolates and *S. aureus* ATCC 25923 were the most susceptible bacteria from the tested gram positive organisms. Both MRSA and *S. aureus* were sensitive to all the four plant extracts with inhibition zone ranging from 15.5 to 27.0 mm and 15.3 to 25.3 mm, respectively. Leaves extract of *B. abyssinica* exhibited the highest potency against MRSA, *S. aureus, S. agalactiae* and *E. faecalis* at a concentration of 200 mg/ml, followed by *X. strumarium* and *R. natalensis*. While extracts of *X. strumarium* displayed the highest level of activity against *S. pneumoniae* and *S. pyogenes*. Compared to standard antibiotics Erythromycin used in the study *X. strumarium* extract showed statistically significantly higher inhibitory activity at 200 mg/ml concentration against *S. pnuemoniae* and

MRSA ($p<0.05$). Similarly, *B. abyssinica* extract showed significantly higher inhibition of MRSA and *E. faecalis* compared to Erythromycin ($p<0.05$). *B. abyssinica* at a concentration of 100 mg/ml have showed similar growth inhibition against MRSA the positive control antibiotics, Erythromycin (15µg) ($p=0.05$). Likewise, *Rhus natalensis* leaves extract also significantly higher growth inhibition zones were recorded against *E. faecalis* with ($p<0.05$) at 100 and 200 gm/ml test concentrations compared to Erythromycin (15µg). The list inhibition zones were observed by leaves ethanol extract of *Z. scabra* in all tested bacteria with 100 gm/ml.

In our experimental results, we have observed that, *E. faecalis* was susceptible with ciprofloxacin 25 mm, intermediately susceptible for erythromycin 15 mm while, it was resistant to standard drugs gentamicin, vancomycin and ceftriaxone with inhibition zones 11, 11 and 10 mm, respectively Table 2.

Two concentrations of each plant extracts 100 and 200 mg/ml were used to see the concentration effects. There were significant difference between plants and concentrations at 100 and 200 mg/ml was seen. All plant extracts displayed significantly higher inhibition activity at 200 mg/ml concentration compared to 100 mg/ml in the majority of tested gram positive bacterial species except, *X. strumarium* against *S. pnuemoniae, E. faecalis, S. agalactiae.*

Table 2. Antimicrobial Susceptibility profile of test organisms to five standard antibiotics used from different class.

Test Organism	**Antibacterial Inhibition Zone Diameter (mm)**				
	Cipro.	Vanco.	Erytro.	Genta.	Ceftra.
S. aureus	*27* mm S	17 mm*	29 mm S	19 mm S	11 mm*
MRSA	22 mm S	18 mm*	25 mm S	23 mm S	12 mm*
S. pyogenes	26 mm*	18 mm S	32 mm S	30 mm	16 mm*
S. agalactiae	20 mm*	21 mm S	32 mm S	22 mm	34 mm*
S. pneumoniae	27 mm*	19 mm S	19 mm I	22 mm	18 mm*
E. faecalis	25 mm S	11 mm R	15 mm I	11 mm*	10 mm*
E. coli	32 mm S	---	10 mm	18 mm S	24 mm S
K. pneumoniae	21 mm S	---	---	14 mm I	11 mm*
P. aeruginosa	28 mm S	---	---	21 mm S	---
S. typhimurium	35 mm S	---	---	23 mm S	30 mm S
S. flexneri	33 mm S	---	---	19 mm S	25 mm S
P. mirabilis	44 mm S	13 mm*	9 mm*	24 mm S	35 mm S
S. dysentery	32 mm S	---	16 mm*	18 mm S	27 mm S
P. vulgaris	33 mm S	---	---	12 mm S	---
Enterobacter	30 mm S	---	---	9 mm R	---
Citrobacter	31 mm S	---	---	15 mm S	---
Acinetobacter	---	---	15 mm*	11 mm R	---

Key notes: T. Orga: Test organisms; MRSA: Methicillin-Resistant *Staphylococcus Aureus;* Cipro: Ciprofloxacin (5µg); Vanco: Vancomycin (30µg); Erytro: Erythromycin (15µg); Genta: Gentamicin (10µg); Ceftra: Ceftriaxone (5 µg); Inhibition zone in millimeter, S: Susceptible, I: Intermediate, R: Resistant, (---) "no inhibition" (*) interpretive criteria are not applicable Labeling as susceptible, intermediate and resistance based on CLSI documents M100S [131].

Table 3: Antibacterial activity of four selected plant extracts against gram positive bacteria using well diffusion method.

Plant Species	**Concentration**	**Inhibition zone Diameter (mm)**					
		S. aureus	*S. pyogenes*	*S. agalactiae*	*S. pneumoniae*	*E. faecalis*	MRSA
B. abyssinica	200 mg/ml	25.33 ±0.22^{b}	18.00±0.00^{c}	20.00 ±0.00^{b}	15.50±0.22^{c}	26.17±0.41^{a}	27.00±0.00^{a}
	100 mg/ml	23.33±0.22^{c}	14.00±0.00^{d}	16.00 ±0.00^{d}	13.50±0.22^{d}	22.00±0.26^{b}	25.00±0.00^{c}
X. strumarium	200 mg/ml	23.67±0.22^{c}	19.00±0.45^{b}	9.00± 0.00^{h}	26.5± 0.22^{a}	15.50±0.67^{d}	26.00±0.00^{b}
	100 mg/ml	22.00±0.00^{d}	17.50±0.22^{c}	9.00± 0.00^{h}	26.50±0.22^{a}	15.67±0.62^{d}	24.33±0.22^{d}
R. natalensis	200 mg/ml	19.50±0.22^{e}	14.00±0.00^{d}	13.00± 0.00^{f}	13.00±0.00^{e}	22.00±0.00^{b}	18.50±0.22^{e}
	100 mg/ml	18.00±0.00^{f}	10.00±0.00^{g}	11.00±0.00^{g}	11.00±0.00^{f}	17.50±0.22^{c}	16.50±0.22^{f}
Z. scabra	200 mg/ml	18.17± 0.17^{f}	12.83±0.17^{e}	17.0± 0.00^{c}	13.00±0.45^{de}	16.67±0.21^{cd}	17.00±0.00^{f}
	100 mg/ml	15.33±0.22^{g}	11.50±0.22^{f}	15.00±0.00^{e}	11.00±0.00^{f}	12.17±0.54^{e}	15.50±0.22^{g}
+ Erythromycin	15µg/ml	29.00±0.00^{a}	32.00±0.00^{a}	32.00±0.00^{a}	19.00±0.00^{b}	15.00±0.00^{d}	25.00±0.00^{c}
- DMSO	5%	8.00±0.00^{h}	8.00±0.00^{h}	8.00±0.00^{i}	8.00±0.00^{g}	8.00±0.00^{f}	8.00±0.00^{h}

Key notes: Inhibition zone diameter including well diameter (8 mm); DMSO 5%: Negative control, Erythro: Erythromycin (15µg) disk as Positive control; Data are expressed in mean ± S.E.M (standard error of mean), N=6; Mean values with different letters in the same column are significantly different ($P < 0.05$).

5.2.2 Activities on Gram Negative Bacteria

Results of antimicrobial activity of six plant species against eleven gram negative bacteria using agar diffusion method at 100 and 200 mg/ml test concentration were recorded in Table 4a and 4b. The results indicated that, extracts of *B. abyssinica, R. natalensis* and *Z. scabra* were potentially effective in inhibiting the growth of majority gram-negative bacteria with a variable zone of inhibition. *B. abyssinica* leaves extract was the most active plant with superior in suppressing the growth of all tested gram-negative organisms. *R. natalensis* was the second most active plants in inhibiting the growth of all gram negative bacteria except, *S. dysentery, K. pneumoniae* at 100 mg/ml in which it has recorded the list zone of inhibition (8.50 ± 0.21^{cd}) mm.

P. mirabilis was the most susceptible organisms to all the plant extracts with inhibition zone diameter ranging from 9.5 to 21.00 mm followed by *Acinetobacter* species. Similarly, *S. flexneri, P. vulgaris, P. aeruginosa* were the other susceptible organisms by most crud extracts of the above six plants. On the other hand, *K. pneumoniae* was the most resistant bacteria in the treatment of all the plant extracts, except for *E. cymosa* to which the organism showed relatively the highest susceptibility followed by and *R. natalensis* followed by *S. dysentery*. The tested standard antibiotics, ciprofloxacin, have recorded statistically higher inhibition of all gram negative bacteria with ($p< 0.05$), except for *Acinetobacter* species where the antibiotics have significantly the lowest activity compared to all the plant extracts with ($p< 0.05$).

Both *C. abyssinica* and *S. abyssinica* showed the lowest efficacy, only moderate inhibitions were recorded against six bacteria while no growth suppression on the remaining five bacterial species namely *S. dysentery, S. typhimurium, K. pneumoniae, Enterobacter* and *Citrobacter* species. *K. pneumoniae*, *S. dysentery* and *Citrobacter* species were resistant to the challenge of exposure to three of the tested plant extracts.

Comparison effects of the concentration of six plant extracts against gram negative bacteria, statistically significant differences between 100 and 200 mg/ml was recorded by *B. abyssinica* against all organisms, except *S. flexneri, K. pneumoniae* and *Acinetobacter* species. The antibacterial activity of *Z. scabra, E. cymosa, C. abyssinica* and *S. abyssinica* plant extracts at 100 and 200 mg/ml concentration were not statistically different against *S. dysentery, K. pneumoniae, Citrobacter* and *Enterobacter* species.

Table 4a: Antibacterial activity of six selected plant extracts against gram negative bacteria using well diffusion method.

Plant Species	Concentration	Inhibition zone Diameter (mm)					
		Ec.	Sdy.	Sfx.	Sty.	Kp.	Pa.
B. abyssinica	200 mg/ml	11.33±0.21^{b}	14.50 ± 0.22^{b}	25.33 ±0.21^{b}	16.50±0.22 ^{b}	9.67 ± 0.21^{d}	22.17±0.17^{b}
	100 mg/ml	10.50±0.22^{cd}	11.33±0.21^{c}	25.00±0.00^{b}	14.83±0.40^{c}	9.50 ±0.22^{d}	20.00±0.00^{c}
R. natalensis	200 mg/ml	12.17±0.17^{bc}	10.83±0.17^{c}	15.50±0.22^{c}	12.00±0.37^{d}	10.50±0.22^{c}	16.83±0.17^{d}
	100 mg/ml	11.33±0.42^{e}	8.50 ± 0.22^{e}	13.33±0.21^{d}	10.50±0.22^{efg}	8.33±0.21^{e}	13.00±0.00 ^{e}
Z. scabra	200 mg/ml	11.33±0.21^{b}	10.83±0.00 ^{d}	11.00±0.00^{e}	10.67±0.33^{ef}	8.00 ±0.00^{e}	12.17± 0.00^{ef}
	100 mg/ml	10.00±0.00^{d}	8.50 ± 0.00^{e}	10.50±0.22^{e}	10.00±0.00^{fg}	8.00±0.00^{e}	8.00±0.00^{h}
E. cymosa	200 mg/ml	8.00± 0.00^{e}	8.00 ± 0.00^{e}	11.17± 0.17^{e}	11.33±0.21^{de}	11.50±0.22^{a}	11.00±0.37^{fg}
	100 mg/ml	8.00± 0.00^{e}	8.00 ± 0.00^{e}	9.17 ± 0.40^{f}	9.50 ±0.22^{g}	8.67± 0.21^{e}	10.50±0.85^{g}
C. abyssinica	200 mg/ml	10.33± 0.21^{cd}	8.00 ± 0.00^{e}	10.83±0.40^{e}	8.00 ± 0.00^{h}	8.00 ±0.00^{e}	10.00 ±0.22^{g}
	100 mg/ml	8.17± 0.17^{e}	8.00 ± 0.00^{e}	8.67±0.21^{fg}	8.00 ± 0.00^{h}	8.00 ±0.00^{e}	8.00 ± 0.00^{h}
S. abyssinica	200 mg/ml	10.00 ± 0.00^{d}	8.00 ± 0.00^{e}	10.00± 0.22^{e}	8.00 ± 0.00^{h}	8.00±0.00^{e}	10.67 ± 0.21^{g}
	100 mg/ml	8.00 ± 0.00^{e}	8.00 ± 0.00^{e}	8.00± 0.00^{g}	8.00 ± 0.00^{h}	8.00 ±0.00^{e}	8.67 ± 0.21^{h}
Ciprofloxacin	5µg	30.00±0.00^{a}	33.00±0.00^{a}	33.00±0.00^{a}	35.00±0.00^{a}	21.00±0.00^{a}	28.00±0.00^{a}
DMSO	5%	8.00± 0.00^{e}	8.00 ± 0.00^{e}	8.00± 0.00^{g}	8.00 ± 0.00^{h}	8.00 ±0.00^{e}	8.00± 0.00^{h}

Key notes: EC: *E. coli*, Sdy*: S. dysentery*, Sfx: *S. flexneri*, Sty: *S. typhimurium*, Kp: *K. pneumoniae*, Pa: *P. aeruginosa*. Inhibition zone diameter including well diameter (8 mm), DMSO 5%: Negative control, Ciprofloxacin disk (5µg): Positive control; Data are expressed in mean ± S.E.M (standard error of mean), N=6; Mean values with different letters in the same column are significantly different ($P < 0.05$).

