Evaluation of *in-vitro* Antibacterial and Antifungal Activities of Crude extract and Solvent Fractions of the leaves of *Justicia schimperiana*Hochst. Ex Nees(Acanthaceae)

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A thesis submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology

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This is to certify that the thesis prepared by Melaku Tesfaye entitled "Evaluation of *in-Vitro* Antibacterial and Antifungal Activity of Crude extract and Solvent Fractions of the leaves of *Justicia schimperiana* Hochst. Ex Nees (Acanthaceae)" and submitted in partial fulfillment of the requirements for the degree of Master of Science in pharmacology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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ABSTRACT

Evaluation of the *in-vitro* Antibacterial and antifungal Activities of Crude extract and Solvent Fractions of leaves of *Justicia schimperiana* Hochst. Ex Nees(Acanthaceae)

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Addis Ababa University, 2017

Management of infectious diseases has been challenged due to antimicrobial resistance and slow rate of new antimicrobial discovery. Therefore, intensive search has to be conducted on the claimed traditional medicinal plants like *Justicia schimperiana* which have been used traditionally for wound healing, skin burn and other medicinal purposes. There is a report confirming antibacterial activity of leave extracts of *Justicia schimpriana*. Based on previousfindings, it was necessary to further investigate antimicrobial activity of the plant. In this study the leaves of *Justicia schimperiana* was extracted with 80% methanol by maceration. Then, the crude extract was fractionated with solvents of increasing polarity: ethyl acetate, n-butanol and water. The antimicrobial activity of the crude extract and solvent fractions was evaluated on eight bacterial (*S. aureus, S. pneumoniae, S. pyogenes, S. typhi, K. pneumoniae, S. flexneri, P. aeruginosa* and *E. coli*) and two fungal (*C. albicans* and *T. mentagrophytes*) species using agar well diffusion method with different concentrations (800 mg/ml, 400 mg/ml and 200 mg/ml). Minimum inhibitory concentrations were determined by broth micro-dilution method. Among the tested bacterial species *S. pneumoniae* was found to be the most susceptible species with MIC of 1.56 mg/ml. Among the solvent fractions, n-butanol fraction showed strong inhibition with mean zone of inhibition ranging from 16.33mm to 28mm for bacterial species and 18mm to 18.33mm for fungal species at concentration of 200mg/ml. In conclusion, all solvent fractions demonstrated antimicrobial activity and the n-butanol fraction was observed to be more active against the tested microorganisms.

KEY WORDS: *Justicia schimpriana*, Antibacterial activity, antifungal activity.

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List of abbreviations/acronyms

ADR - Adverse drug reaction

ANOVA - Analysis of variance

ATP – Adenylyl triphosphate

ATCC – American type cell culture

BHI - Brain heart infusion

CDC - Centers for disease control and prevention

CFU - Colony forming units

DMSO - Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

EPIs - Efflux pump inhibitors

MBC - Minimum bactericidal concentrations

MDR – Multi drug resistance

MFC - Minimum fungicidal concentration

MHB - Muller hinton broth

MIC - Minimum inhibitory concentration

PDA - Potato dextrose agar

SPSS - Statistical package for the social sciences

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1 Introduction

1.1 Overview of infectious diseases

Infectious diseases are defined by World Health Organization as diseases that are caused by viruses, bacteria, parasites or fungi. Infectious diseases are among the major causes of morbidity and mortality in human beings. Throughout human history, infectious diseases have taken a devastating role on the lives and well-being of people around the world. The developing world has been highly affected by infectious diseases than any other parts of the world (Mulder *et al*., 2014).

Infections have distinct characteristics that, when considered together, make them unique from other types of diseases. Among these characteristics, the major one is their unpredictability and their potential for explosive global effect, as exemplified by the 1918 influenza pandemic (Morens & Fauci, 2007) and the pandemic of human immunodeficiency virus (HIV) infection (De Cock *et al*., 2011), among others. The other unique character of infectious disease is the mechanism of transmission of infectious agent to other individuals through different mechanisms. Their transmission mechanisms are relatively few, well understood, and comparatively easy to study, both experimentally and in the field. In addition, most transmission mechanisms are generally amenable to medical and public health interventions (WHO, 2007).

The extraordinary adaptability of infectious pathogens (i.e., their replicative and mutational capacities) is the other unique character which provides them with a temporary evolutionary advantage against pressures aimed at their destruction. These pressures include environmental factors and antimicrobial drugs, as well as the human immune response. These adaptive characteristics of infectious pathogens give them ability to fight back and survive by forming resistant strains (Aminov, 2010). At the same time, such adaptations left us with challenge of responding to new and resistant strains of infectious pathogens which need new or modified anti-infective agents.

