IN VITRO ANTIBACTERIAL ACTIVITIES OF THE BARK AND LEAF EXTRACTS OF *Croton macrostachyus* (Hochst. ex Delile) AND *Justicia schimperiana* (Hochst.ex Nees) AGAINST SOME SELECTED HUMAN PATHOGENIC BACTERIAL SPECIES

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In Vitro Antibacterial Activities of the Bark and Leaf Extracts of *Croton macrostachyus* (Hochst.ex Delile) and *Justicia schimperiana* (Hochst.exNees) against Some Selected Human Pathogenic Bacterial Species

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As research advisors, we hereby certify that we have read and evaluated the thesis prepared by Lire Dindamo under our guidance, which is entitled "***In Vitro* Antibacterial Activities of the Bark and Leaf of Extracts of *Croton macrostachyus*** and ***Justicia schimperiana* against Some Selected Human Pathogenic Bacterial Species**". We recommend that the thesis is accepted as fulfilling the thesis requirement.

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DEDICATION

This thesis work is dedicated to my beloved mother, Abebech Bubamo and my father Dindamo Wachiso for their love and the sacrifice they paid for my education and the success of my life.

STATEMENT OF THE AUTHOR

By my signature below, I declare and affirm that this thesis is my own work. I have followed all ethical and technical principles of scholarship in the preparation, data collection, data analysis and compilation of this thesis. Any scholarly matter that is included in the thesis has been given recognition through citation.

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Upon successful completion of his highschool studies, he joined Dilla University in 2008 and graduated on July 5, 2010, with B.Sc. degree in Biology. Then, he was employed by Hadiya Zone, Soro Woreda, and had been teaching in Gimbichu Senior Secondary and Preparatory School until he joined the Postgraduate Program Directorate of Haramaya University to pursue his M.Sc. degree in Biotechnology in September 2016.

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ACRONYMS AND ABBREVIATIONS

AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
CLSI	Clinical Laboratory Standards Institute
CRD	Completely Randomized Design
EBI	Ethiopian Biodiversity Institute
EPHI	Ethiopian Public Health Institution
MIC	Minimum Inhibitory Concentration
MDR	Multi-Drug Resistant
MHA	Mueller Hinton Agar
NA	Nutrient Agar
SPSS	Statistical Package for the Social Sciences
SEM	Standard Error of the Mean
TAE	Tannic Acid Equivalent
WHO	World Health Organization
ZOI	Zone of Inhibition

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF TABLES

Table **Page**

LIST OF FIGURES IN THE APPENDIX

Figure **Page**

In vitro Antibacterial Activities of the Bark and Leaf Extracts of *Croton macrostachyus* (Hochst.ex Delile) and *Justicia schimperiana* (Hochst.exNees) against Some Selected Human Pathogenic Bacterial Species

ABSTRACT

Plants possess a wide variety of secondary metabolites which have been found to exhibit antimicrobial, antioxidant and antitumor activities. In Ethiopia, a number of plant species are traditionally used for the treatment of many diseases. Among these, Croton macrostachyus and Justicia schimperiana, which are abundantly available in many areas of the Country, are widely known for their traditional medicinal values. Therefore, this study was conducted with the objective of analyzing the profile of secondary compounds in crude extracts of the leaves and barks of the two plant species and to evaluating their antibacterial activities against some selected human pathogenic bacterial species. Extraction was done by maceration using ethanol solvent. Qualitative analysis of phytochemicals was carried out using standard protocols and phytochemicals while the quantitave analysis was done using the spectrophotometric and gravimetric methods. Furthermore, antibacterial activities of the leaf and bark extracts were determined by the disc diffusion and broth dilution methods. Results of the qualitative analysis showed that both plants have alkaloids, tannins, saponins, terpenoids and steroids in their leaves and barks. Whereas flavonoids and phlobatannins were absent in both plant leaves and barks extracts. The concentrations of the crude alkaloids (123.27mg/g, 119.57mg/g), terpenoids (118.33mg/g, 90mg/g) and phenolic constituents (3.82mg/g, 2.92mg/g) were higher in leaf extracts than in bark extracts in both C. macrostachyus and J. schimperiana, respectively, while those of the saponins (144.1mg/g and 135.33mg/g) were higher in bark extracts than in leaf extracts in both plants, respectively. Comparison between extracts and the negative control (with no inhibitory effect) showed that all types of extracts in all tested concentrations significantly inhibited the growth of all test pathogens. However, the isolates showed a sensitivity difference to extracts. When compared with the commercial antibiotic, however, all extracts at all tested concentrations (100mg/ml, 125mg/ml and 150mg/ml) had a low level of inhibitory activity against all test pathogens. The MIC required for inhibiting the growth of the test pathogens ranged from 25-100mg/ml showing variations within bacterial species and extract types. In general, the study revealed that leaf extracts showed more antibacterial activities against all tested bacteria than bark extracts. These findings suggest that C. macrostachyus and J. schimperiana leaf extracts might have great potentials in the development of antimicrobial agents useful for the treatment of diseases caused by the test pathogens provided that additional in-depth studies are made using purified active principles of the crude extracts and recent technologies.

Keywords: Disc diffusion; *Croton macrostachyus*; *Justicia schimperiana*; Antibacterial activities Phytochemicals

1. INTRODUCTION

Plants have been used in traditional medicine for several thousands of years. The use of traditional medicine in most developing countries is a normative basis for the maintenance of good health. Medicinal plants have been used as an exemplary source for centuries as an alternative remedy for treating human diseases because they contain numerous active constituents of immense therapeutic value. Plant-based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds etc. (Gordon and David, 2001). As these plants and their products are known to possess various secondary metabolites, which show a significant inhibitory effect against the growth of pathogens, they should be given proper attention to further investigation and so as to utilize them effectively to combat the disease-causing pathogens.

Secondary metabolites are organic compounds that are variously distributed within the plant kingdom or found only in restricted lineages (Fridman and Pichersky, 2005; Koul, 2008). Such compounds are not directly involved in the normal growth, development, or reproduction of an organism. The major secondary compounds include terpenes/terpenoids, phenolic compounds and alkaloids (Taiz and Zeiger, 2006; Rockwood, 2006). Most of these compounds are not necessary for the basic metabolism of plant cells and do not directly participate in growth and development (Koul, 2008). However, they help as defense molecules against pathogens. The secondary metabolites of the plants are the major source of pharmaceuticals. Most of the drugs being invented belong to secondary metabolites. This fact is mainly linked to folk medicines, which are large of plant origin (Kunle and Egharevba, 2009).

Croton macrostachyus is one of such medicinal plants known in folk medicine in different countries. Different parts of *C. macrostachyus* are used for the treatment of different ailments in humans such as bleeding, liver problem, inflammation of the lymphoid tissues; severe abdominal cramp, Ascariasis, blackleg, malaria, stomach ache, diarrhea, headache, snake bite and rheumatism (Agize *et al*., 2013).

Justicia schimperiana is widely used in folk medicine for the treatment of respiratory and gastrointestinal diseases as well as inflammation including rheumatism and arthritis (Corrêa and Alcântara, 2012). The leaf and stem bark of *J. schimperiana* are traditionally used for management of malaria, wounds, gonorrhea, and rabies (Habtamu *et al*., 2014).

The increasing rate of development of resistance to commonly used antibiotics by pathogenic microorganisms has led to the search for newer, more effective, affordable and readily available sources, in particular, from local medicinal plants or herbs (Adekunle and Adekunle, 2009). Plants are the most effective and cheapest natural sources of drugs (Prince and Prabakaran, 2011).

There is a continuous and urgent need to discover new compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases. In recent years, drug resistance to human pathogenic bacteria has been reported from all over the world. At present, the emergence of multi-drug resistant human pathogenic organisms has necessitated a search for new antibacterial substances from other sources including plants (Adekunle and Adekunle, 2009; Bhatia *et al*., 2012).

Human pathogenic bacteria such as *Salmonella*, *Escherichia coli*, *Vibrio cholera* and *Shigella* spp are common causes of diarrhea in under-five children (Omulo *et al*., 2015). Development of antibiotic resistance by these human bacterial pathogens against easily accessible and commonly prescribed drugs has become a major concern throughout the world, particularly in developing countries of East Africa including Ethiopia (Omulo *et al*., 2015). *Staphylococcus aureus* is also a pathogen of greater concern because of its ability to cause a diverse array of life-threatening infections and its capacity to adapt fast to the different environmental conditions (Bachir and Abouni, 2015). These features have made infection of *S. aureus* increasingly difficult to treat because of the fast rate at which it develops resistance to common antibacterial agents (Onanuga and Temedie, 2011). Furthermore, *Streptococcus pyogenes* is one of the human pathogens responsible for human skin infections. Infectious diseases kill about 15 million people every year, which means 25% of total annual death (WHO, 2014). Effectiveness of antimicrobial chemotherapy is highly threatened by the wide spreading antimicrobial resistance (Rashmi *et al*., 2005). Most common infections are now becoming resistant to the usual treatment. In addition,

new antimicrobial drugs are not being released to the market in the rate comparable to occurrence of antimicrobial resistance (Joseph, 2013). So, there is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action (Bhatia *et al*., 2012).To fill this gap there should be search for new antimicrobials with effective and novel antimicrobial mechanism. Natural products are among the main area for search of new antimicrobial agents (Rashmi *et al.,* 2005).

Identification and quantification of phytochemical constituents of a given medicinal plant is a pre-requisite to the scientific use of the compounds in modern medication. Therefore, the present study was aimed at determining the antibacterial activities of ethanolic extracts of *C. macrostach yus* and *J. schimperiana* against two gram-positive and two gram-negative pathogenic bacterial strains *in vitro*.