Table 4b: Antibacterial activity of six selected plant extracts against gram negative bacteria using well diffusion method.

Plant Species	**Concentration**	**Inhibition zone (mm) in test organisms**				
		Pmr.	*Pvl.*	*Enro.*	*Ctr.*	*Acnt.*
B. abyssinica	200 mg/ml	21.00±0.36^{b}	19.50 ±0.22^{b}	12.67 ±0.33^{b}	12.00±0.00^{b}	21.67 0.21^{a}
	100 mg/ml	18.33±0.21^{cd}	17.00 ±0.00^{c}	11.83± 0.31^{c}	11.00±0.00^{c}	20.67 0.21^{a}
R. natalensis	200 mg/ml	14.33±0.33^{g}	14.50± 0.22^{d}	12.83 ±0.16^{b}	11.00±0.00^{c}	15.50±0.22^{b}
	100 mg/ml	9.50±0.22^{h}	13.00 ±0.00^{e}	11.83± 0.16^{c}	10.17±0.16^{d}	14.00 0.00^{c}
Z. scabra	200 mg/ml	16.17±0.54^{ef}	11.83±0.17^{f}	9.83 ±0.17^{d}	8.00±0.00^{f}	13.00±0.37^{cd}
	100 mg/ml	16.17±0.30^{ef}	11.33±0.21^{f}	8.00 ±0.00^{e}	8.00±0.00^{f}	10.83±0.17^{e}
E. cymosa	200 mg/ml	19.00±0.22^{cd}	14.00±0.00^{d}	10.00±0.00^{d}	9.17±0.17^{e}	15.67±0.21^{b}
	100 mg/ml	17.67±0.21^{de}	12.00±0.00^{f}	8.00 ±0.00^{e}	9.00±0.00^{e}	13.33±0.21^{cd}
C. abyssinica	200 mg/ml	15.50±0.22^{fg}	10.50±0.22^{g}	8.00 ±0.00^{e}	8.00±0.00^{f}	10.17±0.17^{ef}
	100 mg/ml	15.17±0.17^{fg}	10.00±0.00^{g}	8.00 ±0.00^{e}	8.00±0.00^{f}	9.50 ± 0.22^{f}
S. abyssinica	200 mg/ml	19.50±0.22^{bc}	11.33±0.21^{f}	8.00±0.00^{e}	8.00±0.00^{f}	13.67±0.21^{cd}
	100 mg/ml	18.67±0.21^{cd}	10.17±0.16^{g}	8.00 ±0.00^{e}	8.00±0.00^{f}	12.67±0.42^{d}
Ciprofloxacin	5µg	40±0.00^{a}	33.00± 0.00^{a}	30.00 ±0.00^{a}	31.00±0.00^{a}	8.00 ± 0.00^{g}
DMSO	5%	8.00± 0.00^{h}	8.00 ± 0.00^{h}	8.00 ± 0.00^{e}	8.00 ± 0.00^{f}	8.00 ± 0.00^{g}

Key notes: Pmr: *P. mirabilis,* Pvl*: P. vulgaris*, Enro: *Enterobacter spp.,* Ctr*: Citrobacter spp.*, Acnt*: Acinetobacter spp.* Inhibition zone diameter including well diameter (8 mm), DMSO 5%: Negative control, Ciprofloxacin disk (5µg): Positive control; Data are expressed in mean ± S.E.M (standard error of mean), N=6; Mean values with different letters in the same column are significantly different (P< 0.05).

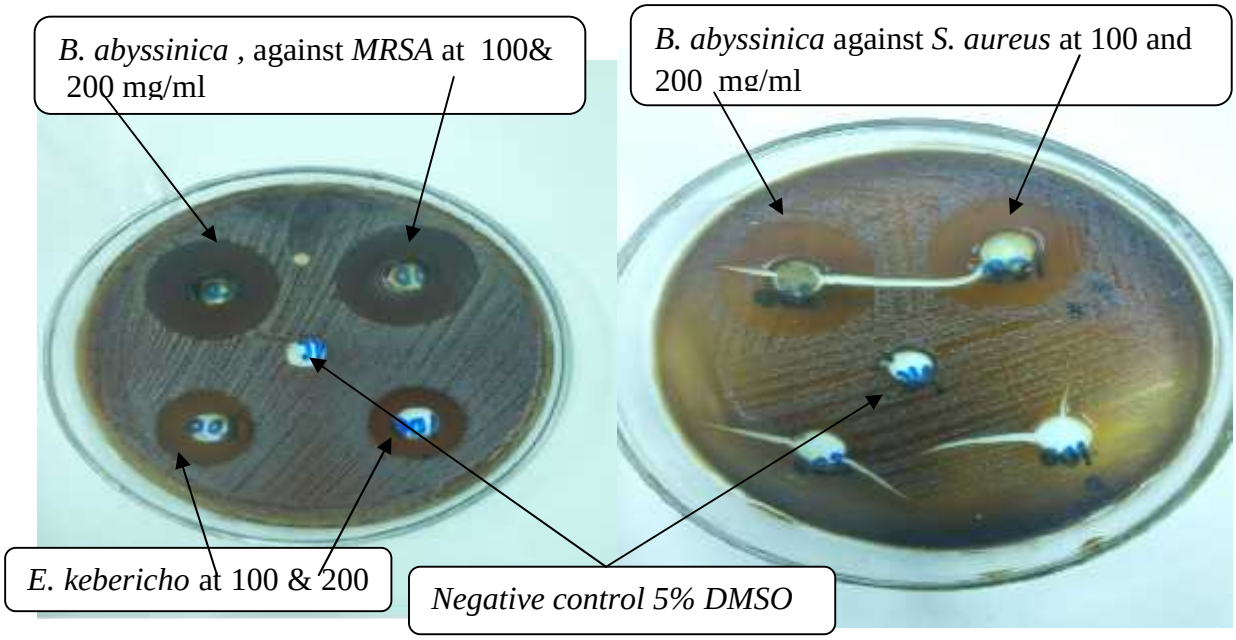

Figure 4 (a): Inhibition Zones of *B. abyssinica* extract against MRSA and *S. aureus* and with well diffusion assay

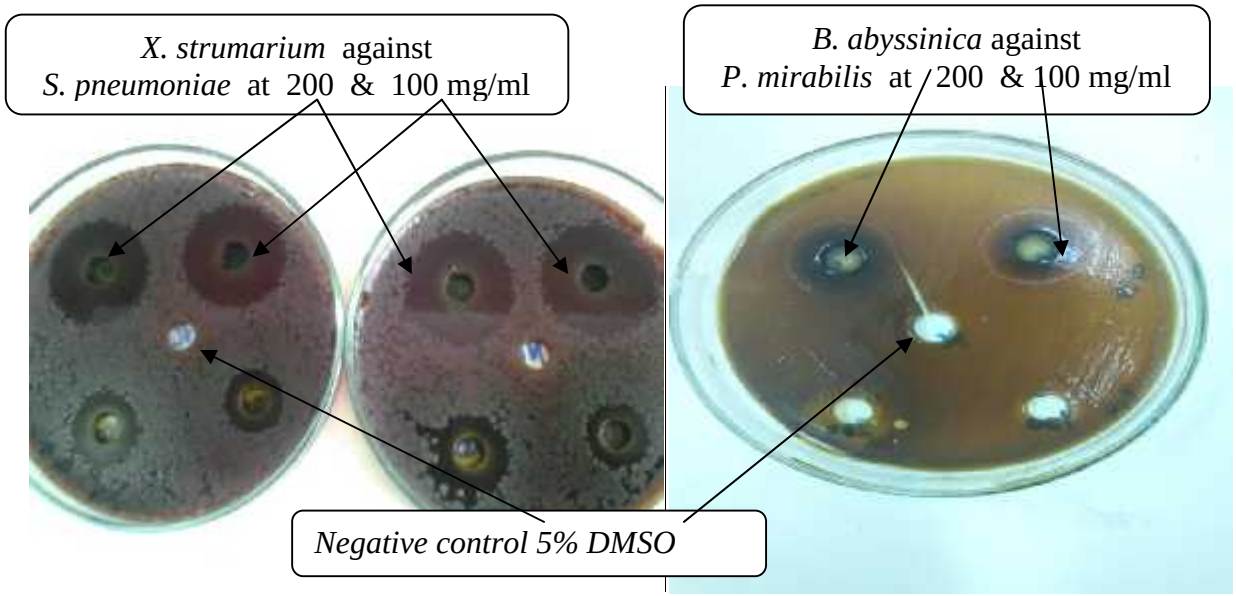

Figure 4b: Inhibition Zones of *X. strumarium* extract against *S. pneumoniae* and *B. abyssinica* leaves extract against *P. mirabilis* with well diffusion assay.

5.3 Antifungal Assay

Three plant species were showed best result from all the 12 crud plans extract in the assessment their antifungal activity against one yeast (*C. albicans*) and four filamentous fungi (*T. mentagrophytes, T. rubrum, A. flavus* and *A. niger*) using the agar well diffusion method. Evaluation of antifungal activity of *B. abyssinica, X. strumarium* and *R. natalensis* extracts were recorded in Table 5., The result indicated that all the 3 plant extracts were potentially effective against the tested dermatophyte *T. mentagrophytes* and *T. rubrum* at both concentrations.

Significantly higher fungal inhibitory activity was demonstrated by both *B. abyssinica* (against dermatophyte) and *X. strumarium* (against *C. albicans* and dermatophytes) at 200 mg/ml as compared to standard antifungal drug Ketoconazole (6.25mg). *X. strumarium* was found to give significantly superior antifungal activity compared to the other two plant extracts in all tested fungal species except in *A. niger* at both tested concentrations. The maximum inhibition was observed against, *T. rubrum* with inhibition zone 46 mm and 48 mm at a concentration of 100 mg/ml and 200 mg/ml, respectively. In other hand, *B. abyssinica* was only effective against dermatophytes, *T. mentagrophytes* was the most susceptible fungi against the challenge of *B. abyssinica* extracts at a concentration of 100 and 200 mg/ml with the inhibition zone of 36 and 42 mm, respectively followed by *T. rubrum*. *B. abyssinica* and *R. natalensis* depicted no inhibitory activity against the growth of *C. albicans, A. flavus* and *A. niger* at a concentration of 100 mg/ml and 200 mg/ml.

Concentration comparison effect of three plant extracts against fungal test organisms at 100 and 200 mg/ml. Statistically significant greater result was seen by *X. strumarium* against all organisms with ($p < 0.05$) except, *A. flavus* and *A. niger*. Likewise extracts of *B. abyssinica* was showed, statistically significantly different results on the two dermatophytic species with ($p < 0.05$) at the 200 mg/ml test concentration.

Table 5: Antifungal activity of three selected plant extracts using well diffusion method.

Plant Species	Concentration	Inhibition Zone Diameter (mm)				
		C. albicans	***T.mentagrophytes***	***T. rubrum***	***A. flavus***	***A. niger***
B. abyssinica	200 mg/ml	8.00 ± 0.00^{c}	42.00±0.00^{c}	35.33±0.21^{c}	8.00±0.00^{d}	8.00±0.00^{b}
	100 mg/ml	8.00 ± 0.00^{c}	36.00±0.00^{e}	31.67±0.42^{d}	8.00±0.00^{d}	8.00±0.00^{b}
R. natalensis	200 mg/ml	8.00 ± 0.00^{c}	15.50± 0.22^{f}	17.50±0.22 ^{e}	8.00±0.00^{d}	8.00±0.00^{b}
	100mg/ml	8.00 ± 0.00^{c}	14.00± 0.00^{g}	15.50±0.22^{f}	8.00±0.00^{d}	8.00±0.00^{b}
X. strumarium	200mg/ml	15.50±0.00^{a}	46.00±0.00^{a}	48.00±0.00^{a}	11.00±0.00^{b}	8.00±0.00^{b}
	100mg/ml	14.50±0.22^{b}	45.00±0.45^{b}	46.00±0.22 ^{b}	10.00±0.00^{c}	8.00±0.00^{b}
+ Keto	6.25mg/ml	8.00 ± 0.00^{c}	38.00±0.00^{d}	32.00±0.00^{d}	42.00±0.00^{a}	13.00±0.00^{a}
- DMSO	5%	8.00 ± 0.00^{c}	8.00±0.00^{c}	8.00±0.00^{g}	8.00±0.00^{d}	8.00±0.00^{b}

Key notes: Inhibition zone diameter including well diameter (8 mm); DMSO 5%: Negative control; Keto: Ketoconazole (6.25mg/ml) as Positive control; Data are expressed in mean ± S.E.M (standard error of mean); N=6; Mean values with different letters in the same column are significantly different ($P < 0.05$).