Infectious diseases are closely dependent on the nature and complexity of human behavior (Morens *et al.,* 2004). Usually, infectious diseases are acquired specifically and directly as a result of our behaviors and lifestyles: social gatherings, travel and transportation, sexual activity,

occupational exposures, sports and recreational activities, what we eat and drink, our pets, the environment, even the way we care for the ill in hospitals and other health care environments. Moreover, microbial colonizing infections that lead to long-term carriage without disease (e.g., within the endogenous human microbiome) may influence the development of infections with exogenous microbes (Kane *et al*., 2011).

The majority of infectious diseases was preventable (E.g. by vaccines), readily treatable (E.g. by antibiotics) or effectively managed using fairly simple, low cost measures such as ensuring adequate sanitation and educational interventions (Plant &Rushworth, 1997). However, the emergence of new diseases and the re-emergence of old ones (Pontier *et al.*, 2009) and infections with resistant strain of infectious pathogens posed a great challenge in our health. Infectious diseases are having a significant burden on global economies and public health (Jones *et al*., 2008). Therefore, it is possible to implicate that researches focusing on how to effectively treat and prevent them from spreading have to be encouraged to effectively lift people out of infection and to build a better world for future generations.

1.2 Management of infectious diseases

Use of substances with antimicrobial properties to combat microbial infection has been practiced for at least 2000 years. Ancient Egyptians and ancient Greeks used specific molds and plant extracts to treat infection (Wainwright, 1989). But, clinical application of penicillin as effective and safe antibiotic agent in 1940s which was followed by isolation of streptomycin, chloramphenicol and tetracycline in 1950s, brought new era in the management of infectious diseases (Aminov, 2010). Antimicrobial drugs have caused a dramatic change not only of the treatment of infectious diseases but of the fate of mankind.

1.2.1 Antibacterial agents

The clinical use of antibacterial agents may have very different effects on bacterial agents due to the differences in the mechanisms by which antibacterial agents affect bacteria: leading to an endpoint of either inactivation or actual death of the bacteria. The antibacterial agents that inhibit multiplication of bacteria are called bacteriostatic and other antibacterial agents which lead to death of bacteria are called bactericidal. Most protein synthesis inhibitors and anti-metabolites antibacterial agents are bacteriostatic, while cell wall synthesis inhibitors, membrane disrupting

agents, nucleic acid inhibitors and aminoglycosides are bactericidal. The bactericidal effects of cell wall synthesis inhibition involve interference in the cell's osmotic defenses and causing it to absorb excess water and burst. Cell membrane disrupting agents cause bacterial death probably by resulting loss of vital metabolites through disrupted membrane (Crofton, 1969). In addition to their specific lethal effects, most bactericidal drugs have been suggested to result death of bacteria by producing highly deleterious hydroxyl radicals as an end product of oxidative cellular damage pathway (Kohanski *et al.,* 2007). Protein synthesis inhibitors usually interfere with the synthesis of protein at an initiation phase and result in insufficient rather than distorted proteins and prevent the growth and proliferation of the bacteria without actually destroying them. Unlike other protein synthesis inhibitors, aminoglycosides are bactericidal as they cause mistranslation by acting both at initial and later phase of protein synthesis and result in distorted proteins in addition to the decrease in the rate of the protein synthesis (Crofton, 1969; Davis, 1987).

1.2.2 Antifungal agents

Among the different groups of fungi, there are three major groups of fungi that cause disease in humans. Moulds (like *Dermatophytes*) are filamentous fungi that grow as long filaments that intertwine to form a mycelium. The other group is true yeasts which are unicellular round or oval fungi like *Candidia neoformans.* The remaining group is yeast like fungi which are similar to yeasts but also form long non-branching filaments. Among them *Candidia albicans* is a common commensal organism in gut, mouth and vagina. It causes wide range of disease including oral thrush, vaginitis, endocarditis and septicemia (Mahmoud *et al*., 1999).

The antifungal agents can be classified as antifungal antibiotics and synthetic antifungals. Antifungal antibiotics are antifungals usually extracted from natural sources. One of such drugs is amphotericin B which is a naturally occurring polyene macrolide antibiotic, produced by *Streptomyces nodosus* and produce its antifungal effect by binding with ergosterol forming a pore on the membrane of susceptible fungi. Griseofulvin is also an antifungal antibiotic which is a narrow-spectrum antifungal agent isolated from cultures of *Penicillium griseofulvum* and causes a fungistatic action by interacting with fungal microtubules and interfering with mitosis. There are other antifungals antibiotics like nystatin and echinocandins.