General objective:

- To determine the antibacterial activities of *Croton macrostachyus and Justicia schimperiana* ethanolic extracts against selected human bacterial pathogens and to identify the major bioactive secondary metabolites found in the crude extracts.

Specific objectives:

- To detect and quantify the presence of major groups of phytochemicals in crude extracts of the leaves and barks of *C. macrostachyus* and *J. schimperiana*.
- To determine the antibacterial activities of the crude leaf and bark extracts of *C. macrostachyus* and *J. schimperiana* against selected pathogenic bacterial species (*Salmonella* Typhi, *Escherichia coli* O157:H7, *Staphylococcus aureus,* and *Streptococcus pyogenes).*
- To determine the Minimum Inhibitory Concentration (MIC) of the crude extracts of the leaves and barks of *C. macrostachyus* and *J. schimperiana*.

2. LITERATURE REVIEW

2.1. Medicinal Plants

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources; many of these isolations were based on the uses of the agents in traditional medicine. This plant-based, traditional medicine system continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care (Owolabi, 2007). According to the World Health Organization (2002), medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety, and efficacy (Nascimento *et al*., 2000).

Medicinal plants represent a rich source of antimicrobial agents. Traditionally used medicinal plants produce a variety of compounds for the treatment of various ailments. These medicinal herbs constitute indispensable components of the traditional medicine practiced worldwide due to the low cost, easy access, and ancestral experience; and they are considered as candidates for developing new antimicrobial drugs (Abdalla *et al*., 2013). Medicinal plants play a key role in the development and advancement of modern studies by serving as a starting point for the development of novelties in drug (Wright, 2005) and various modern drugs were extracted from medicinal plants through the use of plant material as an indigenous cure in folklore or traditional system of medicine (Taye *et al*., 2014). Plants are an important source of potentially useful chemical structures for the development of new chemotherapeutic agents.

Herbal medicine is the use of plants seeds, berries, roots, leaves, bark, or flowers for their therapeutic or medicinal value (Gordon and David, 2001). As reported by the World Health Organization (2010) the majority population of the world, particularly in developing countries presently uses herbal medicine for primary health care. Herbal medicine is a major component in all indigenous people's traditional medicine. According to Maregesi *et al.* (2007) in many countries, traditional medicines are deeply rooted in their cultures. Especially, in the poor communities' medicinal plants have remained the most affordable and accessible source of treatment in controlling several human and animal ailments. This means, about 3.5 to 4 billion,

which is the majority of the global population rely on plants resources for drugs (Lawal *et al*., 2010).

2.2. Medicinal Plants in Ethiopia

The medicinal plant of Ethiopia and the developing countries play major supplementary roles to the limited modern health care available. Ethiopia is endowed with diverse biological resources including about 6, 500 species of higher plants, with approximately 12% endemic, hence making it one of the six plant biodiversity-rich regions (Yineger and Yewhalaw, 2007). In Ethiopia, about 80% of the human population and 90% of livestock is said to be dependent on traditional medicine for primary healthcare services and most of this comes from plants (Kassaye *et al.*, 2006; Yineger and Yewhalaw, 2007). In this country, the long history of use of medicinal plants is reflected in various medico- religious manuscripts produced on parchments and believed to have originated several centuries ago as reported by Kibebew (2001).

Medicinal plants have been used for treating infectious diseases because of their ease of accessibility as well as their lower side effects and toxicity. The impact of microbial diseases is especially important in developing countries such as Ethiopia where there is limited access to modern drugs and prices are mostly unaffordable when the latter are available. Currently, the ever-increasing threat from drug-resistant bacteria calls for a global effort to search for novel solutions (Theuretzbacher, 2017) that can also be based on the natural products from plants that are selected on the basis of documented ethno medicinal use (Verpoorte *et al*., 2005). Numerous *in vitro* studies have been undertaken, and have revealed the antimicrobial potential of herbal medicines traditionally used in various regions of Ethiopia (Taye *et al*., 2014). However, many Ethiopian medicinal plants still await scientific validation of their anti-infective properties. Increased prevalence of resistant bacteria, together with the lack and high cost of new generation drugs has escalated infection-related morbidity and mortality particularly in developing countries like Ethiopia (Borkotoky *et al*., 2013). Numerous biochemical compounds obtained from medicinal plants possess important antimicrobial properties (Abdallah, *et al*., 2013). Application of these compounds is preferred over synthetic drugs as they have long been used in traditional medicine and are considered safe to humans.

2.3. Antimicrobials

An antimicrobial is a substance that kills or inhibits the growth of micro-organisms such as bacteria, fungi, protozoan's, *etc*. On the basis of mode of action, antimicrobials are classified into two broad categories as Microbicidal that kill microbes without leaving any option for their survival and Micro biostatic that cease all the metabolic activities of microbes that are important for their survival so they are called as growth inhibitors of microbes. The history of antimicrobials begins with the observation of Pasteur and Joubert who discovered that one type of microbe could prevent the growth of the other. That growth inhibition was due to secretion of a compound that later on was named as an Antibiotic. Nowadays the term antibiotic is not confined to secretions of microbes only, but also includes all those synthetic drugs that help the body to get rid of any bacterial infection. The discovery of antimicrobials like Penicillin and Tetracycline paved the way for better health of people in the world by curing diseases like Gonorrhea, Strep throat, and Pneumonia (Jahir and Sonali, 2011).

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

2.4. Herbal Antimicrobials

The widespread use of commercially available antimicrobials led to the consequence of the emergence of antimicrobial-resistant pathogens that ultimately led to the threat to global public

health. Since 1980 the introduction of new antimicrobials has declined due to the huge expense of developing and testing new drugs. Most commercially available antibiotics with prolonged use may have negative effects on human health because they kill gut flora, so human beings need to take probiotics to replace the killed gut flora. All the above points clearly show the significance of herbal antimicrobials. The use of plants for treating diseases is old as the human civilization. There are many plants which have been in use as traditional medicine, so they are called medicinal plants. The use of plants for curing diseases is inevitable as is already proven by seeing the problems associated with synthetic antibiotics (Jahir and Sonali, 2011).

2.4.1. *Croton macrostachyus*

Croton macrostachyus (Bisana) belongs to family "Euphorbiaceae" is a deciduous tree, used for traditional medicine to treat rabies, epilepsy, cough, skin disease, dysentery, lung complaints, plain full eyes, toothache *etc*. (Pagadale, 2015). *C. macrostachyus* is a deciduous tree 3-25m high, although more commonly 6-12m; crown rounded and open with large spreading branches. Bark pale gray or gray-brown, finely reticulate, fairly smooth, and finely fissured with age; slash reddish; shoots densely and shortly hairy. Leaves large, green, turning to orange before falling, ovate, base rounded, apex acuminate, satellite hairy but more densely so beneath on long stems crowded at the ends of branch lets. *C. macrostachyus* is common in secondary forests, on forest edges along rivers, around lakes, in moist or dry evergreen upland forests, woodlands, wooded grasslands or clump bush land and along roadsides. It is native to Ethiopia, Eritrea Kenya, Nigeria, Tanzania and Uganda (Yibralign, 2009). *Croton* has been found to possess secondary metabolites such as alkaloids, terpenoids, flavonoids, saponin and compounds such as diterpenoids.

Croton macrostachyus are commonly used for the treatment of non communicable diseases such as diabetes, cancers and other ailments such as digestive problems, dysentery, wounds, fevers, constipation, diarrhea, intestinal worms, malaria, pain ulcers and inflammation. The parts that are used for the treatments of the different kinds of disease are the leaves, the roots, the stem barks, the fruit and the seeds (Yibralign, 2007). Different parts of *C. macrostachyus uses* for the treatment of different ailments in humans and humans like bleeding, liver problem, lymph

inflammation; severe abdominal cramp, Ascaris parasite; blackleg; malaria, stomach ache; diarrhea; a headache, snake bite and rheumatism (Agize *et al*., 2013).

2.4.2. *Justicia schimperiana*

Justicia schimperiana is a shrub with much branched stems 2-3 m high, with the slightly unpleasant smell. It is common shrub growing in most mountain grows forest usually near stream/river, evergreen shrub on hill slopes, waste ground, and village and house hedge from altitude ranging (Hedberg *et al*., 2006). The local name ‘dhumuugaa’in Afan Oromo, ‘Sensel’or ‘simiza‘in Amharic, ‘Tumunga’in Hadiyisa. It belongs to the family of Acanthaceae. It is a leafy shrub with much branched easily breakable stems, simple and opposite, long oval and tip pointed leaves white or yellow flowers.

Justicia schimperiana is widely used in folk medicine for the treatment of respiratory and gastrointestinal diseases as well as inflammation including applications in rheumatism and arthritis (Corrêa and Alcântara, 2012). The traditional use of *J. schimperiana* varies in different geographical area and culture. It is being used for management of wound, gonorrhea, malaria, rabies, and headache by Hadiya people of Southern Ethiopia for centuries (Habtamu *et al*., 2014). The leaf paste of *Justicia schimperiana* topically applied over areas affected by burning and arthritis and the leaf juice given orally for jaundice by traditional healers of Bahirdar zura, Ethiopia (Tamrat *et al*., 2015).The leaf and stem bark of *J. schimperiana* is traditionally used for management of malaria and rheumatism in some area of Ethiopia (Tamrat *et al*., 2015).

2.5. Plant Secondary Metabolites

Secondary compounds or natural products are organic compounds that are variously distributed within the plant kingdom or found only in restricted lineages (Fridman and Pichersky, 2005; Koul, 2008). The major secondary compounds include terpens/terpenoids, phenolics and alkaloids (Taiz and Zeiger, 2006; Rockwood, 2006). Most of these compounds are not necessary for the basic metabolism of plant cells and do not directly participate in growth and development (Koul, 2008). However, they help as defense chemicals against biotic (e.g., pathogens) and abiotic stressors. Researches show that most plants of folk medicine are rich in secondary compounds, though the type and amount vary with plant species. Variation in secondary

compounds may also exist within a species mainly due to plant genotypes, developmental stages and geographical locations (Peñuelas and Llusià, 2001).