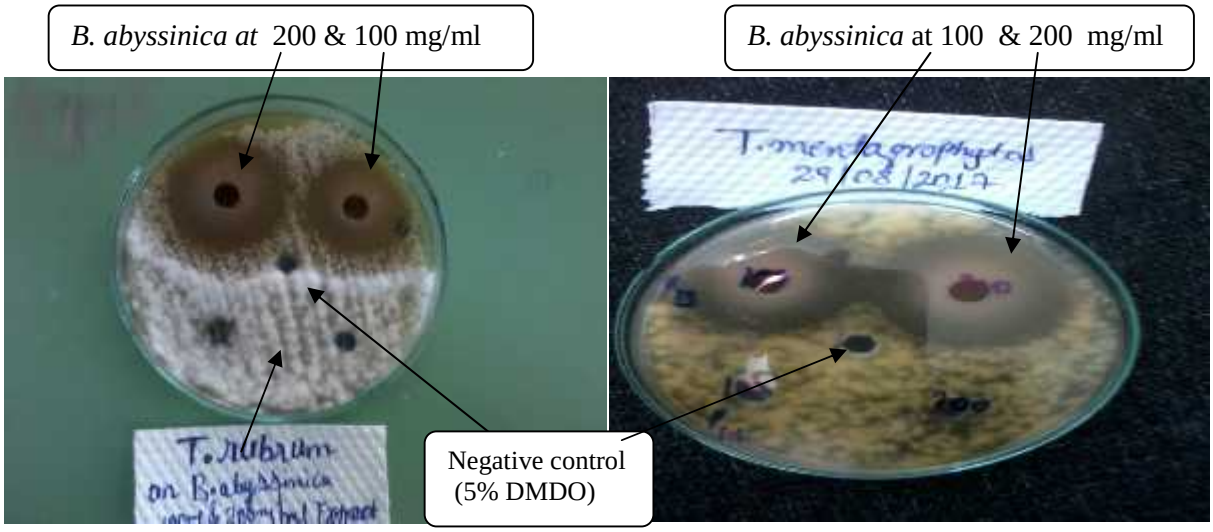

Figure 4c: Inhibition Zones of *B. abyssinica* extract against *T. rubrum* and *T.mentagrophytes* with well diffusion assay

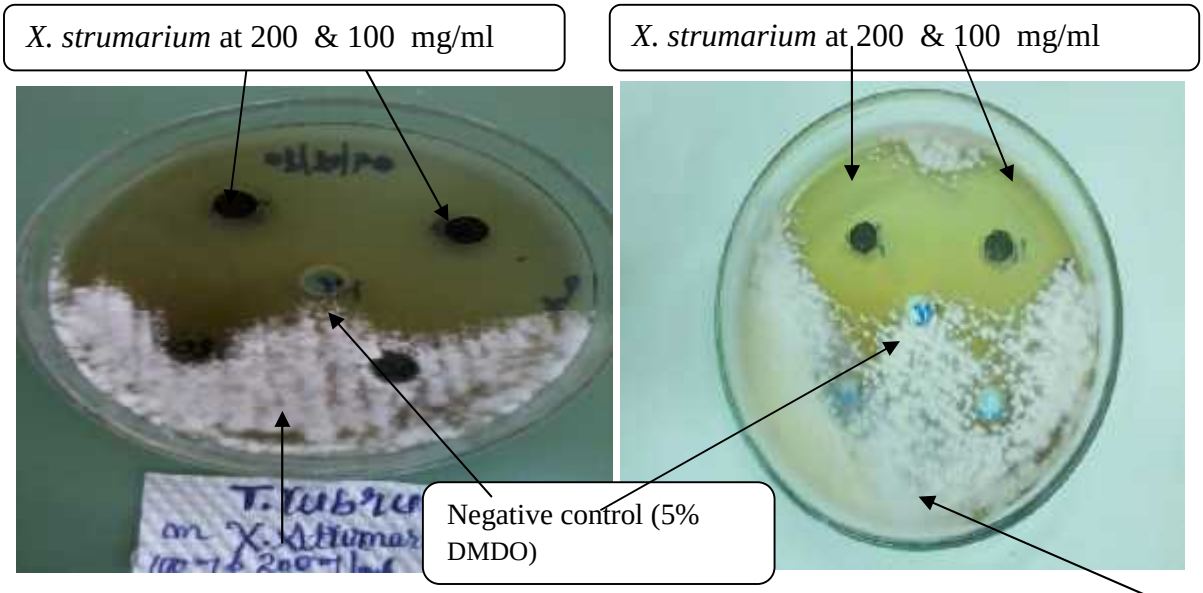

Figure 4d: Inhibition Zones of *X. strumarium* leaf extract against *T. rubrum* (left) and *T. mentagrophytes* (right) using well diffusion assay.

5.4 Determination of MIC, MBC and MFC

5.4.1 Gram Positive Bacterial MICs and MBCs Results

To investigate the antibacterial activities of *B. abyssinica*, all selected gram positive bacterial strains were challenged with different concentrations of the plant extracts and the MIC (defined as the lowest concentration of the antibacterial agent that inhibits the bacterial growth after 24 h of incubation at 37^0C) was determined by agar dilution assay Table 6., *B. abyssinica* ethanol extracts showed excellent activity against all the tested bacteria with MIC ranging from 0.25 to 4 mg/ml. The most susceptible bacteria in the treatment of *B. abyssinica* ethanol extracts were *S. aureus* and MRSA, with MIC value of 0.25 mg/ml. *B. abyssinica* extract showed potentially bactericidal activity against tested gram-positive bacteria with MBC of 1 mg/ml against *S. aureus*, 2 mg/ml against MRSA, 4 mg/ml against *E. faecalis*, 8 mg/ml against *S. pneumoniae*, and 16 mg/ml against *S. agalactiae* and *S. pyogenes*.

Table 6: Minimum Inhibitory (MIC) and Minimum Bactericidal Concentration (MBC) of *B. abyssinica* ethanol extract on different gram positive bacteria by agar dilution assay.

Plant Name	**Saur.**		**MRS.**		**Spy.**		**Spn.**		**Sag.**		**Efec.**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
***B. abyssinica* mg/ml**	0.25	1	0.25	2	4	16	4	8	16	16	2	4
Erythromycin (µg/ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	2	2

Key notes: Saur: *S. aureus*; MRS: MRSA; Spy: *S. pyogenes*; Spn: *S. pneumoniae*; Sag: *S. agalactiae*; Efec: *E. faecalis*.

5.4.2 Gram Negative Bacterial MICs and MBCs Results

To further evaluate antibacterial activities of *B. abyssinica* extract against gram negative bacterial species using quantitative techniques, agar dilution was used and MIC as well as MBC was determined as shown in Tables 7a and 7b., The plant extract demonstrated high antibacterial potential against *P. mirabilis* with 0.5 mg/ml MIC value followed by MIC value of 1 mg/ml against *S. flexneri*, *P. aeruginosa*, *P. vulgaris* and *Acinetobacter* species.

Table 7a: Minimum Inhibitory (MIC) and Minimum Bactericidal Concentration (MBC) of *B. abyssinica* ethanol extract on different gram negative bacteria by agar dilution assay.

Plant Name	**Ec.**		**Sdy.**		**Sfx.**		**Sty.**		**Kp.**		**Par.**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. abyssinica	8	16	8	16	1	2	8	8	16	16	1	2
Cipro. µg/ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Key notes: EC: *E. coli*; Sdy: *S. dysenteriae*; Sfx: *S. flexneri*; Sty: *S. typhimurium*; KP: *K. pneumoniae*; Par: *P. aeruginosa*; MIC, MBC for

The bactericidal potential of *B. abyssinica* was lowest against *Acinetobacter* species with MBC of 1 mg/ml which is similar to the MIC value of the extracts against this organism. Similarly, the same minimal bactericidal concentration of 2 mg/ml was recorded against *S. flexneri P.*

aeruginosa, *P. mirabilis* and *P. vulgaris*. The highest (MIC/ MBC) antibacterial activity of the extract was observed against *K. pneumoniae* and *Citrobacter* species with MIC and MBC value reached 16 mg/ml in both bacteria, followed by *E. coli* and *S. dysenteriae* with MIC of 8 mg/ml and MBC of 16 mg/ml.

Table 7b: Minimum Inhibitory (MIC) and Minimum Bactericidal Concentration (MBC) of *B. abyssinica* ethanol extract on different gram negative bacteria by agar dilution assay.

Plant Name	**Pmr.**		**Pvl.**		**Enro.**		**Ctr.**		**Acnt.**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B.abyssinica	0.5	2	1	2	8	8	16	16	1	1
Mg/ml										
Cipro. µg/ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1280	1280

Key notes: Pvl: *P. vulgaris*; Pmr: *P. mirabilis*; Enro: *Enterobacter* spp., Acnt: *Acinetobacter* spp.; Ctr: *Citrobacter* spp.; Cipro: Ciprofloxacin.

5.4.3 Fungal MICs and MFCs Results

The MIC and MFC of the most effective plant extracts (*B. abyssinica* and *X. strumarium*) in our qualitative methods were subjected to evaluate their fungi static and fungicidal properties Table 8., *X. strumarium* ethanol extract has higher potential of fungal inhibition activity against *T. mentagrophytes* and *T. rubrum* with MIC of 0.5 mg/ml, as compared to the MIC value of 1 mg/ml by *B. abyssinica* against the same fungi. However, the lowest potential of fungicidal activity was recorded by *B. abyssinica* ethanol extract against *T. rubrum* with MFC of 1 mg/ml followed by *T. mentagrophytes* with MFC of 2 mg/ml. MFC of 4 mg/ml of *X. strumarium* ethanol extract was recorded against the tested dermatophytes. Less antifungal activity was recorded by both plant extracts against *C. albicans*, *A. flavus* and *A. niger* with both MIC and MFC value greater than 16 mg/ml.

Table 8: Minimum Inhibitory (MIC) and Minimum fungicidal Concentration (MFC) of *B. abyssinica* and *X. strumarium* ethanol extract against fungal pathogen using agar dilution assay

Plant Name	**Calb.**		**Tm.**		**Trub.**		**Aflv.**		**Anig.**	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
***B. abyssinica* mg/ml**	>16	>16	1	2	1	1	>16	>16	>16	>16
***X.strumarium* mg/ml**	16	16	0.5	4	0.5	4	16	16	16	>16
Ketoconazole (µg/ml)	2	2	0.5	0.5	0.5	0.5	1	1	2	2

Key notes: Calb: *C. albicans*; Tm: *T. mentagrophytes*; Trub: *T. rubrum*; Aflv: *A. flavus*; Anig: *A. niger*.

5.5 Acute Toxicity Study

5.5.1 Toxicity Symptoms

Oral acute toxicity was conducted on extracts showing the best antimicrobial spectrum activities; and it was selected based on the results of agar well diffusions, MICs, MBCs and MFCs evaluation. In this acute toxicity study, intensive observation was conducted on the first 4 to 6 hours after administration of the extract of *B. abyssinica* then followed by 24 h then continued daily thereafter for 14 days. The observation encompasses their physical any signs of toxicity and moribund status.

As depicted in Table 9., the control group did not show any clinical signs of toxicity up to 14 days likewise, in lower doses (150-1200 mg/kg b.wt.) administered groups. However, signs of toxicity any behavioral changes, piloerection, change in breathing (gasping), writhing, diarrhea, muscle weakness (signs of paralysis) observed in few mice at higher doses of (2400-4800mg/kg). Consequently, a dose dependent increase in mortality was observed one at (2400 mg/kg b.wt.) and three mice at a dose of 4800mg/kg b.wt.

Table 9: Acute oral toxicity test of ethanolic leaves extract of *B. abyssinica* on female Swiss albino mice.