The other group of antifungal drugs consists of synthetic antifungals, which are not extracted from natural source. Azole antifungal drugs are a group of synthetic fungistatic agents with a

broad spectrum of activity based on the imidazole or triazole nucleus. Their mechanism of action is inhibition of 14-α-sterol demethylase which resulting in impairment biosynthesis of ergosterol and lead to the accumulation of 14-α-methylsterols which disrupt the close packing of acyl chains of phospholipids, impairing the functions of certain membrane-bound enzyme systems such as ATPase and enzymes of the electron transport system. Flucytosine is also a synthetic orally active antifungal agent which is effective against a limited range (mainly yeasts) of systemic fungal infections, when converted to the antimetabolite 5-fluorouracil by fungal enzyme, 5-Fluorouracil inhibits thymidylate synthetase and thus DNA synthesis. The other synthetic antifungals include terbinafine and naftifine.

1.3 Challenges of infectious disease management

Infectious diseases are usually managed using different antimicrobials which are effective against the pathogenic microorganisms that caused the diseases. However, emerging and re-emerging infectious diseases and infectious disease with resistant strains of infectious pathogens have left us facing a countercharge from infectious pathogens by developing resistance mechanism. In addition to that, slow pace at which novel antimicrobials are discovered, while antimicrobial use is rising, makes the management of infectious diseases challenging.

1.3.1 Antimicrobial resistance

Antimicrobial resistance is resistance of microorganism to an antimicrobial drug that was originally effective for treatment of infection caused by it. The ability of microorganisms to become resistant to the major therapies used against them has long been recognized and is becoming increasingly apparent (WHO, 2014). The rates of resistance for many isolates are rising. WHO (2014) report on global surveillance of antimicrobial resistance revealed that antibiotic resistance is happening across the world, and is challenging the ability to treat common infections in the community and hospitals. The report lists*Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* as the three agents of greatest concern. In most WHO regions, there were countries that reported more than 50 percent *E. coli* resistance to fluoroquinolones and third-generation cephalosporins. In most WHO member countries, *K. pneumoniae* resistance rates to third-generation cephalosporins are above 30 percent and more than 60 percent in some regions (WHO 2014). In all WHO regions MRSA resistance rates exceed 20 percent and above 80 percent in some regions (WHO 2014). Patients who are infected

with drug resistant microbial strains often fail to respond to standard treatment, resulting in worse clinical outcomes and death, and consume more health care resources than patients infected with the same bacteria that are not resistant (Cosgrove & Carmeli, 2003).

Mechanisms of drug resistance

The mechanisms of resistance for antimicrobials differ based on the mechanism of action of the drugs and type of microorganism. The microbial resistance usually results from mutation of genetic material of susceptible microorganism or through acquired genetic material carrying resistance gene. The microbes develop resistance through different mechanisms like: inactivation of drugs *via* hydrolysis (e.g., β-lactamase) or modification (e.g., aminoglycoside resistance), alteration of drug targets within cells thus making them unrecognizable to the drug (e.g., mutation of DNA gyrase in fluoroquinolone resistance) or bypassing the drug target, the use of permeation barriers, preventing access of drugs to the target (e.g., the Gram-negative outer membrane) and active efflux of drugs out of the cell *via* membrane-bound efflux transporters (Nikaido, 1998; Paulsen, 2003).

The development of cellular resistance occurs as a result of mutations to endogenous genes or *via* lateral gene transfer of resistance determinants from other microorganisms. Recent advances in genomics have revealed that many natural ecosystems, including diverse environments such as the human gut and soil, contain large number of genes whose functions can be co-opted to confer resistance to antimicrobials (Perry, 2014; Martinez *et al.,* 2015). These genes are collectively known as the resistome.

The resistome concept is anthropocentric, since the original functions of the genes that comprise the resistome were probably not to confer antimicrobial resistance phenotypes. The recovery of genes that can confer resistance phenotypes from extreme environments that have not been in contact with humans, such as the deep subsurface (Brown& Balkwill, 2009) and permafrost (D'Costa *et al.,* 2011), further suggests that these genes have natural roles other than conferring antibiotic resistance. Resistance mechanisms such as multidrug transporters might have evolved as transporters for naturally occurring substrates, serving as mechanisms to pump toxins from cells, and their ability to also transport antimicrobials may be fortuitous (Paulsen *et al.,* 1996). "Resistance" genes during the pre-antibiotic period were probably chromosomal, and encoded functions of physiological importance. In the post antibiotic period, resistome genes were

laterally transferred to a new host where they lacked their original biochemical and genetic context, and their functions became limited to antimicrobial resistance (Debabov, 2013).

Over the last fifty years research into resistance has mainly focused on clinical aspects of antibiotic resistance, while the possible original functions of resistance genes have been largely overlooked. Understanding the original roles of these resistome elements may aid the development of successful strategies to fight infections caused by antibiotic resistant pathogens.