2.6. Human Bacterial Infection

Infectious diseases are one of the major causes of death in developing countries. Microbes with multi-drug resistance characters are sources of growing concern. The emergence of multidrug resistance results due to indiscriminate uses of antibiotics for controlling such diseases (Westh *et al*., 2004). The term infection is used to refer the presence and multiplication of microorganisms in the body (Godstime *et al*., 20014).

The gastrointestinal tract represents a suitable ecosystem for enteric bacterial pathogens possibly due to the mucous nature and the presence of macro and micro-nutrients on the epithelial cell lining. Some enteric bacterial species can be highly pathogenic when they invade and colonize the digestive tract, thereby causing gastrointestinal disorders which range from diarrhea, gastroenteritis, shigellosis, salmonellosis, to life-threatening consequences (Godstime *et al*., 2014). Enteric bacterial pathogens, the major causes of foodborne gastroenteritis in humans, remain important health problems worldwide. Such infections associated with food contamination are the major public health problems especially in developing countries resulting in morbidity, mortality and socioeconomic impacts such as high rates of hospitalizations and high treatment costs (Teshale *et al*., 2015).

2.6.1. *Staphylococcus aureus* infection

Members of the genus *Staphylococcus* (staphylococci) are Gram-positive, round-shaped bacteria that tend to be arranged in grape-like clusters (Ryan and Ray, 2008). Staphylococci are spherical cells about 1µm in diameter arranged in irregular clusters. Single cocci, pairs, tetrads, and chains are also seen in liquid cultures. Young cocci stain strongly gram-positive; on aging, many cells become gram-negative. Staphylococci are non-motile and do not form spores (Brooker, 2002). *Staphylococcus aureus* is a facultative anaerobe that grows at an optimum temperature of 37ºC and an optimum pH of 7.5 (Ryan and Ray, 2008).

On nutrient agar, following aerobic incubation for 24 hours at 37ºC, colonies are 1–3mm in diameter, have a smooth glistening surface, an entire edge, and an opaque pigmented appearance.

In most strains, pigmentation is golden with orange, yellow and cream varieties. On Mac Conkey agar, colonies are small to medium in size and pink or pink-orange in color (Mackie, 1998.)

Staphylococcus aureus is one of the most versatile organisms. It is found worldwide and causes serious infections of the skin, soft tissues, bone, lung, heart, brain or blood. Diseases caused by Staphylococcal toxins include scalded skin syndrome and toxic shock syndrome (Sherris, 1994).

Most infections with *S. aureus* are localized at the area of entry and are self-limiting and not life-threatening. However, increasingly this bacterium is found to invade deeper into the body causing more serious and even life-threatening infections. Because of many virulence factors it possesses, *S. aureus* is well adapted for causing serious infections, even without the threat of antimicrobial resistance. Now that *S. aureus* has become resistant to so many antimicrobial agents, the infections caused by this bacterium have become even more dangerous. *S. aureus* is commonly found in most environments and may survive on dry surfaces for long periods. These bacteria are susceptible to high temperatures, and many disinfectants and antiseptics. It would seem that we should be able to control this bacterium fairly easily, but with the ability to become resistant to many antimicrobial agents, this is not the case. It is now known that *S. aureus* is the most commonly transferred bacteria among health care workers due to poor hand washing techniques. Studies have shown that by simply improving the hand washing compliance in hospitals, not only are *S. aureus* infections decreased, but antimicrobial susceptible may also be improved (Carboneau *et al.*, 2010; Zoabi *et al.*, 2011).

The past two decades have witnessed two clear shifts in the epidemiology of *S. aureus* infections: first, a growing number of health care associated infections, particularly seen in infective endocarditis and prosthetic device infections, and second, an epidemic of community-associated skin and soft tissue infections driven by strains with certain virulence factors and resistance to ß-lactam antibiotics (Tong *et al*., 2015). It is a pathogen of greater concern because of its ability to cause a diverse array of life-threatening infections and its capacity to adapt fast to the different environmental conditions (Bachir and Abouni, 2015). These features have made infections of *S. aureus* increasingly difficult to treat because of the fast rate at which it develops resistance to common antimicrobial agents. Multiple antibiotics resistance is a major health concern in the treatment of staphylococcal infections, especially infections of methicillin-resistant *S. aureus* (MRSA) which occurs due to the extensive use of antimicrobial agents,

coupled with the transmission of an appreciable proportion of the organism by person-to-person contacts. Multiple antibiotic resistant *S. aureus* is one of the common causes of severe nosocomial infections, and the gastrointestinal tract is an important source of its transmission (Onanuga and Temedie, 2011).

2.6.2. *Escherichia coli* O157: H7 infection

Escherichia coli is a bacterium that normally lives in the intestines of humans and animals. Although most types of these bacteria are harmless, several produce toxins that cause illness. Some strains of *E.coli,* including *E. coli* O157: H7, produce toxins known as Shiga toxins and are called "Shiga toxin-producing" *E. coli* (STEC). These may cause severe diarrhea and kidney damage. *Escherichia coli* O157: H7 was first identified as a possible human pathogen in 1975 in a California patient with bloody diarrhea and was first associated with a food borne (ground beef) outbreak of the disease in 1982. This serotype (defined by its O and H surface antigens) and some non-O157 serotypes of *E.coli* produce toxins, also called Shiga-like toxins because of their similarity to toxins produced by *Shigella dysenteriae* (Mashood, 2006).

Escherichia coli O157: H7 is a toxin-producing bacterium that causes intestinal disease that lasts for about one week in human beings. Diarrhea with blood is a typical severe case that can lead to kidney problems which can be life-threatening, especially in children or the elderly (Mashood, 2006). Human disease from *E. coli* O157: H7 is most often associated with eating improperly cooked meat, particularly ground beef, or from products such as unpasteurized milk and some processed meats. *E. coli* O157: H7 can be found in the healthy animal digestive tract. The *E. coli* strains that can cause disease are the result of Shiga toxins produced. *E. coli* O157: H7 is the most popular strains that are usually related to meat products (Cornick, 2004).

The symptoms usually appear about 3-4 days after exposure but may be as short as one day or as long as 10 days. Most people develop diarrhea (often bloody), severe abdominal cramps, and vomiting. Fever may or may not be present. Some people may have mild diarrhea or no symptoms at all. In some people, particularly children under five years of age, the infection causes a complication called hemolytic uremic syndrome (HUS). This is a serious disease in which red blood cells are destroyed and the kidneys fail. Most people with HUS recover completely after medical treatment, but it can be fatal (Cornick, 2004).

Escherichia coli O157: H7 causes a wide spectrum of human diseases, including bloody and non-bloody diarrhea, hemorrhagic colitis (HC), occasional kidney failure and hemolytic uremic syndrome (HUS) and *E. coli* O157: H7 contamination of drinking, surface, and recreational water has emerged as an important cause of human disease (Chalmers *et al*., 2000; Elder *et al*., 2000) . There is a varying level of antibiotic resistance among *E. coli* O157: H7 isolates. Some *E. coli* O157: H7 isolates have resistance to one or more antibiotics whereas others are multidrug-resistant (Einas *et al*., 2015).

2.6.3. *Salmonella* Typhi infection

The salmonellae belong to a genus of the family Enterobacteriaceae. They are gram-negative, facultatively anaerobic, non-spore forming rods. Motile forms have peritrichous flagella (Adams and Moss, 2008). Salmonellae are recognized as a major cause of enteric fever and gastroenteritis. Many foods, particularly those of animal origin, have been recognized as vehicles for transmitting the organisms to human and to the food processing and preparation environment (Gopinath *et al*., 2012).

Salmonella Typhi is a strain of bacteria that lives only in humans. Humans are the only known reservoir of *Salmonella* Typhi. Infected individuals carry *S.* Typhi in their intestinal tract and bloodstream and periodically shed the bacteria in their stool and less commonly in their urine. Transmission from host to host occurs if food or water contaminated with such fecal matter is ingested. Blood from an infected person also could transmit the bacterium to other people. Following ingestion of contaminated food or water, *S.*Typhi enters the small intestine, multiplies, and spreads into the bloodstream. The resulting infection is systemic. The gall bladder, liver, intestines, and spleen are commonly affected (CDC, 2013; Gopinath *et al*., 2012).

The emergence of antimicrobial resistant (AMR) salmonella strains traced back to during the late 1980s and early 1990s. First-line antibiotics used to treat typhoid fever in humans have historically included chloramphenicol and trimethoprim. However, multidrug-resistant (MDR) strains have emerged (Parry *et al*., 2002). In these years, the occurrence of multiple drug-resistant *S.* Typhi strains, i.e. resistant to chloramphenicol, ampicillin, and co-trimoxazole at a time, led to the use of fluoroquinolones (FQs) for the treatment of enteric fever (Crumb *et al*.,

2004). The widespread use of FQs resulted in an increased rate of *Salmonella enterica* strains with reduced susceptibility to these drugs (Molbak *et al*., 2002).

2.6.4. *Streptococcus pyogenes* infection

Streptococcus pyogenes is a Gram-positive facultative anaerobic bacterium. It is not motile and does not produce spores. It occurs as long chains of cocci and occasionally in pairs. *Streptococcus pyogenes* is classified as Group A streptococcus. Group A streptococci typically have a capsule composed of hyaluronic acid and are beta-hemolytic, which is true for *Streptococcus pyogenes* (Johansson *et al*., 2010). Beta-hemolytic streptococci produce a toxin that forms a clear zone of hemolysis on blood agar, demonstrating its ability to destroy red blood cells. This hemolysis is attributed to toxins formed by Group A streptococci called streptolysins. Streptolysins can destroy not only red blood cells, but also the white blood cells responsible for fighting off bacteria and disease, as well as other body cells (Ortora, 2007).