Observations	**Response**						
	Control	150 mg/kg b. wt.	300 mg/kg b. wt.	600 mg/kg b. wt.	1200 mg/kg b. wt.	2400 mg/kg b. wt.	4800 mg/kg b. wt.
Consciousness	+	+	+	+	+	+	--
Piloerection	--	--	--	--	--	+	+
Movement	+	+	+	+	+	+	--
Diarrhea	--	--	--	--	--	+	+
Urination	--	--	--	--	--	--	--
Food intake	+	+	+	+	+	--	--
Water consumption	+	+	+	+	+	--	--
Writhing	--	--	--	--	--	+	+
Moribund status	--	--	--	--	--	*	***

Key notes: + normal; − absent; * moribund status; b. wt: body weight and all values were expressed mean of 5 animals,

5.5.2 Body Weight Changes Within 14 Days After a Challenge

As revealed in Table 10., the subsequently monitoring for 14 days on those survived female mice after single oral dose extract of *B. abyssinica* administration, development of body weight changes were recorded. Subsequent body weight increment shortly before the extracts administration, then every 5th day thereafter was recorded in both control and treated groups. There was a gradual increment in body weight of the mice while, no body weight change difference observed between experimental and control group. However, very slight weight gain was observed in 4800 mg/kg treated group of mice.

Table 10: Mean n=5 body weights and weight gain of experimental mice before administration (150- 4800mg/kg) and every 5th day of acute oral toxicity study on ethanol extract *B. abyssinica* leaves.

Different experimental groups	**Body weight**	**Duration of treatment (days)**			
		Initial day	5th days	10th days	15th days
Control		147.2 gm	154.1 gm	160.4 gm	162.7 gm
	Weight gain	0	6.9 gm	13.2 gm	15.5 gm
150 mg/kg b. wt.		152.5 gm	157.7 gm	171.4 gm	172 gm
	Weight gain	0	5.2 gm	18.9	19.4 gm
300 mg/kg b. wt.		157.5 gm	163.7 gm	171.3 gm	174.8 gm
	Weight gain	0	6.2 gm	13.8 gm	17.3 gm
600 mg/kg b. wt.		154.1 gm	158.4 gm	162.4 gm	167.1 gm
	Weight gain	0	4.4 gm	8.3 gm	13 gm
1200 mg/kg b. wt.		162.2 gm	167.4 gm	171.5 gm	176.3 gm
	Weight gain	0	5.2 gm	9.3 gm	14.1 gm
2400 mg/kg b. wt.		166.7 gm	137.4 gm	143.9 gm	150.0 gm
	Weight gain	0	5.05 gm	10.6 gm	16.6 gm
4800 mg/kg b. wt.		165.1 gm	68.7 gm	71 gm	71.2 gm
	Weight gain	0	2.7 gm	5 gm	5.2 gm

Key notes: b. wt: body weight and all values were expressed mean of 5 animals

5.5.3 LD50 of the Crude Extract

The data were analyzed statistically by log dose/probit regression line method. Regression line was drawn on the basis of two variables, log dose and empirical probit on a simple graph paper. The test doses were then converted to their logarithm. The empirical probit values, equivalent to percentage mortality were then obtained from the table and plotted against log dose on graph paper. The provisional lines, drawn fitting to the expected values, were read in the values of log dose (X). Used to determine the expected probit necessary for LD50 determination. Therefore, LD50 determination is necessary to establish the scientific basis for

the therapeutic actions of traditional plant medicines as these may serve as the source for the development of more effective drugs.

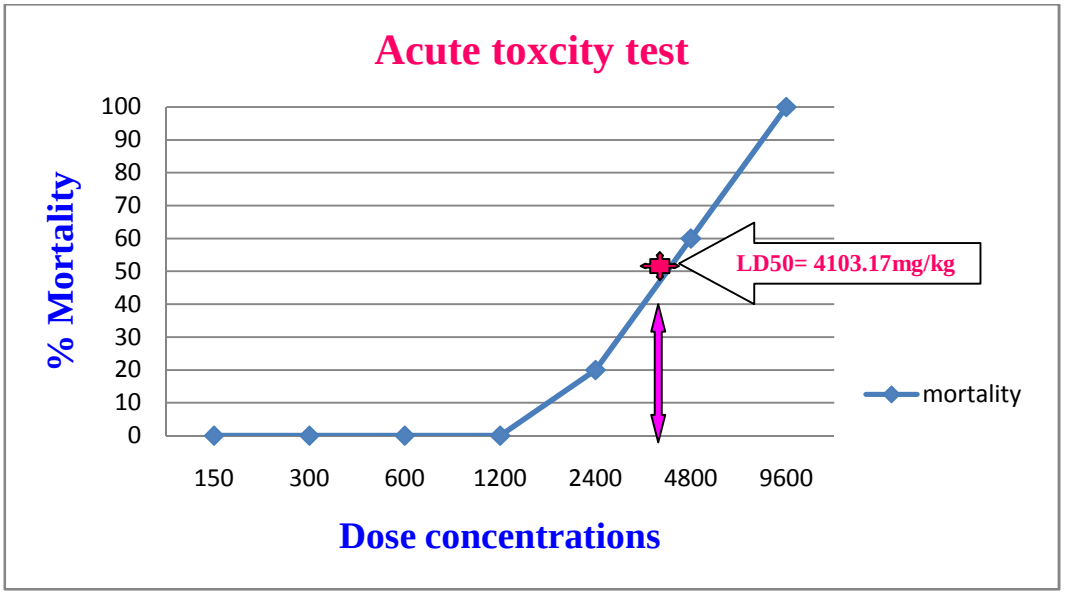

Figure 5: Probit Transformed Dose response of *B. abyssinica* % Mortality (LD50) (4103.175 mg/kg)

6 DISCUSSION

The increasing prevalence of antimicrobial resistance, cost and side effects of the currently existing antimicrobial agents have initiated academic institutions and pharmaceutical companies looking for new, better active, less toxic and cheaper antimicrobial agents [132]. Medicinal plants are one of the important sources of new biologically active compounds. Earlier many research conducted all over the world demonstrated that medicinal plants have antibacterial, antifungal, antiviral, anthelmintic, anticancer, antidiabetic, antioxidant and anti-inflammatory properties [133,134]. Given that, Ethiopia is well known as one of the six biodiversity-rich countries of Africa and one of the most diverse floristic regions in the world as well [21, 26].

A search for biological active compounds from medicinal plants is an active field of research. Therefore, in the present study 12 traditionally used Ethiopian medicinal plants (*Bersama abyssinica Fresen., Xanthium strumarium L, Rhus natalensis., Zehneria scabra., Ehretia cymosa, Clutia abyssinica., Stephania abyssinica., Acmella uliginosa Del., Urtica simensis, Echinops kebericho, Ajuga integerifolia, Artemisia abyssinica.*) have been screened for their antibacterial and antifungal activities. Crude leaves extracts of the majority of medicinal plants have shown potent antimicrobial activity against a variety Gram -positive and Gram-negative bacteria as well as yeast and dermatophytic fungi.

6.1 Effects of B. abyssinica Extract on Gram Positive Bacteria

Among the medicinal plants screened, to meet our objectives and selecting one plant with the best and potent broad spectrum activity. Ethanolic leaves extract of *B. abyssinica* was the most active against the majority (86%) of bacterial and fungal test organisms. It is one of the best plants with remarkable broad spectrum antimicrobial activity, producing 9.67 mm diameter zone of inhibition against *K. pneumoniae* to 42.00 mm diameter zone of inhibition against *T. mentagrophytes.* However, crude extract of this plant was less active against *A. flavus, A. niger* and *C. albican.* The zone of inhibition diameter of crude extract of the plant at a concentration of 200 mg/ml against MRSA, *E. faecalis, S. aureus* and *S. agalactiae* was 27.00 ± 0.00, 26.17 ± 0.40, 25.33 ± 0.21 and 20.00 ± 0.45 mm) respectively. The inhibition zone diameter produced by the extract were significantly greater inhibition zones with ($p<0.05$) than produced by standard drug Erythromycin at a concentration 15 µg (25.00 ± 0.00, 15.00± 0.00 mm) against MRSA and *E. faecalis* respectively.

However, the activity of the extract against *S. pyogenes* and *S. pneumoniae* were moderate (18.00 ± 0.00 and 15.50 ± 0.22 mm). The results of our study revealed that *E. faecalis* was susceptible to Ciprofloxacin, intermediately with Erythromycin (25 and 15.00 mm) zone of inhibition and resistant to standard antibiotics Gentamicin, Vancomycin, and Ceftriaxone with a diameter zone of inhibition 10 to 11 mm. Intrinsic and acquired antibiotic resistance, their survival in adverse environmental conditions, high heat tolerance to 45^0c, has made the bacterium resistant to many of the existing drugs. The pathogen has remained a major cause of nosocomial infections [135]. Fortunately, in our study, significantly best result (26.17 ± 0.40 mm) zone of inhibition was obtained by this crude plant extract with ($p<0.05$). Therefore, the cured extract of *B. abyssinica* could be a potential source to develop a drug that is better active against the bacterium.

With regard to quantitative assay, the MIC and MBC of crude extract obtained from *B. abyssinica* was evaluated using the agar dilution method against different test organisms. The MIC values against *S. aureus*, MRSA, *E. faecalis, S. pneumoniae*, *S. pyogenes*, and *S. agalactiae* were 0.25, 0.25, 2, 4, 4, 16 mg/ml, respectively, while with MBC values were 1, 2, 4, 8, 16, 16 mg/ml respectively. The best antibacterial activity with (0.25 mg/ml or 250µg/ml) of MICs and (1 and 2 mg/ml) MBCs were observed against *S. aureus* and MRSA. Moreover, moderate antibacterial activities with MIC (2, 4, 4 mg/ml) were seen against *E. faecalis, S. pyogenes*, *S. pneumoniae.* Whereas, the highest MBCs (8, 16, 16 mg/ml) were observed against *S. pneumoniae*, *S. pyogenes* and *S. agalactiae.* This finding is in agreement with Tanzania study [136], on leaf methanol extract resulted with MICs 1.56 mg/ml on *S. aureus, S. pyogenes*. The MIC values in most cases lower than or equal to MBC values, suggesting that the plant's crude extract have bacteriostatic effects at low concentrations and bactericidal at high concentrations [137]. The MBC/MIC ration is possible to specify the modality of a substance. If the MBC/MIC is ≤ 2, the substance is said to be bactericidal and MBC/MIC ≥ 4, the substance is said to be bacteriostatic effect [90, 138].

This research is supported by previous works in Côte D'Ivoire, [90] on an aqueous stem bark extract of *B. abyssinica* against *S. aureus* ATCC 25923 and MRSA using agar diffusion and a broth microdilution method revealed that, a maximum inhibition of (15.1±0.1 and 18.0±0.3 mm) at 100 mg/ml concentration. At the same time, (1.563, 1.563 mg/ml) MICs and MBCs against *S.*

aureus and (0.195, 1.563mg/ml) against MRSA were observed, respectively. Likewise, a study done in Ethiopia, by Mathewos on methanol leaves extract of this plant, resulted 16 mm inhibition on *S. aureus* [139]. Their results were much lower than ours findings (25.33±0.21, and 27.00±0.00 mm) inhibition with (0.25, 1 mg/ml) MIC, (0.25, 2 mg/ml) MBCs observed on similar test organisms, respectively. However, in both studies better inhibition results were observed against MRSA than *S. aureus* ATCC. Repeated experiments were performed by other studies on different plants spp., the exact reason why larger zone of inhibition to MRSA compared to *S. aureus* is not known [132, 137]. However, it might be antibiotic resistance does not interfere with the antibacterial action of plant extracts. Moreover, these extracts might have different modes of action on the test organisms [140, 141].

In another three studies conducted in Ethiopia 2014 and 2015 by Lulekal E, and Teka A, on root and seed ethanol extracts to determine MIC, by using a broth microdilution method, no observable effects were seen against ATCC *S. aureus*, *S. pyogenes*, *E. faecalis* at (512 to 4 µg/ml) test concentrations [101, 142]. Likewise, Geyid et al., [100] study at (500- 2000 µg/ml) root bark extracts by agar dilution method, no observable effects were seen on *S. pyogenes* and *S. pneumonia* except on *S. aureus*.

The possible explanation for the differences noticed by earlier and our studies could have been resulted from difference in the methodologies used for the determination of MIC/MBC, parts the plant, geographical and seasonal variation where the plant material was collected, organic solvent and extraction procedure, as well as concentration of crude extracts and strains of test bacteria used. Since, organic solvent like ethanol, methanol is best solvent for extracting bio-active antimicrobial compounds from medicinal plant than aqueous extraction. Studies have shown that, most antimicrobial compounds have polar characteristics and beastly extracted by polar hydroalcoholic extraction solvents than the non-polar extracting solvents like hexane, benzene [121, 137, 141].