Measures to control antimicrobial resistance

The challenge of antimicrobial resistance now requires urgent measure from all stakeholders to fight back. There are possible solutions that are proposed to tackle this global problem. The first one is rational use of antimicrobial drugs. Ideal antimicrobial use involves use of correct drug by the best route in right dose at optimum intervals for the appropriate period and after an accurate diagnosis. The high prevalence of resistant bacteria seems to be related to irrational antimicrobial usage: i) easy availability without prescription at drug outlets, ii) injudicious use in hospitals, and iii) uncontrolled use in agriculture, animal husbandry, and fisheries. Many traditional practitioners are also using allopathic drugs irrationally (Holloway, 2000). The other recommended solution is preserving existing effective antimicrobial agents for the future use. In the prevailing situation of antimicrobial resistance, it is advisable to preserve the existing antimicrobials for future use and the new molecules should only be used if the old ones are ineffective (Rashmi *et al*., 2005).

The antimicrobial resistance is a natural process in microorganism, which is used to defend themselves from any pressure against their survival. As long as, antimicrobials are being used, the eradication of antimicrobial resistance is unachievable and it is not possible to totally avoid the use of antimicrobials as long as pathogenic microbes are present. The antimicrobial resistance prevention ways mentioned above can slow down the occurrence of antimicrobial resistance, not totally prevent antimicrobial resistance. Therefore, the development of new antimicrobial agents is not an option. There is need for new groups of antimicrobials and newer compounds from the old groups of antimicrobial drugs (Paudel, 2008; Ozkan, 2015). One of the areas for search for effective antimicrobial agents is looking for natural products that can serve as antimicrobial agent or lead compound for development of antimicrobial agent from plants that are traditionally used as remedy for microbial infection related diseases (Baker *et al.*, 1995).

1.3.2 Dry antibiotic pipeline

The pharmaceutical companies have been reluctant to invest in research and development of antimicrobial drugs. The lack of interest to invest in antimicrobial drugs discovery and development come from several reasons. One of the reasons for industries not to invest is the existence of some generic drugs on market which are still effective against most infections and the health institutes of many countries advocate these drugs as first treatment choice. The newly developed more effective antimicrobial drugs are usually saved for severe infections which means this new drugs are not frequently dispensed and used only in small case as last line of defense. Therefore, there is small market gain from new antimicrobial for pharmaceutical companies.

The second reason is the short and limited duration of antimicrobial regimens. Unlike the medication for chronic diseases, which are used for long period of time (usually lifelong), the antimicrobial medications are usually curative treatment used for short period of time. This makes antimicrobials less profitable marketed product compared to other medications (Projan, 2003).

The other reason for reduced focus of pharmaceutical companies on antimicrobial is the rapid development of resistance by pathogenic agents to the antimicrobials. This means the clinical lifespan of the drug will be short. So, companies fear rapid development of resistance can result in loss of capital invested on the drug.

The negotiations with public purchasers don't tend to take into account the relative health gain from effective antibiotic treatment, often granting higher prices to drugs (such as cancer drugs) that offer only a few months of additional life could be another reason (Outterson *et al.,* 2007).

To facilitate the research and development of antimicrobial drug there should be initiations from all stakeholders. There should be incentives for the industries to be involved in development of new antimicrobial drugs. In addition to that, cooperation between industries and research institutes also would have positive role in solving this problem.

1.4 Potential antimicrobial targets

1.4.1 Novel antibacterial targets

There are approximately 265–350 genetically validated antibacterial targets in bacterial genome. Among them about 60% are broad-spectrum targets which are available in many bacteria (Hutchinson *et al.,* 1999). Surprisingly, targets used by currently marketed drugs are around 20 (Singh & Barrett, 2006) and thus provide wide opportunity for the discovery and development of new antibacterial agents with novel mode of action with no cross-resistance. However, this target based search for novel antibacterial agent has not been productive. Among the reasons for unproductive search of novel antibacterial agent with this method, the major reasons could be: agents target single enzyme are highly susceptible to resistance (Silver 2007; Brotz-Oesterhelt and Brunner 2008) so that, research institutes are not willing to spend on such agents and the second possible reason could be the chemical libraries currently available mainly contain chemicals that could not be used as antibacterial agent, especially because of the difficulty of compound entry into bacterial cells (Silver 2011; Brown *et al.,* 2014). Here are some of potential target for antibacterials which could be used for development of novel antibacterial agents.