Streptococcus pyogenes is a coccus which causes a wide range of diseases in humans, from mild to life-threatening ones, such as pharyngitis, scarlet fever, tonsillitis, cellulitis, impetigo, erysipelas, proctitis, vulvovaginitis, pneumonia, endocarditis, meningitis, sepsis, necrotizing fasciitis, and myonecrosis (Shirin, 2015). *Streptococcus pyogenes* is the most common bacterial cause of a sore throat. A painful, red throat with white patches on your tonsils is characteristic of pharyngitis, otherwise known as strep throat. It is usually accompanied by swollen lymph nodes, fever, and headache. Occasionally it is also accompanied by nausea, vomiting, and abdominal pain (Lamagni, *et al*., 2008).

2.7. Antimicrobial Resistance

Antimicrobial resistance is resistance of microorganism to an antimicrobial drug that was originally effective for treatment of infection caused by it. Antimicrobials are an extremely valuable resource across the spectrum of modern medicine to treat and prevent infectious diseases. Their development has been associated with dramatic reductions in communicable disease mortality and has facilitated technological advances in cancer therapy, transplantation, and surgery (Dixon and Duncan, 2014). However, after a certain time of antibacterial usage, some bacterial pathogens became unresponsive to many of the first effective drugs. For example,

the resistance feature or evolution of *Staphylococcus aureus* had not been stopped to the first effective drug, benzylpenicillin, but continued to be resistant to different antibiotics. For example, this bacterium became resistant to methicillin and vancomycin by developing a different mechanism of resistance such as the production of a β-lactamase and increased cell wall production; which in turn decreased the usefulness of such drugs for serious staphylococcal infections (Howden *et al*., 2014; Livermore, 2000). In addition, the antibacterial resource is threatened by the dwindling supply of new antimicrobials and the global increase in antimicrobial resistance (Dixon and Duncan, 2014).

Even though the antibiotic resistance is a global threat, its burden is higher in developing countries like Ethiopia because of the high prevalence of bacterial diseases and the presence of risk factors for its emergence and spread (Huynh *et al*., 2015). Rates of antimicrobial resistance among hospital and community pathogens have increased considerably. This is especially more common in clinic settings in which resistant strains are frequently found before they spread to the community (Canton *et al*., 2013). Hence, antibacterial resistant infections would be likely more common in immuno-compromising conditions like HIV/AIDS patients due to their frequent encounters with the health care system, need for empiric antimicrobials, and immune dysfunction (McNeil, 2014). The increasing prevalence of hospital and community-acquired infections caused by multi-drug- resistant (MDR) bacterial pathogens is the limiting option for effective antibiotic therapy. Moreover, this alarming spread of antimicrobial resistance has not been paralleled by the development of novel antimicrobials (Cassir *et al*., 2014).

Antimicrobial resistance (AMR) results in reduced efficacy of ant-bacterial, anti-viral and anti-fungal drugs, making the treatment of patients difficult, costly. The development of AMR is a natural phenomenon in microorganisms and is accelerated by the Selective pressure exerted by the use and misuse of antimicrobial agents in humans and animals. The current lack of new antimicrobials on the horizons to replace those that become ineffective brings added urgency to the need to protect the efficacy of existing drugs (WHO, 2014).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The experiment was conducted in the laboratory of the School of Biological Sciences and Biotechnology at Haramaya University, Oromia region, Ethiopia. Phytochemical analysis and extraction were carried out in General Biology Laboratory while antibacterial activity tests were carried out in Microbiology Laboratory. Haramaya University is located 5 km away from Haramaya town in East Hararge Zone and about 17 km from the city of Harar, 40 km from Dire Dawa and 510 km east of Addis Ababa, the capital city of Ethiopia. Geographically, it is located at a latitude and longitude of 9°26′N and 43°3′E, respectively, with an elevation of 2047 meters above sea level (Samuel Sahle, 2008).

3.2. Study Design

The experiments were carried out in a completely randomized design (CRD) using laboratory-based bioassay. The treatments included two plant species (*Croton macrostachyus and Justicia schimperiana*) and crude extracts of two parts of both plants (leaves and barks) in triplicates. Amoxicillin and pure ethanol were used as positive and negative controls, respectively.

3.3. Collection and Identification of Plant Materials

The fresh plant leaves and barks of *Croton macrostachyus* and *Justicia schimperiana* were collected from Bate and Gandaboy village, Haramaya District, East Hararge, Ethiopia in November 2017. They were collected using plastic bags with appropriate labeling. The collected leaves and barks were authenticated by a botanist and a Herbarium keeper at Haramaya University and voucher specimens were deposited at HU Herbarium. The collected leaves and barks were washed under running tap water to remove dust. Thereafter, leaves and barks were dried under the shade at room temperature for 15 days (Shah and Yadav, 2015).

3.4. Preparation of Crude Extracts

Ninety-five grams of leaves and barks of *C. macrostachyus* and *J. schimperiana* were ground to fine powder using an electric grinder. The resulting powder obtained was packed in a polyethylene bag to prevent it from mixing with the surrounding materials until the experiment

was conducted. The bag containing the powder was then stored in air tight container and kept in the refrigerator at 4 ºC until used for analysis.

The dried powder was extracted with 97% ethanol in 1:5 ratio. Eighty grams of powder and 400 ml of ethanol were added to 500 ml Erlenmeyer flask and the mixture was mixed very well by shaking. Then, the flask was wrapped in aluminum foil to avoid evaporation. Thereafter, it was shaken on a platform shaker for 3 days at room temperature. Then, the extract was filtered by Whatman No 1 filter paper. A portion of the resulting extract was concentrated by heating on a hot plate at about 30 - 40 ºC for 30 min and used for qualitative analysis. The remaining portion was left at room temperature to dry for four days and preserved at 4 ºC until used for qualitative and quantitative analysis, as well as for anti-bacterial activity tests (Shah and Yadav, 2015).

3.5. Analysis of Phytochemical Composition of the Selected Plants

3.5.1. Qualitative analysis of major secondary metabolites

Qualitative analysis of major secondary metabolites (flavonoids, alkaloids, tannins, saponins, terpenoids, steroids and phlobatannins) of *Croton macrostachyus* and *Justicia schimperiana* were carried out using the concentrated and solidified ethanolic extracts as well as the powdered specimens following standard procedures as described below.

Test for flavonoids: 2 ml of each of the concentrated ethanolic extract was added into different test tubes. Then, 4 drops of 10 % NaOH solution were added and heated in a water bath for 10 min. An intense yellow color formation which becomes colorless on the addition of 10 drops of 1 % HCl showed the presence of flavonoids (Adachukwu *et al*., 2013).

Test for alkaloids: One point five ml of 1% HCl was added to 4.5 ml of each concentrated ethanolic extracts in different test tubes. Each mixture was heated for 2 min in a water bath while stirring continuously. It was then cooled and filtered. The resulting filtrate was tested with Mayer's Reagent for the presence of alkaloids as described by Adachukwu *et al.* (2013). One ml of the filtrate was added to 0.4 ml of Mayer's reagent. Formation of cream yellow precipitate indicated the presence of alkaloids.

Test for tannins: This test was carried out following the methods described by Ajayi *et al*. (2011). In this method, one gram of each powdered sample was separately added into test tubes each containing 20 ml of distilled water. Then, the mixture was boiled in a water bath for 7 min and filtered while hot into the Erlenmeyer flask. After cooling, 1ml of the filtrate was added to 5ml of distilled water and then a few drops (2-3) of 10% ferric chloride were added to it. Formation of blue-black or brownish-green precipitate indicated the presence of tannins.

Test for saponin: One gram of each powdered sample was placed into separate test tubes and mixed with 10 ml of distilled water. Then, the mixture was boiled in a water bath for 10 min and filtered while hot into Erlenmeyer flask. After cooling, the following additional tests were carried out as described by (Ajayi *et al*., 2011).

i) Foam test: Two point five ml of filtrate was added to a test tube and diluted to 10 ml with distilled water. It was then shaken vigorously for 2 minutes. Formation of froth confirme d the presence of saponin in the filtrate.
ii) Emulsion test: Two drops of olive oil were added to the frothing dilution of the foam test and the mixture was shaken vigorously for a few minutes. Formation of a fairly stable emulsion indicated the presence of saponin.

Test for terpenoids (**Salkowski test**): Five ml of each concentrated ethanolic extract was mixed with 2 ml of chloroform in separate test tubes, and then 2 ml of concentrated sulfuric acid was added carefully and shaken gently to form a layer. Formation of a reddish brown coloration in the inter-phase confirmed a positive result for the presence of terpenoids (Biswas *et al*., 2013).

Test for steroids (**Lieberman-Bur chard's Test**): Two ml of chloroform and 10 drops of acetic acid were placed in a test tube. 0.5 ml of each concentrated ethanolic extract was added to the test tube and mixed with the above-mentioned reagents. Then, 1.5 ml of concentrated sulphuric acid was added from the side of the test tube. A change from red color through blue to the green color indicated the presence of steroids (Gayathri and Kiruba, 2014).

Test for phlobatannins: Zero point five gram of each solidified extract was placed into separate test tubes and mixed with 20 ml of distilled water. The mixture was boiled in a water bath for 10 min. After cooling, each mixture was separately filtered through a Whatman No 1 filter paper.