6.2 Effects of *B. abyssinica* Extract on Gram Negative Bacteria

The activity of *B. abyssinica* extract against gram- negative bacteria were variable. The extract was more active against *S. flexneri* with an inhibition diameter zone of (25.33 ± 0.21 mm) but it was less active against *K. pneumonia* with a diameter zone of inhibition of (9.83 ± 0.17 mm) at 200 mg/ml extract concentration. Moreover, the extract inhibited the growths of an array of pathogenic bacteria such as *P. aeruginosa*, *P. mirabilis, Acinetobacter spp., P. vulgaris, S. typhimurium* and *S. dysenteriae* producing 22. 20 mm, 21. 00 mm, 21. 67 mm, 19. 50 mm, 16. 5 mm and 14. 5 mm diameter zone of inhibition, respectively. In Mathewos A. *et al.,* 2015 study in Ethiopian, on methanol leaves extract of this plant, by disc diffusion method at 100 mg/ml resulted in (15, 14 and 13 mm) inhibition of *S. typhi, E. coli* and *K. pneumoniae* [139].

In similar other study in Côte D'Ivoire [98] on root ethanol extract of this plant with macrobroth dilution, resulted in (10, 20 mg/ml) MIC and MBC values against *S. typhi, S. typhimurium.* While, (2.5, 2.5 mg/ml) on *P. aeruginosa* respectively. Their finding was relatively lower than the current study (8 mg/ml) both MIC and MBC against *S. typhimurium* while, (1, 2 mg/ml) on *P. aeruginosa* respectively. A study in Tanzania, resulted in 0.78 mg/ml MIC on *E. coli, S. flexneri* and *K. pneumonia* with micro broth dilution method and 1.56 on *P. aeruginosa* [136].

In the previous three Ethiopian studies, [100, 101, 142]., no observable effects were seen against *E. coli, P. aeruginosa* at (512 to 4 µg/ml) test concentration. Fortunately, in our study result better MICs (1 mg/ml) was seen against *P. aeruginosa* and comparable (1 mg/ml) on *S. flexneri* and lower (4 to 16 mg/ml) on *E. coli* and *K. Pneumonia* was seen. The possible reason for the difference might be the method, test concentration used, the plant material and environmental variation. Moreover, the leaves of this plant contain higher amount and various kinds of phytocompounds than the seed and root bark [92].

Previous studies have been reported that, extracts of this plant material posses various kinds of bioactive compounds which is responsible for its therapeutic and pharmacological applications. Phytochemical analysis conducted in Ethiopia revealed, leaves of *B. abyssinica* confirmed to have secondary metabolites like alkaloids, flavonoids, glycosides, phenols, tannins, coumarin, anthraquinones, triterpenoids, phytosterols, and sterols' [139]. Moreover, study conducted in Tanzania (Arusha), using Gas chromatography coupled with mass spectrometer (GC-MS) for the analysis of constituents compounds *B. abyssinica*. A total of 24 phytocompounds were identified

from the leaves, 21 compounds from stem bark and 19 from root barks. Compounds like, terpene alcohol (3,7,11,15-Tetramethyl-2-hexadecen-ol), alcoholic compound (7,8-Epoxylanostan-11-ol,3-acetoxy), Pyrogallol (1,2,3-Benzenetriol), fatty acid (Capric ether and 2,3-Dimethylfumaric acid), Aldehyde (2-furancarboxaldehyde,5-methyl), Steroid (Ethyl iso-allocholate) were some of the compound confirmed antimicrobial [91, 99]. Plants containing alkaloids, flavonoids, terpenoids, steroids, Xanthone, mangiferin, phenolic and polyphenols, compounds have been reported to have highly anti-microbial activity [100, 121, 143]. The other important subclass groups of compounds have been known to be synthesized by plants in response to microbial infection found to have antimicrobial activity include phenolic acids, quinones, flavones, flavonols. Hence, this is the main basis for *in vitro*, to be effective against a wide array of microorganisms [121].

Fortunately, the current study demonstrates that, gram-negative bacteria such as *S. flexneri, P. aeruginosa, P. mirabilis, Acinetobacter, P. vulgaris, S. typhimurium* were very susceptible gram negative bacterial specious with 25.33 mm to 16.50 mm growth inhibition and with MICs, MBCs 0.5 mg/ml to 16 mg/ml were observed in this experimental study by crude extract *B. abyssinica* leaves. Hence, these could be the best possible evidence for explaining the potency of this medicinal plant for further in-depth investigation to isolate and develop promising antimicrobial phytomedicine.

6.3 Antimicrobial Effects of *Rhus natalensis*

R. natalensis was the other effective plant extract with the broad inhibitory activity of all gram positive bacteria with 13 to 22 mm range of inhibition zone. Likewise, it is also the second most active plant extract in inhibiting the growth of all gram negative bacteria 10.50 to 16.83 mm at 200 mg/ml test concentration. In Kenyan study [144]., using disc diffusion method revealed an inhibition of 9.7 and 12.0 mm against *E. coli* and *S. aureus* respectively, of the root juice the same plant. Likewise, a study in Western Cape of South Africa [145]., on methanol extract using the agar diffusion method resulted with inhibition of 6, 14, 17 mm against *E. coli, P. aeruginosa, S. aureus*, and 10, 7 mm against *T. mentagrophytes, C. albicans* respectively. However, far better results were obtained on similar test organisms with 12.17, 19.50, 16.83 mm and 15.5 mm inhibition zones, respectively. To the best of my knowledge, no previous studies were conducted

in Ethiopia to demonstrate efficacy and the broad spectrum antimicrobial activities of this promising plant extracts of *R. natalensis*.

6.4 Antimicrobial Effects of *X. strumarium*

Based on the findings of the current study, *X. strumarium* leaves crude extract was the other effective medicinal plant against gram positive bacteria than gram negative with 23.67, 26.00, 26.50, 19.00, 15.50, 9.00 mm growth inhibition zones against *S. aureus*, MRSA, *S. pneumoniae, S. pyogenes, E. faecalis* and *S. agalactiae,* respectively. This result is in agreement with Hassan et al., study [110]., in Ontario, Canada. No observable effects were seen against *E. coli* by the leaves ethanol extracts and with a similar method. However, best results were depicted on other gram positive bacteria which is not covered in the present study, as *B. cereus, M. avium*. On the other study conducted in Ethiopia by Belay and Kinde [111, 112]; using leaf ethanol extract of *X. strumarium* at 10% concentration using agar disc diffusion methods. Their results depict that, 20.67 and 21.33 mm mean zone of inhibition, on *S. aureus* and *S. agalactiae* isolated from cows bovine mastitis respectively.

However, disagreement was seen with the India study, [109], 100 mg/ml crude methanolic extract resulted 12 to 15 mm inhibition against *P. vulgaris*, *P. aeruginosa* and *K. pneumoniae* by using, disc-diffusion method. In Bangladesh study, [108] on leaf chloroform extracts of this plant at 50 mg/ml concentration, with disc diffusion method resulted in 14 mm inhibition on *S. aureus* and *S. dysenteriae*, 9 mm on *E. coli*, 25 mm against *A. niger* and 20 mm on *C. albicans* were depicted. In the current study relatively better outcome was seen on *S. aureus* 23.67 mm and other gram positive bacteria and 15.5 mm on *C. albicans.* While, no inhibitory effects on gram negative at 100 and 200 mg/ml test concentrations. The difference might be the method, microbial strain used and the environment, where the plants grow and collected. Consequently, the observed antibacterial effects of this plant extracts associated with the presence of major phytochemicals compounds such as xanthol, xanthenin, xanthanolides, alkaloids, flavonoids, sesquiterpenoids tannins, saponins and phenolic acids is proved to be a potential source active compounds.

6.5 Antimicrobial Effects of *Z. scabra, E. cymosa, S. abyssinica*

In the current study, *Z. scabra, E. cymosa, S. abyssinica* and *C. abyssinica,* were the other effective plant extracts with range of growth inhibitions of 8.50 to 19.50 mm were obtained by these plant extracts at 100 and 200 mg/ml as depicted in Table 4a and 4b. Whereas, less to non effective against *S. dysentery, S. typhimurium, K. pneumoniae*, *Enterobacter, Citrobacter.* However, disagreement was seen with Geyid et al., study [100] in Ethiopia, where there were no noticeable effects were seen in their study. Likewise, Lulekal et al., [101] report no observable effects were seen by *Z. scabra* and *C. abyssinica* on *E. faecalis, S. aureus, S. pyogenes, E. coli, P. aeruginosa* and *C. albicans* at (512 to 4 µg/ml) test concentrations except, *C. abyssinica* against *S. pyogenes, C. albicans* and *Z. scabra* against *C. albicans* at 512 µg/ml. The present study on *C. abyssinica* agree with Kenyan Korir et al., [146] study on, *P. aeruginosa, K. pneumonia* and *C. albicans, T. mentagrophytes*. But, disagree about, MRSA, *S. aureus*, *E. coli,* and on methane extracts using disk diffusion method.

The possible explanation for this discrepancy in results may be the concentration they choose to test MIC and the methods (microdilution broth). Activities *E. cymosa* crude extract, was also considered as a relatively the best antimicrobial plant for *K. pneumonia* with 11.5 mm followed by *R. natalensis* 10.5 mm and *B. abyssinica* 9.67 mm at 200 mg/ml test concentration. Besides, this extract also the third possible choice for *S. aureus*, *P. mirabilis, P. vulgaris*, *S. flexneri, S. typhimurium, Enterobacter, Acinetobacter, Citrobacter spp.* 22 to 9.17 mm inhibition zones and only in effective to *S. pyogenes, S. pneumoniae, E. faecalis, E. coli* and *S. dysentery* at 200 mg/ml.

As we observed from the current investigation, gram positive bacteria were relatively more susceptible by the selected plants species than gram negative bacteria such as, *E. coli, K. pneumonia, Citrobacter* and *Enterobacter* spp., which were less susceptible bacterial species. Likewise, in the previous different research gram-negative bacteria are frequently having developed multi drug resistance to many of the antibiotics currently available in the market of which *E. coli, K. pneumonia, P. aeruginosa* were the most prominent [142]. The main reason raised for the difference in sensitivity, between gram-negative and gram positive bacteria is the structure of the cell wall. Since, gram-negative bacteria possess envelope, consisting of three principal layers: outer membrane lipopolysaccharide/endotoxin, which is absent on gram

positive, serve as a permeability barrier, excluding certain antimicrobial agents from penetrating and retaining in the cell. It is also the main contributing factor to the intrinsic antibiotic resistances observed in Gram-negative bacteria [56, 141].

6.6 Antifungal Activities

In the present investigation among the selected 12 plant species involved in screening for antimicrobial potential against the six fungal pathogens, the leaves of *X. strumarium* and *B. abyssinica* have showed the most potent antifungal activity. Maximum growth inhibition (46, 48 mm) at 200 mg/ml concentrations of *X. strumarium* against *T. mentagrophytes* and *T. rubrum.* While; 42 mm and 35 mm by the crude extracts of *B. abyssinica* were obtained on both dermatophyte respectively. Similarly, minimum results 15 mm and 11 mm against *C. albicans* and *A. flavus* were obtained by *X. strumarium* ethanol extract.

Relatively minimum antifungal activity (17.50, 15.50 mm) growth inhibition zones were observed by *R. natalensis* against *T. rubrum* and *T. mentagrophytes.* The results obtained by both plant extracts, particularly on the above two dermatophytes were very significant compared to that of the antifungal standard drug Ketoconazole (38 mm, 32 mm) inhibition zones on *T. mentagrophytes, T. rubrum, A. flavus*. Unfortunately, all the plant extracts tested in this experiment did not show any growth inhibitory effects on *A. niger* at 100 and 200 mg/ml test concentrations. This finding in agreement with the previous study in Ethiopia by Hailu et al., [147] *A. niger* was insensitive to all plants species they selected and tested. To the best of our knowledge, no previous studies have been published on the antifungal activity of *B. abyssinica* against the two dermatophytes, *T. mentagrophytes* and *T. rubrum.* Accordingly, the on the base the current result showed this medicinal plant extract is the best candidate option and very promising for the discovery of antimicrobial medicine in the future especially for these dermatophytes.