Topoisomerase II

The topoisomerase II is an enzyme complex comprising four subunits: GyrA/ParC, GyrB/ParE. Each pair of subunits is usually inhibited by single dual targeting agent. Topoisomerase II Gyr A/ParC subunit pair is target for currently available antibacterial agents like quinolones and novobiocin (Silver, 2011). The other pair of subunit (GyrB/ParE) could be target for antibacterial agents with no cross resistance with quinolones. Here are some of recently found antibacterial leads which target Gyrb/ParE subunit of topoisomerase II. Benzimidazole carbamate lead which is optimized to benzimidazole urea lead, VRT-752586 with significant improvement of potency and Gram-positive spectrum. VRT-752586 at dose less than 50 mg/kg showed *in-vivo* efficacy in murine thigh model of *S. aureus* infection (dosed iv) and murine lung pneumonia model of *S. pneumoniae* infection (dosed po) (Charifson *et al.,* 2008). The other one is tricyclic gyrase B inhibitor that showed potent antibacterial activity against *K. pneumoniae, A. baumannii* and *P. aeruginosa*. It was effective in different murine infection models (Tari *et al.,* 2013).

Bacterial protease

Bacterial proteases are collection protein degrading enzymes that have vital roles in cell viability (Emiola *et al.,* 2015) and pathogenicity (Moayeri *et al.,* 2015). Proteases are broadly classified as extracellular and intracellular proteases. Extracellular proteases mainly involve in virulence, where they are responsible for the destroying of host tissue and the degradation of host defense proteins such as immunoglobin A1 (Wandersman, 1989). On the other hand, intracellular proteases involve in essential genes required for cell viability and some participate in virulence. These essential roles of proteases make them a potential target for antibacterial drug development. However, bacterial proteases are not yet target for any clinically used antibacterial agent. There are different specific proteases being investigated as potential antibacterial agent. AAA+ family proteolytic complexes and signal peptidases (SPs) among the site investigated for potential antibacterial targets. Caseinolytic protease (ClpP) belongs to the AAA+ family of proteases, whose activity is tightly regulated by associated AAA+ ATPases. β-lactones irreversibly inhibiting proteolytic activity of ClpP (Böttcher & Sieber, 2008). β-lactone has been able to reduce virulence factor activity, including α-hemolysin and listerolysin, in *S. aureus* and *L. monocytogenes,* respectively (Böttcher & Sieber, 2009). SPs are required to cleave the targeting peptide, which used to lead the protein to its destination, following protein secretion. Both type I and type II SPs have been successfully targeted by chemical inhibitors (Inukai *et al.,* 1978; Craney & Romesberg, 2015).

Efflux inhibitors

The mechanism of resistance for MDR gram-negative strains is closely related to efflux pumps of the resistance nodulation division (RND) family (Ruggerone *et al.,* 2013). The major RND efflux system of *E. coli* (AcrAB-TolC) consists of a cell membrane transporter (AcrB), an inner membrane channel (TolC) and an adaptor protein (AcrA) (Yamaguchi *et al.,* 2015). Different particles reach periplasmic space through porin or diffusion through lipid bilayer first captured by AcrB. The AcrB transporter takes the compounds into the TolC channel and to the exterior using the proton motive force (Ma *et al.,* 1993). Therefore, the AcrAB-TolC efflux pump can be inhibited by inhibition of AcrB function. Efflux pump inhibitors (EPIs)/AcrB inhibitors could be used with antibiotics to improve the antibacterial potency and to infections with resistant strain. Here are some of efflux inhibitors which could be used to enhance antibacterial effect of antibiotics: artesunate (AS), an antimalarial agent, had no direct antibacterial activity by itself,

but it could significantly enhance the antibacterial effects of beta-lactam antibiotics against *E. coli* ATCC 35218 *in-vitro*, which might be associated with the efflux pump AcrB (Li *et al.*, 2011).

1.4.2 Novel antifungal targets

The development of antifungal drug is more of a challenge compared to development of new antimicrobials targeting bacteria. This is mainly because fungal pathogens are more closely related to the mammalian cell. There are many proposed potential antifungal target. Among these targets, it is not possible to definitely predict which target could be used to develop antifungal drug in the near future. However, it is possible to list available sites that can be selectively targeted by antifungal drugs.

Protein N-myristoyl transferases

N-myristoylation is a process of addition of the 14-carbon fatty acid myristate to proteins terminal glycine residue at the stage of translation. Such proteins carrying the terminal lipid moiety are involved in many of essential cellular processes (Farazi *et al.,* 2001). The enzyme involved, myristoyl-CoA - protein N–myristoyltransferase (Nmt1p), was found experimentally to be essential both for *C. neoformans* (Lodge *et al.,* 1994) and *C. albicans* (Weinburg *et al.,* 1995). Derivatives of benzofuran inhibitors have been synthesized with enhanced inhibitory activity against Nmt1p, some of which showed antifungal activity against fungi *in-vitro* and even shown antifungal activity in mouse models of *C. albicans* infection (Masubuchi *et al.,* 2001; Ebiike *et al.,* 2002; Masubuchi *et al.,* 2003).