Thereafter, 2 ml of 1% aqueous hydrochloric acid was added to each mixture and shaken to develop red precipitate that indicated the presence of phlobatannins (Shaik *et al.,* 2011).

3.5.2. Quantitative determination of the chemical constituents

The stored powder and solid crude extracts of *Croton macrostachyus* and *Justicia schimperiana* were used for standard quantitative estimation of the secondary metabolites. All analyses were done in triplicates.

Determination of phenolic content: Spectrophotometric method was used to quantify total phenol content in leaf and bark extracts as described by Cavalcanti de Amorim *et al*. (2012).

Stock solution of tannic acid (0.1 mg/ml, w/v) was prepared by dissolving 10 mg of tannic acid in 100 ml of 80% ethanol. Then, 0.1, 0.2, 0.3, 0.4 and 0.5 ml volumes of stock solution were pipetted and transferred into separate pint flasks. 500 µl of 10% Folin-Ciocalteu solution was added to each of the pint flasks and mixed to homogeneity for 10 seconds. Then, they were allowed to stand for 5 minutes. Thereafter, 1 ml of 7.5% Sodium carbonate was mixed to neutralize the mixture and homogenized for 30 seconds. Then, the final volume was adjusted to 10 ml with distilled water in order to obtain the final standard tannic acid concentration of 1, 2, 3, 4 and 5 µg/ml. These standard reaction mixtures were allowed to stand for 30 minutes after which their absorptions were measured at 760 nm using distilled water as a blank. Then a calibration curve was constructed using the generated data.

Stock solution of an extract (1 mg/ml, w/v) was prepared by dissolving 10 mg of the solid extract in 10 ml of 80% ethanol. 500 µl stock solution of the extract was then transferred to a test tube followed by addition of 500 µl of the Folin-Ciocalteu solution and 1 ml of the sodium carbonate solution. The final volume was adjusted to 10 ml by adding 8 ml of distilled water. The sample solutions were kept at room temperature for 30 minutes and their absorptions were measured at 760 nm using distilled water as a blank. The total phenolic content was calculated as tannic acid equivalent (TAE) by the following equation:

$$TPC = C*V/M$$

Where T is the total phenolic content in mg/g of the extracts as Tannic Acid Equivalence (TAE), C is the concentration of tannic acid obtained from the calibration curve in mg/ml, V is the volume of the extract solution in ml and M is the weight of the extract used in g.

Determination of alkaloid content: Alkaloid content was determined using Harborne (1973) method. In this method, three grams of the powder was weighed and added into a 50 ml Erlenmayer flask. Then, 20 ml of 10% acetic acid in ethanol was added into a well-covered flask and the solution was allowed to stand for 4 hrs. Afterward, the solution was filtered and concentrated ammonium hydroxide was added drop wise into the filtrate until the formation of a precipitate was stopped or completed. The precipitate was then allowed to settle. After decanting the supernatant, the precipitate was washed with dilute ammonium hydroxide and then filtered. The residue obtained on the filter paper was dried and weighed. Finally, the alkaloid content was calculated as mg per g of the sample powder used.

Determination of saponin content: Saponin content was determined according to the methods described in Obaboni and Ochuko (2001). Three grams of each grounded sample was put into a conical flask and 15 ml of 20% aqueous ethanol was added. Then the flask was heated in a hot water bath for 4 hr with continuous stirring at about 55°C. Then, the mixture was filtered. After filtering, the residue was again extracted (re-extracted) with another 30 ml of 20% ethanol. The combined extracts were reduced to 10 ml on a water bath at about 90°C. Then, it was transferred into a 250 ml separatory funnel and 5 ml of diethyl ether was added and shaken vigorously. The aqueous layer was then recovered while the diethyl ether layer was discarded. This purification process was repeated. Thereafter, 15 ml n-butanol was added to extract saponin. The combined n-butanol extracts were washed twice with 2.5 ml of 5% aqueous sodium chloride. Then, the washed n-butanol extracts were transferred to pre-weighed Petri plate and heated in a water bath for evaporation. Then, from the resulting material, samples were taken and dried in the oven at 60 °C to a constant weight and measured. The saponin content was calculated as mg per g of the sample powder used.

Determination of terpenoid content: Two grams of powder was soaked in 50 ml of 97% ethanol for 24 hrs. The extracts were filtered by Whatman No 1 filter paper and the filtrate was added into a separating funnel followed by addition 50 ml of petroleum ether. The resulting mixture was shaken and allowed to stay for 5 minutes for layer formation. Then, the bottom layer was drained and discharged while the top petroleum ether layer was collected and concentrated to dryness using a rotary evaporator at 40 ºC for 18 hrs. The mass of dry extract, which was

considered to represent crude terpenoids, was measured and its content was calculated as mg per the sample powder used (Furguson, 1956).

3.6. Anti-bacterial Assay

3.6.1. Collection of test organisms/ bacterial strains

For antibacterial bioassay, selected human pathogenic bacteria such as *E. coli* O157 H:7 (ATCC 25922), *Salmonella* Typhi (ATCC 13311), *Staphylococcus aureus* (ATCC 25923) and *Streptoco ccus pyogenes* (ATCC 196151) were obtained from Ethiopian Public Health Institution (EPHI) and Ethiopian Biodiversity Institute (EBDI), Addis Ababa, Ethiopia. Two Gram negative pathog enic bacteria (*E. coli* O157: H7 and *Salmonella* Typhi) and two Gram-positive pathogenic bacteria (*S. aureus* and *S. pyogenes*) were used as test pathogens in this study.

3.6.2. Sub - culturing and preparation of inoculum

Twenty-eight grams of nutrient agar (28 g of NA) medium was mixed with one liter of distilled water in a volumetric flask. Then the mixture was heated on a hot plate until a clear solution was produced and subsequently autoclaved at 121^0C for 15 minutes using an autoclave to sterilize. The resulting autoclaved medium was transferred into separate Petri plates and allowed to solidify. Each of the selected bacterial pathogens obtained from EPHI and EBI was cultured on separate nutrient agar plates and incubated for 24 hr at 37°C to obtain colonies. Two to three colonies formed on the plate were picked up with a sterile inculcating loop and transferred into a test tube containing a sterile normal saline solution and vortexed thoroughly. This was repeated until the turbidity of each bacterial suspension matched the turbidity of the 0.5 McFarland standards as described by the Clinical Laboratory Standard Institute (CLSI, 2012). The resulting suspension was used as inoculum for the test pathogen to be used in the antibacterial susceptibility test.

3.6.3. Antibacterial susceptibility test

Different concentrations of plant extracts were prepared using ethanol as solvent. The stock solutions (200 mg/ml) were prepared by reconstituting 1 g of each of the dried crude powder in

5ml of ethanol. From this stock solution, 100 mg/ml, 125 mg/ml and 150 mg/ml working solution was prepared (Alabi *et al*., 2012).

Antibacterial activities of the extracts were analyzed using the disc diffusion assay according to the technique described by Jackie (2016). Several plates of Mueller-Hinton agar were prepared and labeled according to the type and concentration of the extract. These included the plates used as negative and positive controls.

Preparation of antibiotic-impregnated discs: Whatman No.1 filter paper discs of 6 mm diameter were punched out with the aid of paper punch and were placed in the Petri dishes. They were then sterilized by autoclaving at 121°C for 15 min. After that, the discs were cooled and they were impregnated with 0.01 ml of the prepared test solutions of each extract (Taura *et al*., 2014).

Thirty-eight grams of Mueller-Hinton agar medium was mixed with one liter of distilled water in a volumetric flask and autoclaved at 121°C for 15 minutes and the medium was allowed to cool to about 45°C before dispensing into Petri dishes. The medium was poured into a sterile 90 mm Petri dish in the safety hood (CLSI, 2012).

Inoculation of Mueller Hinton Agar (MHA) Petri Plates: Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This removes excess fluid from the swab. Then, the dried surface of Mueller Hinton Agar plate was inoculated by streaking the swab over the entire sterile agar surface from the center to the rim of the agar plate. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of the inoculum. Then, the MHA plates were left open for three to five minutes to allow for any excess surface moisture to be absorbed (CLSI, 2012).

Following this step, the impregnated discs with 0.01 ml (10µl/disk) of prepared test solutions of each extract were dispensed on to the surface of the inoculated agar plate using sterile forceps. Each disk was pressed down to ensure complete contact with the agar surface. The discs were distributed evenly so they are no closer than 24 mm from center to center (CLSI, 2012).

Amoxicillin impregnated discs (5µg) were used as positive controls while pure ethanol impregnated discs were used as negative controls.

The MHA plates were sealed with parafilm and incubated at 37°C for 24 hrs. After incubation, the diameter of the zone of inhibition around each disc was measured to the nearest millimeter along two axes (i.e. 90° to each other) by using a transparent ruler and the mean of the two readings was recorded. The experiment was carried out for each selected pathogen in parallel and with three replications (Biswas *et al*., 2013).

3.6.4. Determination of minimum inhibitory concentration

Two ml of nutrient broth was added into each of the five test tubes containing 0.1 ml of each of the prepared extract concentrations (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml). After properly mixing, a standardized inoculum of 0.1 ml of the test pathogen was dispensed into the test tubes containing the suspension of nutrient broth and the extract. Then, all test tubes were properly corked and incubated at 37°C for 24 hrs. At the end of incubation, the presence of growth was evaluated by comparing turbidity of the cultures containing the extracts with the negative control. The lowest concentration without visible growth of organisms was regarded as the MIC. All the experiments were performed in triplicates for each bacterium. The average value was taken for the MIC of test plant materials (Taura *et al*., 2014).