A similar study conducted in Bangalore indicate, [148] using leaf ethanol extract of *X. strumarium* against *T. rubrum* and *T. mentagrophytes*, *showed* susceptibility at 20000 µg/ml MIC and 40000 µg/ml MFC by employing broth dilution methods. Their finding was in agreement with the current study. However, best results were achieved in our findings (0.5 mg/ml and 4 mg/ml), which is equivalent to 500µg/ml MIC, 4000 µg/ml MFC on both dermatophyte respectively. Previous study depicts that, a compound like Xanthatin, Cinnamic

acid can be isolated from *X. strumarium* extracts have contributed to the antimicrobial property [104].

The observed strong antifungal activities of *X. strumarium, B. abyssinica* and *R. natalensis* against *T. mentagrophytes* and *T. rubrum, A. flavus, C. albicans* correlates with different ethnobotanical reports. Their traditional practice of the local people to treat different ailments such as wound, skin infection, scabies, dandruff, dysentery and others [87, 142, 149]. In the investigation of new antifungal agents, low toxicity and broad spectrum fungicidal activities are needed for effective infections management [150, 142].

6.7 Acute Toxicity Studies

As the WHO estimates, about 80% of people living in developing countries rely almost exclusively on traditional medicine for their primary health care needs. Medicinal plants are the "backbone" of traditional medicine, which means more than 3.3 billion people utilize medicinal plants on a regular basis [151]. Even though, medicinal plants are widely accepted, in usages and assumed to be safe, however, it can potentially be toxic [152]. The organization for economic co-operation and development (OECD) panel of experts has defined acute toxicity, "the adverse effect occurring within a short time of (oral) administration of a single dose of a substance or multiple doses given within a span of 24 hours [126]. The objectives of the current acute toxicity studies is to determine the LD_{50} values which help in determining and estimating the safe dose range at which leave extracts of *B. abyssinica* can be used without any harm or lethal effect. Generally, human toxicity is estimated based on test results on rats and other animal models [123].

In this acute toxicity study, intensive observation was conducted on the first 4 to 6 hours after administration of the extract *B. abyssinica* then followed by 24 h then continued daily thereafter for 14 days. The observation encompasses their physical and any moribund status condition during the 24 h period and within 14 days.

As depicted in Table 9, the control group did not show any clinical signs of toxicity up to 14 days likewise, in the lower doses (150-1200 mg/kg) administered groups. Whereas, at higher doses of (2400-4800 mg/kg), some signs of toxicity like piloerection, change in breathing (gasping), (writhing) like signs of abdominal cramping, diarrhea, muscle weakness (signs of paralysis) observed in few mice. These signs of toxicity were in agreement with previous studies

done in Ethiopia on a hydroalcoholic root bark extract of *B. abyssinica* resulted with LD50 of 5044 mg/kg body weight [95]. However, in our case dose dependent increase in mortality was observed at a dose range (2400-4800 mg/kg) one mouse at (2400 mg/kg) and three mice at a dose of (4800 mg/kg) with an LD50 (4103.2 mg/kg) body weight. This discrepancy in LD50 value might be due to the difference in parts of the plant and animal sex types used in our study. Since females are generally slightly more sensitive to chemical exposure and the preferred and recommended species by OECD than male [126].

In the principle toxicity categorization of Hodge and Sterner, [96, 153] LD50 extremely toxic = < 1 mg/kg; highly toxic = 1–50 mg/kg; moderately toxic = 50–500 mg/kg; slightly toxic = 500–5000 mg/kg; practically nontoxic= 5000–15,000 mg/kg; relatively harmless = > 15,000 mg/kg. Accordingly, leaves of *B. abyssinica* extract is classified as slightly toxic medicinal plant.

All the surviving mice were also monitored daily 14 days for recovery and existence of weight gain by recording shortly before administration of the extracts, then every 5th day thereafter. As displayed in Table 10, the extracts did not interfere with food, water intake and the normal metabolism of animals that is why no observable effect on their growth throughout the duration of the study period on the lower dose challenged groups.

The subsequent body weight increment was seen in both control and treated groups, while there was no significant body weight change observed between experimental and control groups. However, very slight weight gain was observed in the two survived mice in 4800 mg/kg treated group. Since, observable changes on both food and water consumption have been used as indicators of adverse effects of drugs and chemicals. Similarly, According to previous studies, after some exposure to potentially toxic substances, slight body weight reduction is expected [154]. Hence, in this preliminary acute toxicity study on extract of *B. abyssinica*, suggests the need for further more studies to clarify the toxicity for the benefits of humans and animals uses.

7 STRENGTH AND LIMITATION

7.1 Strength of the Study

1. This study tried to cover and investigate on 22 pathogenic organisms, their spectrum of antibacterial and antifungal activity of the selected medicinal plants.

2. The other strength of this study isolated best plant for further in-depth study for the discovery of new medicinal products, even for drug resistance bacteria like MRSA, Acinetobacter spp, dermatophytes *T. mentagrophytes* and *T. rubrum*

7.2 Limitation of the Study

- The study has a complete discussion for the beast efficacies plant extract of the screened plant species. Since all the experiments were performed based on the objectives following standards SOPs guidelines and different controls, there was no bereaved limitation in this assay.

8 CONCLUSIONS

Our study demonstrated broad spectrum antimicrobial activity of 12 Ethiopian traditionally used medicinal plants against pathogenic bacteria and fungi, proving traditional claim. The study offers a preliminary scientific information and could be the basis for the traditional use of these plants. This result can pave the way towards the move for the discovery of new efficacious, less toxic and inexpensive plant based medicines used for controlling pathogenic microorganisms. Moreover, it might give the answer to the currently existing and newly emerging fear of multidrug resistance pathogenic micro-organisms.

Moreover, our findings have indicated that *B. abyssinica, X. strumarium, R. natalensis* were the most potent plants out of the selected medicinal plants. These plants can be the best candidate for further studies in the development of new antimicrobial agent, following isolation and characterization of active compounds from the plants which can serve as leading compounds in antimicrobial drug development for the treatments of bacterial and fungal origins.

9 RECOMMENDATION

- Similar studies should be conducted on other resistant clinical isolate pathogenic bacterial and fungal test organism
- *In-vivo* further studies should be conducted on animal model to have levels of efficacy, margin of safety, for the benefits of producing commercially available products.
- In-vitro antimicrobial studies should be conducted on isolated, characterized and formulated bioactive compounds of these plants like *B. abyssinica* in order to maximize its efficacy towards the discovery of new potent antibiotics.
- Based on this primarily acute toxicity study results of *B. abyssinica*, sub chronic and chronic toxicity study should be conducted for further usage.
- Strong activity obtained by *X. strumarium* against gram-positive bacteria and fungal species could make this plant one of the best candidate for further studies for the discoveries potent antimicrobial compound.
- Future studies should also focus on the mechanism of action of those crude plant extracts and isolated compounds.

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ANNEXES

Annex I: Standard Operating procedures (SOPs)

CLSI, OECD, SOPs of TMMDR were strictly followed for the analysis of different culturing and testing of antimicrobial activates employed for medicinal plant extrications and for acute toxicity study's using animal models [116,120, 126].

1. SOP for Extraction of Medicinal Plants Using Cold Organic Solvents (Maceration)

Purpose: - This procedure provides instructions how to separate the components with medicinal properties that can be dissolved in organic solvents.

Materials

Reagents: Organic solvents (alcohol, Chloroform, Hexane, pet ether etc…)

Reagent preparation: Take the appropriate amount of the solvent and dilute with distilled water if required.

Supplies: Required for the extraction of medicinal plant

Filter paper, Volumetric flasks (1000 ml), Beakers (250 ml), Funnels, Spatula, Erlenmeyer Flasks (500 ml), Measuring cylinder (500ml), Plastic containers, Glass rod, Washing bottles Evaporating flasks (1000ml).

Equipment: Analytical Balance, Water bath, Refrigerator, Rotary evaporator, Shaker

Principle

In the extraction practice for articles of botanical origin, the constituents of interest are completely or partially separated from other components with the aid of water, alcohol, alcohol-water mixtures or other suitable solvents. This extraction process involves the removal of the desired constituents from the plant matter with suitable solvents, the evaporation of all or nearly all of the solvent and the adjustment of the residual fluids, masses, or powders to the prescribed standards. Extracts may be subjected to processes that increase the content to characterize constituents, decrease the content of unwanted constituents, or both. Extracts may be defined as preparations with liquid, solid, or semisolid consistency [114, 115, 155].

Clinical Utility: the extraction of biologically active compounds from natural products that can be used interchangeably as drugs or as supplements.

Procedure:

Step	Action
1	Weigh the required amount of plant material using beam balance
2	Put the weighed plant material in an extraction flask
3	Soak the plant material with the appropriate amount of cold organic solvent [1:10]
4	Put the soaked material on a shaker and shake at the speed of 100rpm for 72hrs
5	Stand the soaked or macerated plant sample until it settles
6	Decant and then filter the supernatant using Whatman No 1 filter paper
7	Concentrate the filtrate to extract using the rotary evaporator
8	Pour the extracted solution in a labeled container and put in the a water bath ($40^{O}C$)
9	After the extract is dried, calculate the weight percentage and place it in a refrigerator

SOP- 2

2). Acute Oral Toxicity Test

Purpose: This procedure provides instruction how to perform acute oral toxicity test in mice.

Materials and Reagents: - Diluting solution

Supplies: Gastric feeding needles (gavages), Glove, Distilled water, Detergent, Alcohol Beaker 25, 50, 100, 250mL.

Sample:

Sample type	Amount required	Transport and Storage	Stability
Plant extract	1ml/100g of body weight:	At room temp	none applicable

Principle: Acute toxicity studies in animals are usually necessary for all medicinal plants intended for human use. The information obtained from these studies is useful in choosing doses for a repeated-dose studies, providing preliminary identification of target organs of toxicity, and occasionally revealing delayed toxicity.

Procedure:

Step	Action
1	Use female Albino mice, weighting 28-30g.
2	Acclimatize the animals with the working environment.
3	Divide the mice's randomly into groups based and body weight (five mice in each group).
4	Withdraw food, but not water, for 3-4 hours prior to the experiment
5	Administer the diluting solution for one group as a negative control.
6	For the other groups administer the plant extract orally via gavage as required dose (Graded dose).
7	Observe signs of toxicity for the first two hours and in two hours interval for six hours.
8	Observe for mortality, if any, after 24 hours and for 14 days after administration

Procedure Note:

- ❖ The use of vehicle control groups should be considered. For compounds with low toxicity, the maximum feasible dose (5000-10000 mg/kg) should be administered.
- ❖ Animals should be observed for 14 days after administration. All mortalities, clinical signs, time of onset, duration, and reversibility of toxicity should be recorded.

Calculation: Determine LD50

Result Interpretation

- Response data and dose level for each animal (i.e. Animals showing signs of toxicity including mortality, severity and duration of effects)
- Individual weights of animals at the day of dosing, at every fifth intervals thereafter, and at time of death or sacrifice**.**
- Date and time of death if prior to scheduled sacrifice.
- The time course of onset of signs of toxicity and whether these were reversible for

Clinical Utility

Acute toxicity studies used to have a preliminary safety information of a substance as well as for conducting pharmacological experiment such as anti-microbial, (bacterial, malarial, anti-helmentic) experiment intended for drug discovery.etc…

The information obtained from these studies is useful in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity and occasionally, revealing delayed toxicity [126].

3). SOP- for the Determination for MIC

Principle: Agar plates, with twofold dilutions of the extracts and antibiotics are inoculated with a standardized inoculum of the bacteria and fungus incubated under standardized conditions following CLSI guidelines. The next day, the MIC is recorded as the lowest concentration of antimicrobial agent with no visible growth after overnight incubation. MIC-determination performed as agar dilution is regarded as the gold standard for susceptibility testing.

Purpose: In drug discovery programs the first step is often a screening of candidates' antimicrobial agents for MICs against microbial interest. The MICs are usually the starting point for evaluations [116, 117].

Material and Instruments:

No	Materials
1	Mueller Hinton Broth (MHB), Mueller Hinton Agar
2	Nutrient Agar (NA), SD -Agar
3	Sterile 20 ml screw cap test tubes, standard organism and antibiotics
4	250 ml conical flask, 250 ml measuring cylinder
5	Inoculating loop, 1 ml and 0.1 ml micro pipette
6	10 ml glass pipette, Beaker, Marker, Bunsen burner, Borer
7	Small and large petri plate, Vortex mixture, Shaking, Micropipette
8	Electrical digital balance, Spatula, pH meter, Spectrophotometer
9	Incubator adjuster at 37^0C, and 25^0c, BSC II, Autoclave

Procedure:

Step	Action
1	Prepare the stock solutions 10x the concentrations to be tested and make twofold serial dilutions of these stock solutions.
2	Add the antimicrobial solutions to a molten MHA agar at 50ºC.
3	Allow the agar to solidify

4	Inoculate the strains to be tested in MHB to make a suspension with turbidity equivalent to 0.5 McFarland standard and standardized it.
5	Using 2µl loop, inoculate a [10^4CFU/spot] of the suspension on plates of different concentrations of antibiotics starting from low concentration.
6	Incubate the plates at 35ºC and 25 ºC for 24 hours.
7	Examine the plates, both in reflected and transmitted light.
8	The lowest concentration on which the strain showed no growth is taken as the minimum inhibitory concentration for that strain.