DNA topoisomerases

DNA topoisomerases are involved in the unbundling of DNA supercoils. *C. albicans* DNA topoisomerase I was investigated as a potential antifungal target on biochemical grounds (Foster *et al.,* 1992). Gene disruption experiments showed the topoisomerase I gene was essential for unbinding of DNA supercoils in *C. neoformans* (Del Poeta *et al.,* 1999) and *C. albicans* (Jiang *et al.,* 1997). Inhibitors of microbial topoisomerases have shown antifungal activity *in-vitro* (Anizon *et al.,* 1997). Eupoleuridine, an alkaloid having fungistatic activity in *C. albicans* (Hufford *et al.,* 1987), also inhibited the activity of *S. cerevisiae* DNA topoisomerase I.

H^+ ATPase

Fungal H^+ ATPase has been described as potential antifungal targets by different researchers for years (Setoyoung *et al.,* 1997). The plasma membrane ATPase proton pump in *C. neoformans* has been considered as a particular target with the discovery of fungicidal effects from an ATPase antagonist, Ebselen (Soteropoulos *et al.,* 2000) and a conjugated styryl ketone, NC1175 (Manavathu *et al.,* 2001). Comparative genomic analyses suggest that H^+ ATPase encoded by PMA1 have homologues gene in different fungi which could mean, it may provide broad-spectrum antifungal target (Soteropoulos *et al.,* 2000).

1.5 Experimental plant

Justicia schimperiana

Local name _'dhumuugaa' in Afan Oromo, _'Sensel' or _'simiza' in Ahmaric, 'Tumunga' in Hadiyisa. It is common shrub growing in moist mountain forest, usually near streams and rivers, in evergreen scrub on hill slopes, forest clearings, different plantations, waste ground or planted. 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 and white or yellow white flowers. It is up to 4 m high, with slightly unpleasant smell (Hedberg *et al.,* 2006).

Botanical background

The family Acanthaceae is a taxon of dicotyledonous flowering plants containing almost 250 genera and 2500 species. Most are tropical herbs, shrubs or twining vines, some are epiphytes. Only a few species are distributed in temperate region. The four main centers of distribution are Indonesia, Malaysia, Africa, Brazil and Central America (Reddy *et al.,* 2013). *Justicia* is the largest genus of Acanthaceae, with approximately 600 species that are found in pantropical and tropical regions (Corrêa and Alcântara, 2012; Hedberg *et al.,* 2006). The species of *Justicia* can be easily recognized by their bilabial corolla, with a posterior lip that is generally two-lobed, an anterior lip that is three lobed, two stamens, a capsule with four seeds, and a basal sterile portion (Corrêa and Alcântara, 2012). The presence of compounds with diverse chemical class including alkaloids, lignans, flavoinoids and terpenoids was reported (Corrêa and Alcântara, 2012). Other chemical class of chemicals like essential oils, vitamins, fatty acids and salicylic acid, steroids also reported to reside in the Justicia (Corrêa and Alcântara, 2012)

Ethnopharmacological use of *Justicia schimperiana*

The traditional use of *Justicia 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 arthrithis and the leaf juice given orally for jaundice by traditional healers of Bahirdar zuria, Ethiopia (Muthuswamy and Solomon, 2009). The dried root of *Justicia schimperiana* is traditionally used for management of seizure in some areas of Ethiopia (Tamrat *et al.*, 2015). The traditional healers use this plant also as treatment agent for patient with rheumatism (Haile *et al.*, 2008). The leaves of *Justicia schimperiana* traditionally used for management of malaria and coccidiosis (Getaneh *et al.*, 2014). In addition to the above aliments, *Justicia schimperiana* is traditionally used for treatment of bilirubinemia (Ivo *et al.*, 2014) and intestinal parasites (Fisseha *et al.*, 2009).

The medicinal activity of *Justicia schimperiana* against different diseases has been tested in many researches. Its aqueous leaf extract has shown antihyperglycemic activity and improvement in glucose tolerance in streptozotocin induced diabetic mice (Andualem *et al.*, 2016). The antimalarial activity of this plant has been tested and strong antimalarial activity was observed in methanol and aqueous extract of the leaf (Jemal *et al.*, 2014). Umer *et al.* (2010) reported the hepatoprotective activity of leaves of *Justicia schimperiana*. Murthy *et al.* (1993) screened the antibacterial activity of different parts (leaves, stem, bark and root) of *Justicia schimperiana* and found it to be active against the tested bacterial species (*S. aureus, E. coli, B. cereus, P. mirabilis, K. pneumonia* and *P. aeruginosa*). Moreover, Kothai and Thirunalasundri (2011) reported antibacterial effect of aqueous and ethanol root extracts against both clinical and standard strains of *S. pyogenes*. By contrast, Tamirat *et al*. (2015) reported the absence of detectable zone of inhibition of root extract of *Justicia schimperiana* against *S. pneumoniae, S. pyogenes and S. typhi* (Tamirat *et al.*, 2015).