3.7. Method of Data Analysis

Statistical Package for Social Sciences (SPSS), Version 20 (SPSS; Chicago, IL, USA), was used to analyze the data. The data recorded from the determination of phytochemical contents in leaf and bark extracts as well as those recorded from the disc diffusion tests were analyzed using one-way Analysis of Variance (ANOVA) to examine the significant difference between means. Differences between data were considered statistically significant at $p< 0.05$.

4. RESULTS AND DISCUSSION

4.1. Qualitative and Quantitative Determination of phytochemicals

4.1.1. Qualitative analysis of phytochemicals in Crude Extracts

The qualitative analysis of phytochemical from leaves and barks of *Croton macrostachyus* and *Justicia schimperiana* revealed the presence of major secondary metabolites which include alkaloids, tannins, saponin, terpenoids, and steroids. However, flavonoids and phlobatannins were absent in both plant extracts.

Previously, different researchers have done phytochemical screening from methanol extracts of *C. macrostachyus* and *J. schimperiana* macerated leaves. As in the present study, Melaku (2017) found alkaloids, saponin, tannins, and terpenoids, but did not detect phlobatannins and flavonoids in methanolic extracts of *J. schimperiana* leaf. Aylate *et al*. (2013) reported the presence of tannins, alkaloids, saponins, flavonoids, terpenoids and the absence of phlobatannins in methanolic leaf extract of *C. macrostachyus.* In contrast, Jemal Abdela (2014) detected the presence of alkaloids, saponins, and flavonoids in methanolic leaf extracts of *J. schimperiana,* but did not detect steroids, terpenoids and tannins in the extracts. Jayapriya and Gricilda (2015) also detected in methanolic extracts of *J. schimperiana* leaves alkaloids, flavonoids, saponins and terpenoids but not phlobatannins and tannins.

Some researchers also reported the presence of some phytochemicals from *C. macrostachyus* leaf ethanolic extracts. Getacew (2016) detected flavonoids and tannins. Tesso *et al*. (2015) also detected alkaloids, saponins, tannins, steroids, and terpenoids, but not phlobatannins and flavonoids. In addition, Kiran and Deenadayalan (2017) detected alkaloids, tannins, saponins, steroids and terpenoids but they could not detect flavonoids and phlobatannins in ethanolic extract of *C. macrostachyus* barks. Finally, Amsalu *et al.* (2016*)* also detected tannins, flavonoid s, alkaloids and saponins in the extracts, but they could not detect steroids and terpenoids in the methanolic extracts *C. macrostachyus* leaves.

The phytochemical composition and content from the same plant species and plant part varies. The variation may be due to differences in methods of extraction, such as percentage and volume of the solvent used, temperature, period used for extraction, concentration of the solvent used,

the moisture content of extracts, plants' age, genotype of the plant, physiological and environmental conditions (Wendakoon *et al*., 2012; Obaineh and Shadrach, 2013).

Table 1: Results of the phytochemical screening from ethanolic extracts of the leaves and barks of *C. macrostachyus* and *J. schimperiana*

Screened phytochemicals	*Croton macrostachyus*		*Justicia schimperiana*	
	Leaf	Bark	Leaf	Bark
Alkaloids	+	+	+	+
Flavonoids	–	–	–	–
Saponin	+	+	+	+
Steroid	+	+	+	+
Tannin	+	+	+	+
Terpenoid	+	+	+	+
Phlobatannin	–	–	–	–

"+" indicates the presence of the phytochemicals while "–" indicates the absence of the phytochemicals.

4.1.2. Quantitative analysis of phytochemicals

The results of the quantitative analysis of phytochemicals are presented in Table 2 below. Analysis of variance showed that there was no significant difference between leaves and barks of both plants in the amount of alkaloid and terpenoids they possess while there was a significant difference between the plant parts studied in the phenol and saponin content in both plant species. Saponin was found to be the highest in terms of amount in both *Croton macrostachyus* and *Justicia schimperiana* barks followed by terpenoid, alkaloids and phenols. In contrast, total phenol content was the lowest in both plant barks and leaves. Furthermore, the identified groups of secondary metabolites also varied in an amount between each other in different parts of both species (Table 2).

The quantitive analysis showed that the difference in the amount of alkaloids (123.27 mg/g, 121.10 mg/g) and terpenoids (118.33 mg/g, 103.33 mg/g) of *C. macrostachyus* leaf and bark extracts, respectively, were insignificant ($p>0.05$) (Table 2). Similarly, alkaloids (119.57 mg/g, 114.77 mg/g) and terpenoids (90 mg/g, 86.67 mg/g) of *J. schimperiana* leaf and bark extracts, respectively, were found to show no significant difference in amount ($p>0.05$). In contrast, the

phenolic (3.82 mg/g, 3.0 mg/g TAE) and saponin contents (123.43 mg/g, 144.10 mg/g) of *C. macrostachyus* leaf and bark extracts, respectively, and the phenolic (2.92 mg/g, 2.14 mg/g TAE) and saponin (122 mg/g, 135.33 mg/g) contents of *J. schimperiana* leaf and bark extracts showed significant difference ($p<0.05$). The saponin content (135.33 mg/g) of *J. schimperiana* bark extract was found to be significantly higher than the crude saponin content (122 mg/g) of *J. schimperiana* leaf extract ($p<0.05$). Similarly, in *C. macrostachyus*, the saponin content (144.10 mg/g) of the bark extract was significantly higher than the saponin content (123.43 mg/g) of the leaf extract ($p<0.05$). Leaf extracts had generally higher content of phytochemicals, with the exception of saponin than bark extracts (Table 2). This may be due to the difference among plant parts in their roles in physiology and survival of the plant in different regions and seasons which influence the accumulation of secondary metabolites. In line with this, Agati *et al*. (2013) reported that plant leaves regulate the antioxidant system by synthesizing phenolic compounds to act as absorbers of surplus radiation in the epidermal layers, while Asghari *et al*. (2014) reported that alkaloids were mainly located in barks and roots in June, but the leaves were major storage sites of alkaloids in July.

Outcomes of the current study have been compared with previous studies conducted using similar standard methods for quantitative determination of phytochemicals from macerated leaves and barks of both plants. Kiran and Deenadayalan (2017) found significantly lower contents of crude terpenoids, alkaloids, and phenols in 97% ethanol extracts of the leaves and barks of *C. macrostachyus*. Oboso (2016) also reported significantly lower content of phenol in leaf extracts of *J. schimperiana.* Similarly, Murthy (2013) reported significantly lower phenol content in methanol leaf extracts of *C. macrostachyus*.

In general, comparison of the current study with previously conducted studies showed that phytochemical composition and content vary within plant species and plant parts. The variation may be due to the difference in plant material and extract preparation process such as extraction methods, percent and volumes of the solvent used, temperature and time period used for extraction, or dryness of extracts. These explanations are supported by the findings of previous researchers. Agbafer *et al*. (2011) reported that a successful recovery of the biologically active compound from plant material is largely dependent on the type of solvent used in the extraction procedure. Wendakoon *et al*. (2012) revealed that the bioactive components and contents of plant

extracts depend on the concentration of solvent, time period and temperature used in the extraction process.

Other factors that contribute to the variation of phytochemical composition and content of plant extracts are related to biological and environmental factors as well as biochemical, physiological, ecological, and evolutionary processes. In connection with this, Compean and Ynalvez (2014) reported that the type and amount of secondary compounds vary with plant species and part of plants. Penuelas and Llusia (2001) also indicated that variation in secondary compounds may exist within species mainly due to differences in plant genotypes, developmental stages and geographical locations. Genes and the associated biosynthetic pathways are also underlying causes of plant secondary metabolite variation. Most plant secondary metabolites originate from a small group of precursor compounds, which eventually become modified into diverse end-products in well-regulated mutations. The most important mechanism in diversifying secondary metabolites is the whole genome and local gene duplication (Moore *et al*., 2014).

Table 2. Quantity of phytochemicals in ethanolic extracts of *C. macrostachyus* and *J. schimperiana* leaves and barks

Amount of phytochemicals (mg/g)	*Croton macrostachyus*		*Justicia schimperiana*	
	Leaf	Bark	Leaf	Bark
Phenolic	3.82 ± 0.82^{A}	3.01 ± 0.07^{B}	2.92 ± 0.63^{A}	2.14 ± 0.13^{B}
Alkaloid	123.27 ± 0.37^{A}	121.1 ± 0.49^{A}	119.57 ± 0.59^{A}	114.77 ± 0.58^{A}
Saponin	123.43 ± 1.00^{A}	144.10 ± 0.26^{B}	122.00 ± 0.51^{A}	135.33 ± 0.37^{B}
Terpenoids	118.33 ± 4.40^{A}	103.33 ± 4.40^{A}	90.00 ± 2.88^{A}	86.67 ± 1.67^{A}

The values in the table show mean ± SEM (n=3). Superscripts in capital letters compare between means within the row, and means with similar capital letters represent no significant difference whereas means with different capital letters are significantly different at $p< 0.05$.

4.3. Antibacterial Activity Test

The antibacterial effects of *C. macrostachyus* leaf and bark crude extracts showed effective bacterial growth inhibition against all test bacterial species. The *C. macrostachyus* bark with lowest tested concentration (100 mg/ml) extract showed that as low 7.50 mm to as high 16.17 mm diameter inhibition zones in highest tested concentration (150 mg/ml) of *C. macrostachyus*

leaf (Table 3). The highest inhibition zone (16.17 mm) of *C. macrostachyus* leaf was recorded against *S. aureus*; while the least inhibition zone was seen against *Salmonella* Typhi (7.50 mm). Similarly, the maximum inhibition zone for *J. schimperiana* leaf with the highest tested concentration (150 mg/ml) extract seen against *S. aureus* (15 mm) in while the minimum was against *S.* Typhi (7.33 mm) in the lowest tested concentration (100 mg/ml) of *J. schimperiana* bark.