4). SOP- for the Determination of MBC

Principle: The MBC test determines the lowest concentration at which an antimicrobial agent kills a particular microorganism. The MBC is determined using a series of steps, undertaken after a MIC test has been completed.

Purpose: MBC testing is useful for comparing the germ-killing activity of several antimicrobial agents. The MBC test can be a good and relatively inexpensive tool to rank a great number of antimicrobial agents by potency, for screening purposes [113, 121].

Step	**Action**
1	The agar plate was incubated at 37^0C for 24 hours.
2	Which is bacteriostatic for the test organism in the MIC.
3	The agar plate was examined for visible growth (cloudy)
4	For determination of MBC, the concentration which was bactericidal
5	Subculture the test organism from the MCI plate
6	Then inoculate into the antibiotic/extract free Mueller Hinton agar
7	The plates will be incubated at 37^0C for 24 hours.
8	Plates that did not show any growth considered to be the MBC for the crude extract used

Annexes II.

All medicinal plants which claimed of having antibacterial and antifungal properties, were collected from different parts of Ethiopia from March 03/2017 to 05/04/2017. Leaves of *Stephania abyssinica* and *Clutia abyssinica* from Oromia region Bale Zone around 430 km Southeastern part of Ethiopia and Leaves of *Rhus natalensis* and *Xanthium strumarium* from Shashamane, 240 km from the capital of Addis Ababa. Leaves of *Zehneria scabra,* and roots of *Urtica simensis* from Amhara region (North Shewa Zone Ankober district) which is 172 km northeast of the capital Addis Ababa and 46 km away from Debre Brihan town. Leaves of the leaves of *Acmella uliginosa* from Southern Ethiopia Wondo Genet, around 265 km, *Ehretia cymosa* from Mierab Abaya Woreda around Arba Minch 495 km away from Addis Ababa. Leaves of *Bersama abyssinica*, *Echinops kebericho*, *Ajuga integerifolia*, *Artemisia abyssinica* were collected from the botanical garden of Ethiopian Public Health Institute (EPHI) Traditional and Modern Medicine Research Directorate (TMMRD) Addis Ababa.

Antimicrobial effects of *Ajuga Integerifolia, Artemisia Abyssinica*

Antimicrobial efficacy *A. integerifolia* and *A. abyssinica* were found to have moderate antibacterial and fungal activity as demonstrated in the **Annex II, Tables (1-3).** When we see the antimicrobial activity of *A. abyssinica* this plant extract also effective against gram positive bacteria with growth inhibition 12.5±0.55 mm on *S. aureus* and 10 mm on both *S. agalactiae, S. pneumoniae* and 14 mm MRSA no effect on *S. pyogenes*. In addition, this plant extract has good activity on gram negative bacteria test organisms 11 mm and 10.5 mm *S. flexneri, P. aeruginosa*, 16.5 mm on *P. mirabilis*, 13 mm against *Acinetobacter spp*., were seen at 200 mg/ml test extracts concentration. This result is in agreement with Oskay M et al., study on *A. arborescens* with similar method resulted 15 mm MRSA, 20 mm on *S. pneumoniae, no on E. coli* but, differ *P. aeruginosa* on effects and 20 mm *S. pyogenes K. pneumonia* 12 mm [140]. Whereas, ***A. integerifolia*** also effective with 15.5 ±0. 55 mm inhibition on both *S. aureus,* MRSA and *S. agalactiae* and 10.67 mm on *Acinetobacter.* It also have anti fungal activity of 11.5 ± 0.55mm *T. rubrum*.

Antimicrobial Effects of *Echinops kebericho, Acmella uliginosa*

Likewise, *E. kebericho* extract was also effective against *S. aureus,* MRSA with 11.5 and 19.0 inhibition zones and 17 ±0. 55 mm on *S. fluxionary*, 10.67 mm on *P. aeruginosa*, 13.33± 2.58 mm *P. mirabilis*, 11 mm on *P.* vulgaris were seen at 200 mg/ ml test concentration. *A. uliginosa,* crude extract has a promising growth inhibitory effect 10.5 mm on MRSA and *Acinetobacter*. While, (14.5 ±0. 55, 12.33 ± 0.52 mm) against *T. mentagrophytes*, *T. rubrum* at 200 mg/ml. *U. simensis* crude extract also have antimicrobial effect (10.5 mm) and 9 mm on MRSA and *Acinetobacter* at 200 mg/ml which is the least effective plant species in this study.

Table 1. Group mean Results of all the test plants against Gram positive Bacterial test organisms all from excel mean result at 100 and 200mg/ml test concentration**.**

Plant	Concnt.	*S. aureus*	*S. pyogenes*	*S. agalactiae*	*S. pneumoniae*	*E. faecalis*	MRSA
A. uliginosa	100	8.0 ± 0.0	8.0 ± 0.0	8.0 ± 0.0	9.0 ± 0.0	8.0 ± 0.0	8.0 ± 0.0
	200	8 ± 0.0	8 ± 0.0	**10 ± 0.0**	8 ± 0.0	8 ± 0.0	**10.5 ± 0.5**
U. simensis	100	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0
	200	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	**10.5 ± 0.55**
R. natalensis	100	**17.5 ± 0.55**	**10 ± 0.0**	**11 ± 0.0**	**12 ± 0.0**	**17.5± 0.55**	**16.5 ± 0.55**
	200	**19.5 ± 0.55**	**14 ± 0.0**	**13 ± 0.0**	**14 ± 0.0**	**22 ± 0.0**	**18.50 ± 0.55**
***C. abyssinic*a**	100	10 ± 0.0	8± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	**12 ± 0.0**
	200	10.5± 0.55	8 ± 0.0	8 ± 0.0	9.83 ±0. 41	8 ± 0.0	**13 ± 0.0**
X. strumarium	100	**22 ± 0.0**	**17.5± 0.55**	9 ± 0.0	**26.5± 0.55**	**15.67±1.50**	**24.33 ±0.52**
	200	**23.67± 0.52**	**19 ± 1.09**	9 ± 0.0	**27 ± 0.0**	**15.5±1.64**	**26 ± 0.0**
S. abyssinica	100	8 ± 0.0	8 ± 0.0	8 ± 0.0	9 ± 0.0	8 ± 0.0	**12.5 ± 0.55**
	200	**11 ± 0.89**	8 ± 0.0	8 ± 0.0	10 ± 0.0	8 ± 0.0	**15.5 ± 0.55**
Z. scabra	100	**15.33±0.52**	**11.5± 0.55**	**15 ± 0.0**	**11 ± 0.0**	**12.17±1.32**	**15.5 ± 0.55**
	200	**18.17±0.41**	**12.83±0.41**	**17 ± 0.0**	**13 ± 1.10**	**16.67±0.52**	**17 ± 0.0**
B. abyssinica	100	**23.33±0.52**	**14 ± 0.0**	**16 ± 0.0**	**13.5 ± 0.55**	**22 ± 0.63**	**25 ± 0.0**

	200	**25.4± 0.52**	**18 ± 0.0**	**20 ± 0.0**	**15.6 ± 0.52**	**26.4±0.98**	**27 ± 0.0**
E. kebericho	100	**11.33±0.82**	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	**19 ± 0.0**
	200	**11.5 ±0.55**	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	**19 ± 0.89**
A, integerifolia	100	**10 ±0.89**	8 ± 0.0	9 ± 0.0	8 ± 0.0	8 ± 0.0	**14 ± 0.0**
	200	**15.5±0.55**	8 ± 0.0	10 ± 0.0	8 ± 0.0	8 ± 0.0	**15.5 ± 0.55**
A. abyssinica	100	**10± 0.89**	8 ± 0.0	9 ± 0.0	9 ± 0.0	8 ± 0.0	**11.5 ± 0.55**
	200	**12.5±0.55**	8 ± 0.0	**10 ± 0.0**	**10 ± 0.0**	8 ± 0.0	**14 ± 0.0**
E. cymosa	100	**21 ± 0.0**	8 ± 0.0	9 ± 0.0	8 ± 0.0	8 ± 0.0	**19.5 ± 0.55**
	200	**22.5±0.84**	8 ± 0.0	10 ± 0.0	8 ± 0.0	8 ± 0.0	**19 ± 0.0**
Erythromycin (15µg)		**29 ± 0.0**	**32 ± 0.0**	**32 ± 0.0**	**19 ± 0.0**	**15 ± 0.0**	**25 ± 0.0**
DMSO 5%		8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0

Key notes: P1--P12: Plants, P1: *Acmella uliginosa*, P2: *Urtica simensis* ,**P3**: *Rhus natalensis*, P4: *Clutia abyssinica*, **P5:** *Xanthium strumarium*, **P6:** *Stephania abyssinica*, **P7:** *Zehneria scabra*, **P8:** *Bersama abyssinica*, **P9:** *Echinops kebericho*, **P10:** *Ajuga integerifolia*, **P11:** *Artemisia abyssinica* , **P12:** *Ehretia cymosa*, Bacterial strains; Sa: *Staphylococcus aureus*, Sy: *Streptococcus pyogenes*, Sp: *Streptococcus pneumoniae*, Efc: *Enterococcus faecalis*, MRSA: methicillin resistant *Staphylococcus aureus*, Inhibition zone diameter in mm, including well diameter (8 mm), test medium: MHA, dose: 100 and 200 mg/ml, mean values n= 6, DMSO: Dimethyl sulfoxide.

Table 2. Group mean Results of all the test plant against Gram Negative organisms

Plant	*Conct*	*Ec.*	*Sdy.*	*Sfx.*	*Sty.*	*Kp.*	*Pa.*	*Pmr.*	*Pvl.*	*Entr.*	*Ctr.*	*Acnt.*
A. uliginosa	100	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0
	200	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	9± 0.0
U. simensis	100	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0
	200	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	8± 0.0	**9± 0.0**
R. natalensis	100	11.3±1.03	8.5±0.6	13.3 ±0.52	10.5 ±0.55	8.3±0.52	13±0.0	14.5±1.64	13 ±0.0	11.8±0.41	10.2±0.41	14 ±0.0
	200	12.17± 0.41	10.83± 0.41	15.5±0.55	12± 0.89	10.5±0.55	16.83± 0.41	14.3±0.82	14.5±0.55	12.83±0.41	11 ±0.0	15.5 ±0.55
C. abys	100	8.17±0.41	8 ±0.0	8.67±0.52	8 ±0.0	8 ±0.0	8 ±0.0	15.17± 0.41	10 ±0.0	8 ±0.0	8 ±0.0	9.5 ±0.55
	200	10.33± 0.52	8 ±0.0	10.83±0.98	8 ±0.0	8 ±0.0	10.5±0.55	15.5±0.55	10.5±0.55	8 ±0.0	8 ±0.0	10.17±0.41
X.strumar	100	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0
	200	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0
S.abyssin	100	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8.67 ±0.52	18.67± 0.52	10.17 ±0.41	8 ±0.0	8 ±0.0	**12.67± 1.03**