Fig 1:-*Justicia schimperiana* Hochst. Ex in its natural habitat

1.6 Rationale for the study

Infectious diseases kill about 15 million people every year, which means 25% of total annual death (WHO, 2013). 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(Rojas *et al.,* 2003).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).

Plants which are traditionally used for medicinal purpose are candidates for search of new antibacterial agent and usually they are safe for human use because, they have long history of use by humans without major adverse effect (Vermani and Garg, 2002). In most of the cases, these practices are handed down from generation to generation empirically without knowing the plausible mechanisms, safety, and efficacy of herbal treatments (Mujumdar *et al.,* 2001). In order to get scientific ground for the traditional use of herbal medicine and to search for new, effective and safe antimicrobial agent, scientific studies have to be conducted on the herbal medicines. The leaf of *Justicia schimperiana* is traditionally used for management of wound, gonorrhea (Habtamu *et al.,* 2014) and skin burn (Muthuswamy and Solomon, 2009). Extracts obtained from different parts of *Justicia schimperiana* had been tested for antimicrobial activity. Murthy *et al.* (1993) studied the antibacterial activity of aqueous, methanol and chloroform extracts of different parts (leaf, bark, stem and root) of *Justicia schimperiana* and reported the plant to have significant activity against *S. aureus, E. coli, B. cereus, P. mirabilis, K. pneumoniae* and *P. aeruginosa*. By contrast, Tamirat *et al.* (2015) did not detect any antimicrobial activity with the ethanol extract of root of the plant. As the methanol extract was the one with the highest activity (Murthy *et al.,* 1993), the present study attempted to further investigate the antimicrobial activity (determination of zone of inhibition, MIC and MBC) of the 80% methanol leaf extract and different solvent fractions against a wider microbial species, including bacteria and fungi.

2 Objectives

2.1 General objective

- To evaluate the *in-vitro* antibacterial and antifungal activity of crude extract and solvent fractions of leaves of *Justicia schimperiana* on selected bacterial and fungal species.

2.2 Specific objectives

- To determine zone of inhibition of the crude extract and solvent fraction of the leaves of *Justicia schimperiana* on selected bacterial and fungal species using agar well diffusion method.
- To determine the minimum inhibitory concentration of the crude extract and solvent fraction of the leaves of *Justicia schimperiana* against selected bacterial and fungal species using broth dilution method.
- To determine the minimum bactericidal and fungicidal concentration of the crude extract and solvent fractions of the leaves of *Justicia schimperiana* against the selected bacterial and fungal species.
- To qualitatively determine the phytochemical constituents of the crude extract and solvent fractions of leaves of *Justicia schimperiana*.

3 Materials and Methods

3.1 Chemicals, drugs and media

The following chemicals, drugs and media (bacteriological and mycological media) were used in the present study: absolute methanol and n- butanol (Carlo Erba reagents, France), ethyl acetate (HiMedia Laboratories Pvt. Ltd., India), distilled water, sterile sheep blood (Ethiopian Public Health Institute, Ethiopia), 0.5 McFarland equivalence/standards (Remel, Lenexa Kansas 66215, USA), Dimethyl sulfoxide (DMSO) (Uni-Chem, India), resazurin sodium salt (Serva Feinbiochemica Heidelberg, New York), Mueller-Hinton Agar (MHA), Mueller-Hinton Broth (MHB), brain heart infusion agar, blood agar base, manitol salt agar and XLD medium (Oxoid Ltd, Basingstoke, Hampshire, England), brain heart infusion broth (Becton Dickinson and , Cockeysville), violet red Bile glucose agar (Research-lab Fine Chem. Industries, India), Potato Dextrose Broth (PDB) (HiMedia Laboratories Pvt. Ltd. India), Potato Dextrose Agar (PDA) (Sisco Research Laboratories Pvt. Ltd. India), ketoconazole 50µg/disc, nystatin 100 units/disc, ceftriaxone 10ug/disc ampicillin 10 µg/disc (Oxoid Ltd, Basingstoke, Hampshire, England) and ciprofloxacin 5 µg/disc (Becton, Dickinson and Company, USA). All the chemicals and solvents were of analytical grade.

3.2 Plant material collection and preparation

The leaf of *Justicia schimperiana* was collected from its natural habitat in January, 2016, around Hossana Town, Hadiya zone, 232 km away from Addis Ababa, Ethiopia. Identification of the plant specimen was done by a taxonomist at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, where a voucher specimen was deposited for future reference with voucher No. of 004/HR .