According to disc diffusion, the growth inhibition of the crude extracts of *J. schimperiana* bark with the lowest tested concentration (100 mg/ml) did not against gram-negative bacteria (*E. coli* and *S.* Typhi). On the other hand, *J. schimperiana* bark Crude extracts with the lowest tested concentration (100 mg/ml) were against gram-positive bacteria (*S. aureus* and *S. pyogenes)* significantly smaller area than positive control ($P<0.05$). The inhibition of crude extracts against *S.* Typhi, *E. coli*, *S. pyogenes* and *S. aureus* were significantly smaller than the inhibition of positive controls even with the highest tested concentration (150 mg/ml). While the inhibition zone against *S. aureus* was slightly comparable to positive controls with a concentration of 150 mg/ml. By comparing the concentrations of extracts used with positive controls, still, positive controls were much more potent than the extracts.

The crude extracts of *C. macrostachyus* leaf with the highest tested concentration (150 mg/ml) on *S. aureus* inhibited significantly greater area than against *E. coli, S.* Typhi and *S. pyogenes*. Among tested bacterial species, *S.* Typhi and *E. coli* showed lesser susceptibility to crude extracts of barks of both plants. These could be because of the inherent ability of this bacterial species to produce resistance mechanisms like efflux pump, reduced permeability or biofilm formation which could hinder the antibacterial activity of the bioactive compounds (Harriet and Nandita, 2014). Inhibition area of the crude extracts increased with concentration, which implies that the antibacterial activity of the crude extracts was dose-dependent; this proportional increase in inhibitory activity as the concentration of extract increase was also reported in other studies on other plants (Shetty *et al*., 2016).

Among the test bacterial strains, gram-positive bacteria were more susceptible to the crude extract. The mean zones of inhibitions of the crude extracts were higher for gram positives than gram negatives at their comparable concentration. These differences in susceptibility between gram positive and gram negative bacteria could be because of their difference in their cell wall

composition (Nikaido and Vaara, 2013). The gram-negative bacteria lipolipopolysaccharide rich outer cell membrane may partially hinder the passage of active phytochemicals to the cell unlike gram-positive which have no outer cell membrane. Moreover, the crude extracts showed antibacterial activities against both gram-positive and gram negative bacteria which could indicate that the presence of broad-spectrum bioactive metabolites in the study plant (Srinivasan *et al*., 2011).

Previous studies showed the antibacterial activities of leaf and bark extracts of *C. macrostachyus* and *J. schimperiana*. For example, using disc diffusion method, Jackie *et al.* (2016) reported nearly the same inhibition zone of 150 mg/ml of ethanolic bark extract against *S. aureus, S.* Typhi and *E. coli*. Alemu *et al.* (2014) also used disc diffusion method and reported nearly similar inhibition zone 75 mg/ml methanolic leaves extract against *S. aureus*, but smaller inhibition zone against, *E. coli*. Supporting the result of this study, Jackie *et al.* (2016) reported that ethanolic *C. macrostachyus* bark extract had more effective antibacterial activity against gram-positives than gram-negatives. Agree on the result of this study, Wagaw *et al*. (2015) found that ethanolic leaf and stem bark extracts were more effective against gram-positives than gram-negatives. Romha *et al*. (2018) also reported that methanolic leaf extract had more effective antibacterial activity against gram-positives than gram-negatives.

The antibacterial activity of both plant leaf and bark extracts were showed highest inhibition zones in all concentration against a gram-positive bacteria (*S. aureus* and *S. pyogenes*) than gram-negative bacteria (*E. coli* and *S.* Typhi) in the present study. The extracts from leaves *C. macrostachyus* in earlier studies also inhibited highest zones on *S. aureus* and *S. pyogenes* than *E. coli* all tested bacterial species (Igor, 2016; Romha *et al*., 2018) which agrees with the current finding. Usually, gram-negative bacteria are resistant to plant extract because they have cell wall having effective permeability barrier, a thin lipopolysaccharide exterior membrane which restricts penetration of extruding plant extract (Biswas *et al*., 2013).

Table 3: The antibacterial activities of the leaf and bark extract of *C. macrostachyus* and *J. schimperiana* against some standard human pathogenic bacterial species

source of anti bacterial agent	Con. (mg/ml)	plant part	Inhibition zone (mm)			
			E. coli	*S.* Typhi	*S. aureus*	*S. pyogenes*
C. macrostachyus	100	Leaf	8.67 ± 0.17^{Aa}	7.83 ± 0.17^{Ab}	9.83 ± 0.44^{Ba}	9.17 ± 0.44^{Ab}
		Bark	8.00 ± 0.29^{Aa}	7.50 ± 0.29^{Ab}	9.50 ± 0.29^{Ba}	8.40 ± 0.21^{Ab}
	125	Leaf	12.17 ± 0.17^{Aa}	11.17 ± 0.72^{Ab}	14.43 ± 0.23^{Ba}	11.47 ± 0.24^{Ab}
		Bark	11.40 ± 0.83^{Aa}	9.40 ± 0.37^{Ab}	12.83 ± 0.33^{Bb}	10.90 ± 0.45^{Ab}
	150	Leaf	14.70 ± 0.15^{Aa}	13.33 ± 0.60^{Ab}	16.17 ± 0.67^{Ba}	14.17 ± 0.17^{Ab}
		Bark	12.83 ± 0.93^{Aa}	12.37 ± 0.75^{Ab}	14.50 ± 0.29^{Ab}	13.77 ± 0.15^{Ab}
J. schimperiana	100	Leaf	7.50 ± 0.00^{Ab}	7.33 ± 0.17^{Ab}	9.50 ± 0.29^{Ba}	8.00 ± 0.29^{Ab}
		Bark	0	0	8.50 ± 0.29^{Aa}	7.67 ± 0.17^{Bb}
	125	Leaf	9.83 ± 0.16^{Aa}	9.70 ± 0.15^{Ab}	13.67 ± 0.17^{Ba}	10.33 ± 0.33^{Ab}
		Bark	8.33 ± 0.44^{Aa}	7.70 ± 0.15^{Ab}	12.43 ± 0.23^{Ba}	9.83 ± 0.17^{Ab}
	150	Leaf	13.17 ± 0.17^{Aa}	12.33 ± 0.17^{Ab}	15.00 ± 0.29^{Ba}	13.17 ± 0.17^{Ab}
		Bark	11.83 ± 0.93^{Aa}	11.77 ± 0.93^{Ab}	14.17 ± 0.17^{Aa}	12.33 ± 0.17^{Ab}
Amoxicillin	5µg		$18.00\pm.00^{A}$	16 ± 0.00^{B}	19 ± 0.00^{A}	18.50 ± 0.00^{A}
Ethanol			0	0	0	0

The values are Mean ± SEM (n=3). Superscripts in capital letters compare between means in a row; and means with similar capital letters represent no significant difference, whereas means with different capital letters are significantly different at $P<0.05$. Small letter superscripts compare between means within a column; and means with similar small letters show no significant difference, whereas means with different small letters show a significant difference at $P<0.05$.

4.4. Minimum Inhibitory Concentration

The results of the minimum inhibitory concentrations (MICs) of the extracts are shown in Table 4. The MIC against the test bacteria varied with bacterial species and extract type. As it can be seen from the table, the highest MIC of the leaf and bark extracts of both plants were observed against *Salmonella* Typhi and *E.coli* while the lowest MIC was recorded against *Staphylococcus aureus and Streptococcus pyogenes.* The leaf and bark extracts of each plant showed also

different MIC against the test bacteria. Generally, the broth dilution assay showed that in both plants leaf extracts had more antibacterial activities against all test bacteria than bark extracts.

The lowest MIC values of both leaf and bark extracts were observed against *S. aureus*. The results obtained in the broth dilution method were consistent with the results obtained from the disc diffusion method in this study. The smallest MIC of the *C. macrostachyus* leaf crude extract was recorded for *S. aureus,* which was 25 mg/ml. For unknown reasons, among the tested bacterial species, *E. coli* and *S.* Typhi did not respond to *J. schimperiana* bark extracts while *S. pyogenes and S. aureus* were inhibited with *J. schimperiana* bark crude extract.

Table 4. The minimum inhibitory concentration (mg/ml) of the leaf and bark ethanolic crude extracts (0.1ml) of *C. macrostachyus* and *J. schimperiana* against the bacterial species

Bacterial strain	MIC (mg/ml) values for crude extracts			
	C. macrostachyus		*J. schimperiana*	
	Leaf extract	Bark extract	Leaf extract	Bark extract
Escherichia coli	50±0.00	66.66±16.67	100±0.00	0
Salmonella Typhi	66.66±16.67	83.33±16.67	100±0.00	0
Staphylococcus aureus	25±0.00	50.00±0.00	66.67±16.67	83.33±16.67
Streptococcus pyogenes	41.67±8.33	66.67±16.67	66.67±16.67	100±0.00

Values are expressed as Mean ± SEM (n=3)

5. SUMMARY, CONCLUSION, AND RECOMMENDATION

5.1. Summary and Conclusion

Croton macrostachyus and *Justicia schimperiana* are widely used in traditional medicine. They are believed to have active components that help to treat and manage various infectious diseases. Therefore, this study was aimed at screening the active secondary compounds known to have anti-bacterial activities from *C. macrostachyus* and *J. schimperiana* leaf and bark extracts; quantifying some of the detected active secondary compounds and assaying the anti-bacterial activities of the crude ethanolic extracts.