	200	10 ±0.0	8 ±0.0	10.5 ±0.55	8 ±0.0	8 ±0.0	10.67± 0.52	19.5±0. 55	11.33± 0.52	8 ±0.0	8 ±0.0	13.67 ±0.52
Z, scabra	100	10 ±0.0	8 ±0.0	10.5 ±0.55	10 ±0.0	8±0.0	8 ±0.0	16.17± 0.75	11.33± 0.52	8 ±0.0	8 ±0.0	10.83±0. 41
	200	11.33± 0.52	10 ±0.0	11 ±0.0	10.67±0. 82	8 ±0.0	12.17± 0.41	16.17± 0.75	11.83± 0.41	9.83±0.4 1	8 ±0.0	13 ±0.89
B. abys	100	10.5±0. 55	11.33± 0.52	25 ±0.0	14.83±0. 98	9.5±0.5 5	20±0.0	18.33± 0.52	17 ±0.0	11.83±0. 75	11±0.0	20.67±0. 52
	200	**11.4±0. 52**	**14.6±0. 55**	**25.4±0.5 2**	**16.6±0.5 5**	**9.8±0.5 2**	**22.2±0 .41**	**21.2±0. 89**	**19.6±0. 55**	**12.8±0.8 2**	**12±0.0**	**21.8±0.5 2**
E. keberich o	100	8 ±0.0	8 ±0.0	16.5±0.5 5	8 ±0.0	8 ±0.0	9±0.89	11±0.5 5	10.5±0. 55	8 ±0.0	8 ±0.0	8 ±0.0
	200	8 ±0.0	8 ±0.0	17 ±0.55	8 ±0.0	8 ±0.0	10.67± 0.52	13.3±2. 58	11 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0
A. integ	100	8 ±0.0	8 ±0.0	8.5 ±0.55	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	10.5±0.5 5
	200	8 ±0.0	8 ±0.0	10 ±1.09	8 ±0.0	8 ±0.0	9.67±0 .52	8 ±0.0	8 ±0.0	8 ±0.0	8 ±0.0	10.7±0.8 2
A. abys	100	8 ±0.0	8 ±0.0	9 ±0.0	8 ±0.0	8 ±0.0	9.83±0 .41	16 ±0.89	8 ±0.0	8 ±0.0	8 ±0.0	12.33 ±0.52
	200	8 ±0.0	8 ±0.0	11 ±0.0	8 ±0.0	8 ±0.0	10.5±0 .55	16.5 ±0.55	8 ±0.0	8 ±0.0	8 ±0.0	13 ±0.0

E. cym	100	8 ±0.0	8 ±0.0	9.17±0.9	9.5±0.55	8.67±0.52	10.5±2.07	17.67 ±0.52	12 ±0.0	8 ±0.0	9 ±0.0	13.33±0.52
	200	8 ±0.0	8 ±0.0	11.17±0.41	11.33±0.52	11.5±0.55	11±0.89	19±0.89	14 ±0.0	10 ±0.0	9.17±0.41	15.67±0.52
Positive cont. Ciprofloxacin (5µg)		**30 ±0.0**	**33 ±0.0**	**33 ± 0.0**	**35 ± 0.0**	**21± 0.0**	**28±0.0**	**40± 0.0**	**32.5±0.0**	**30 ± 0.0**	**31±0.0**	**8 ± 0.0**
DMSO 5%		8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0

Key Notes: Bacterial strains; P1--P12: Plants, P1: *Acmella uliginosa*, P2: *Urtica simensis*, P3: *Rhus natalensis*, P4: *Clutia abyssinica*, P5: *Xanthium strumarium*, P6: *Stephania abyssinica*, P7: *Zehneria scabra*, P8: *Bersama abyssinica*, P9: *Echinops kebericho*, P10: *Ajuga integerifolia*, P11: *Artemisia abyssinica*, P12: *Ehretia cymosa*, Ec: *E. coli*, *Sdy: S. dysentery*, Sfx: *S. flexneri*, *Sty: S. typhimurium*, Kp: *Klebsiella pneumoniae*, Pa: *Pseudomonas aeruginosa*, Pmr: *P. mirabilis*, Pvl*: P. vulgaris*, Enro: *Enterobacter spp.*, Ctr*: Citrobacter spp.*, Acnt*: Acinetobacter spp.* Inhibition zone diameter in mm, including well diameter (8 mm), test medium: MHA, dose: 100 and 200 mg/ml, mean values n= 6, DMSO 5% Dimethyl sulfoxide, Negative control, Positive control. Ciprofloxacin (5µg).

Table 3. Group mean Results of all the test plant against Selected Fungal spp. at 100 and 200mg/ml test concentration

Plant	**Concentration**	***C. albicans***	***T. mentagrophytes***	***T. rubrum***	***A. flavus***	***A. niger***
Acmella uliginosa	100	8 ± 0.0	**12.5 ±0.55**	**9 ± 0.0**	8 ± 0.0	8 ± 0.0
	200	8 ± 0.0	**14.5 ±0.55**	**12.33 ± 0.52**	8 ± 0.0	8 ± 0.0

U. simensis	100	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0
	200	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0
R. natalensis	100	8 ± 0.0	**14 ± 0.0**	**15.50 ±0.55**	8 ± 0.0	8 ± 0.0
	200	8 ± 0.0	**15.50 ± 0 .55**	**17.50 ±0.55**	8 ± 0.0	8 ± 0.0
C. abyssinica	100	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0
	200	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0
X. strumarium	100	**14.5 ± 0.55**	**45 ± 1.10**	**46 ± 0.0**	**10 ± 0.0**	8 ± 0.0
	200	**15 ± 0.0**	**46 ± 0.0**	**48 ± 0.0**	**11 ± 0.0**	8 ± 0.0
S. abyssinica	100	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0
	200	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0
Z, scabra	100	8 ± 0.0	9.5 ± 0.55	8 ± 0.0	8± 0.0	8 ± 0.0
	200	8± 0.0	**13 ± 1.10**	8 ± 0.0	8 ± 0.0	8 ± 0.0
B, abyssinica	100	8 ± 0.0	**36 ± 0.0**	**31.67 ± 1.03**	8 ± 0.0	8 ± 0.0
	200	8 ± 0.0	**42 ± 0.0**	**35.4 ± 0.55**	8 ± 0.0	8 ± 0.0
E, kebericho	100	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0
	200	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0
A. integerifolia	100	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0
	200	8 ± 0.0	8 ± 0.0	**11.5 ± 0.55**	8 ± 0.0	8 ± 0.0

A. abyssinica	100	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0
	200	8 ± 0.0	8 ± 0.0	**13 ± 0.0**	8 ± 0.0	8 ± 0.0
E. cymosa	100	8 ± 0.0	**14 ± 0.0**	8 ± 0.0	8 ± 0.0	8 ± 0.0
	200	8 ± 0.0	**15 ± 1.10**	8 ± 0.0	8 ± 0.0	8 ± 0.0
Ketoconazole 6.5 mg/ml		**8 ± 0.0**	**38± 0.0**	**32± 0.0**	**42.7±0.0**	**13± 0.0**
DMSO 5% Negative control		8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0	8 ± 0.0

Key Notes: Fungal test organisms:- *C. albicans, T. mentagrophytes, T. rubrum, A. flavus, A. niger,* **P1--P12**: Plants, P1: *Acmella uliginosa,* P2: *Urtica simensis,* **P3**: *Rhus natalensis,* P4: *Clutia abyssinica,* **P5:** *Xanthium strumarium,* **P6:** *Stephania abyssinica,* **P7:** *Zehneria scabra,* **P8:** *Bersama abyssinica,* **P9:** *Echinops kebericho,* **P10:** *Ajuga integerifolia,* **P11:** *Artemisia abyssinica* , **P12:** *Ehretia cymosa,* Positive control, Ketoconazole **6.5** mg/ml**,** DMSO 5% Dimethyl sulfoxide Negative control. Inhibition zone diameter in mm, including well diameter (8 mm), test medium: SDA, dose: 100 and 200 mg/ml, mean values n= 6.

Annexes III. Photographs illustrating selected medicinal plants from different parts of Ethiopia

Figure 1. **(**a) *Bersama abyssinica* Fresen (b) *Rhus natalensis Bernh.*

Figure 1. (c) *Clutia abyssinica*

Figure 1. (d) *Artemisia abyssinica*

Figure 1. (e) *Ehretia cymosa* Figure 1. (f) *Ajuga integerifolia*

Figure1.(g) *Echinops kebericho*. (h) *Stephania abyssinica* (I) *Zehneria scabra*

Annexes III. Photographs illustrating materials, Instruments and culture results

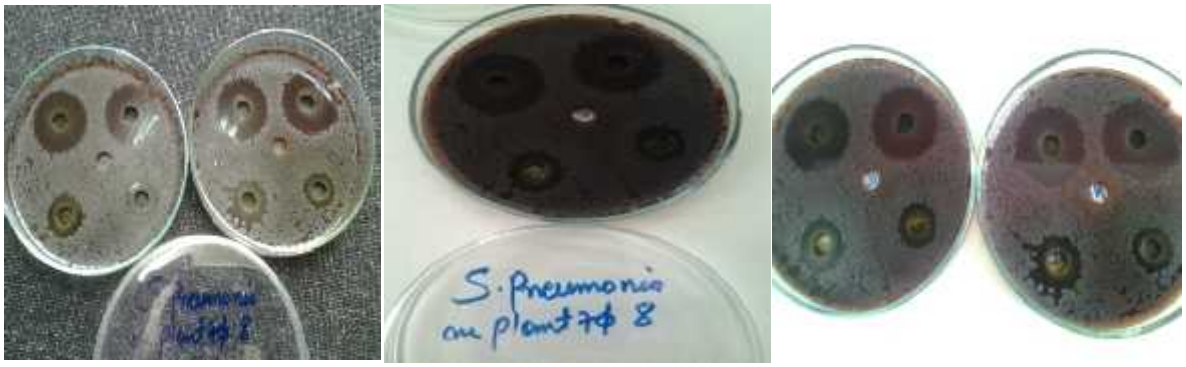
During drying in the shade during maceration Rota vapor used for extraction processes

During culturing and sensitivity testing

B. abyssinica against *T. rubrum* and *T. mentagraphyte* results, Ketoconazole on *A. flavus*

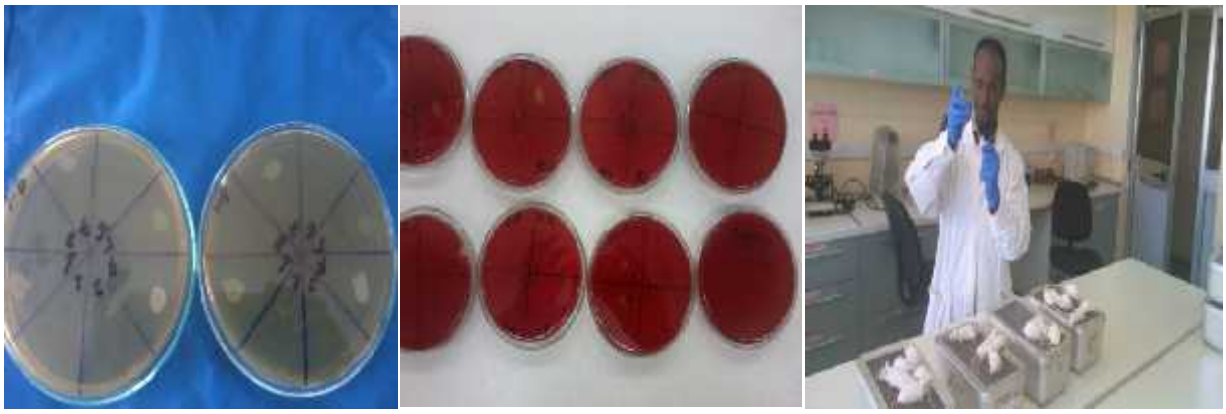
X. strumarium extract against *T. rubrum* and positive controls on MRSA

B. abyssinica against MRSA at 100 and 200mg/ml test concentrations



X. strumarium leaf extracts against *S. pneumonia* at 100 and 200mg/ml

During MIC, MBC, MFC testing



During MIC, MBC, MFC testing

During Acute toxicity testing

Definition of Terms

Maceration: Involved soaking plant materials (coarse or powdered) in a stopper container with a solvent and allowed to stand at room temperature with a continuous shaking for a period of 3 days.

Extraction: is the separation of medicinally active portions of plant using selective solvents through standard procedures.

MIC: is the lowest concentration of antimicrobial that can inhibit visible growth of a microorganism after overnight incubation

Agar diffusion: refers to the movement of molecules through the matrix that is formed by the jelling of agar. When performed under controlled conditions, the degree of the molecule's movement can be related to the concentration and the sizes of the molecule. Used to determine the susceptibility or resistance of bacterial and fungal strain to an antibacterial agent.

Herbal medicines: include herbs, herbal materials, herbal preparations and finished herbal products that contain active ingredients parts of plants or combinations. Herbal medicine involves the use of plants for medicinal purposes.

DECLARATION

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for a degree in Addis Ababa University or any other universities, colleges or institutions seeking for similar degree or other purposes. I also declare that all sources of materials used for the thesis have been duly acknowledged.

Name of M.Sc. Candidate: **Abiy Abebe (BSc)**

Signature_____

Date of submission: _____

Place: Addis Ababa University Department of Medical Laboratory Sciences, Ethiopia

This thesis has been submitted with our approval as university advisor.

Name of advisor: **Adane Bitew (MSc, PhD, Associate prof.)**

Signature: _____

Date:_____

Place: Addis Ababa University, Department of Medical Laboratory Sciences, Ethiopia

Name of advisor: **Negero Gemeda (MSc, Researcher)**

Signature: _____

Date: _____

Place: Ethiopian Public Health Institute, Traditional and Modern Medicine Research Directorate