Croton macrostachyus and *Justicia schimperiana* leaf and bark were collected, washed, dried and ground. Then, the obtained dry powder was extracted by maceration using ethanol as solvent. The phytochemical screening was performed following standard procedure and quantity of screened crude alkaloids, terpenoids and saponin were determined by following the standard procedure of gravimetric method. Besides, the total phenolic content in the bark and leaf extracts of both plants were determined using techniques of the spectrophotometric method. Anti-bacterial activities of the extracts against four human bacterial pathogens, *E. coli* O157: H7 *S.*Typhi, *S. aureus* and *S. pyogenes* were determined using disc diffusion and broth dilution methods.

The results of qualitative phytochemical screening of leaf and bark extracts of both plants revealed the presence of alkaloids, saponins, steroids, tannins and terpenoids in both leaf and bark extracts. Whereas, flavonoids and phlobatannins were absent in both plant extracts. Quantitative analysis revealed that generally, leaf extracts had the highest concentration of phytochemicals. Concentration crude of alkaloid, terpenoid and phenolic were found in larger in leave than bark extracts in both plants. There was no significant difference between the concentrations of crude alkaloid and terpenoids found in leaf and bark extracts of both plants. The crude saponin concentrations were found significantly higher in bark extract than leaf extracts in both plants. The result of antibacterial activity test showed each extract type had a varying degree of growth inhibition to the four human pathogenic bacterial species, i.e., *Escherichia coli* O157: H7, *S.* Typhi, *S. aureus*, and *S. pyogenes*. Both leaf and bark extracts exhibited the most effective antibacterial activity against *S. aureus*. Antibacterial activities of

both plant extracts were significantly less than that of amoxicillin used as positive control. Likewise, each bacterial species showed varying sensitivity to the different extract types. Compared to a commercial antibiotic (amoxicillin), all extract types had a lower inhibitory effect.

The MIC required inhibiting growth ranged from 25-100 mg/ml and the required suppressing growth of the pathogen varied with bacterial species and extract types. The leaf and bark extracts of each plant also showed different MIC to suppress the growth of tested bacteria. Broth assay showed that in both plants, leaf extracts showed more antibacterial activities against almost all tested bacteria than bark extracts.

The results of a present study that showed *C. macrostachyus* and *J. schimperiana* leaf and bark extracts have the ability to inhibit the growth of both the gram-positives and gram-negatives human pathogenic bacterial species with its composition and abundant content of bioactive secondary metabolites. Many evidences which confirmed the identified phytochemicals to be bioactive gathered in earlier studies. Presence of these medicinally important constituents in the studied extracts might be responsible for the observed antibacterial activity. Even though antibacterial activities of both plant part extracts were significantly less than that of amoxicillin, the activity of the extracts was broad and encouraging. Therefore, the *C. macrostachyus* and *J. schimperiana* leaf and bark ethanolic extracts could have been as a good source for useful drugs and an alternative medicine in the treatment of disorders which caused by these tested pathogens.

5.2. Recommendations

Based on these findings (results of the present study), the following recommendations were forwarded.

- Evaluation of the *in vivo* antibacterial activity against human bacterial pathogens using experimental animals should be conducted.
- Isolation and identification of the specific bioactive compound responsible for the antibacterial activity should be carried out.
- The antimicrobial activities of both plants should also be tested on other microbial species which were not addressed in this study.

- *C. macrostachyus* leaf ethanolic extract should be a potential raw material for extraction and purification of therapeutic alkaloids and terpenoids.
- *J. schimperiana* and *C. macrostachyus* bark ethanolic extracts should be a potential raw material for extraction and purification of therapeutic saponins.
- The results of the study indicated that ethanolic extracts of both plant leaf result was better antibacterial activities as compared to bark extracts. Hence, ethanolic extracts of both plants leaf should be used for the treatment of human infection caused by *Staphylococcus aureus*.

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7. APPENDIX

7.1. Appendix Figure

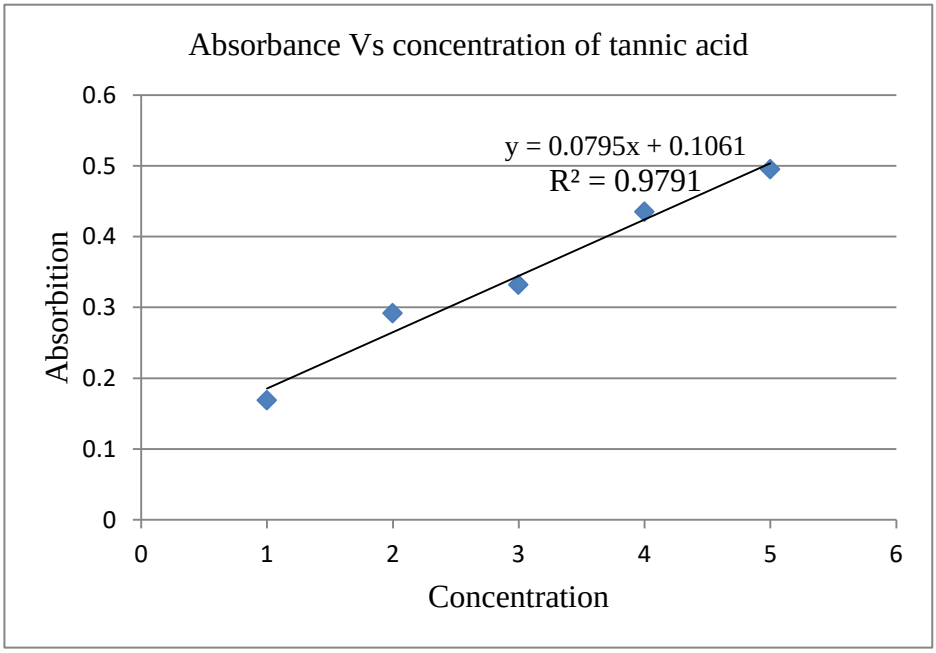

Figure 1: Standard curve for estimation of tannic acid

Figure 2: During plant sample Collection and drying time

Figure 3: During crude sample preparation

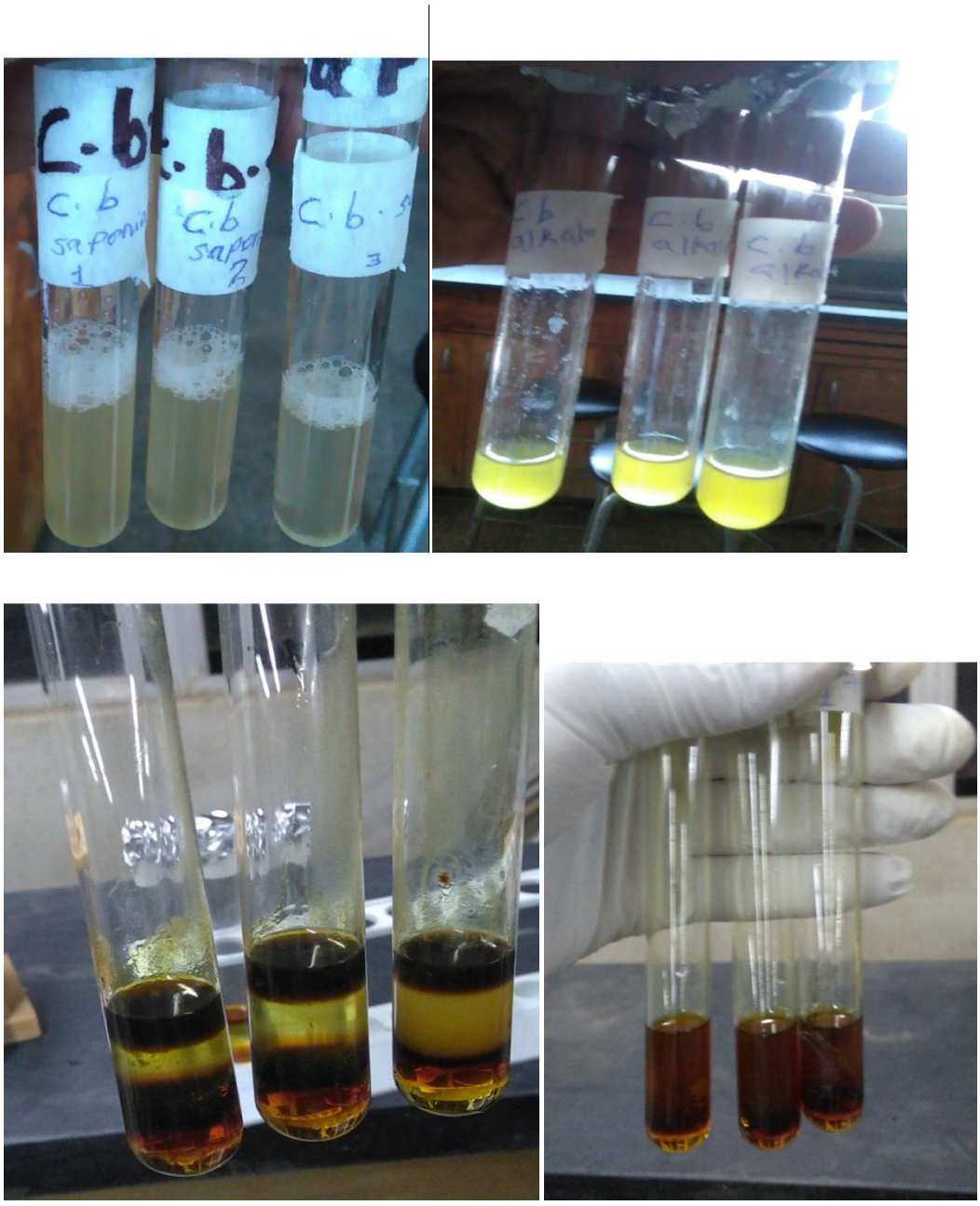

Figure 4: Qualitative test results for phytochemicals

Figure 5: Phenol Content Determination by using UV- Spectrophotometer

Figure 6: Antibacterial susceptibility test