Fresh leaves of *Justicia schimperiana* was washed with tap water to remove dirt. Then it was dried under shade at room temperature. The dried leaf was pulverized using a mortar and pestle to get a coarse powder used for the extraction.

3.2.1 Preparation of the crude extract

Extraction was carried out by macerating the dried leaf power of *Justicia schimperiana* with 80% methanol. 500 g of coarsely powdered leaf was macerated with 80% methanol (200 g in 1600 ml) i.e. in the ratio of 1:8 in Erlenmeyer flask for 72 h at room temperature. The extraction

process was facilitated by using an orbital shaker (Bibby Scientific Limited Stone Staffo Reshire, UK) at 160 rpm with occasional stirring. After 72 h, the extract was separated from the marc using gauze and further filtered by Whatman filter paper No. 1 (Maidstone, UK). The marc was re-macerated twice using the same volume of 80% methanol to exhaustively extract the plant material. After exhaustive extraction, the methanol portion of the solvent was removed by evaporation under reduced pressure using rotary evaporator (Buchi Rota vapor, Switzerland) in distillation flask at 72 rpm and 40 °C. And the water was removed by freeze drying using lypholizer (Operon, Korea vacuum limited, Korea). The yield of crude extract found was 108 gram which is 21.6% w/w. Portion of the extract was used for the antimicrobial activity testing and the remaining crude extract was subjected to fractionation.

3.2.2 Fractionation of the crude extract

The crude extract was fractionated by subjecting it to a successive extraction using separator funnel with ethyl acetate and n-butanol in increasing order of their polarity and finally the remaining solution was used as aqueous fraction. Briefly, the 90gram of dried crude extract of the plant was suspended in 600mlof distilled water then slightly shaken to mix completely with solvent. The mixture was transferred in to a separator funnel, and then 500ml ethyl acetate was added to it. The new mixture shaken gently to mix and allowed to settle for some times until it forms two layers. Then the ethyl acetate fraction was collected. More ethyl acetate was added to take the entire ethyl acetate fraction and repeated the procedure twice as described above. Finally, the ethyl acetate fraction was placed in an oven set at 45 ºC until the solvent was completely removed. To the remaining aqueous portion in separating funnel,500ml n-butanol was added followed by vigorous shaking as described for the preceding solvent fractionation. The upper layer was n-butanol, which was separated from aqueous portion and the procedure repeated twice. Then separated n-butanol portion was taken and evaporated using a rotary evaporator (Buchii model R-200, Switzerland), then the filtrate was placed in an oven set at 45 ºC until the remaining solvent completely removed and left dried powder of n-butanol fraction . The remaining aqueous layer was collected and freeze dried in a lyophilizer (Operan, Korea vacuum limited, Korea) and left powder of aqueous fraction. The percentage yield of the solvent fractions for ethyl acetate and n-butanol solvents fraction were 42.1% and 15.2% respectively and the remaining 41.5% was aqueous fraction.

3.3 Antimicrobial activity assay

3.3.1 Test organisms

Bacterial strains

The standard American type cell culture (ATCC) bacterial species of *Streptococcus pyogenes* (ATCC 19615), *Streptococcus pneumoniae* (ATCC 49619), *Shigella flexneri* (ATCC 12022), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC) 25922) and *Salmonella typhi* (ATCC 13062) were obtained from Aklilu Lemma Institute of Pathobiology while, *Staphylococcus aureus* (ATCC 25923) and *Klebsiella pneumoniae* (ATCC700603) were collected from Ethiopian Public Health Institute (EPHI) microbiology laboratory. All bacterial test organisms were inoculated into brain heart infusion agar plate except the fastidious Streptococcus species which was inoculated into blood agar base enriched with 5% sterile sheep blood. Then, in order to avoid the growth of contaminant microorganisms and to obtain a pure colonies of bacterial species, bacterial test organisms were inoculated in to selective medium i.e. violet red bile glucose agar (*E. coli*), XLD media (*S. flexneri and S. typhi*) and for *S. aureus* manitol salt agar.

Fungal strains

Similar to bacterial test organism, the *Trichophyton mentagrophytes* (ATCC 18747) and *Candida albicans* (ATCC 10535) fungal test organisms were obtained from EPHI and then, inoculated to previously prepared Potato Dextrose Agar (PDA). The selection of above mentioned fungal species was done based on the availability of those species and by considering species of organisms most likely to cause systemic and topical infections.

3.3.2 Media preparation and inoculum standardization

All (fungal and bacterial) media were prepared and used according to the manufacturer's protocol. The powder media was added to a flask containing measured volume of distilled water and placed on hot plate with stirrer until it boils. Then the media was sterilized at 121 ^{0}C for 15 minutes using an autoclave (with the exception of violet red bile glucose agar and XLD medium). The medium was cooled to 45-50 ºC inside water bath (for fastidious *Streptococcus* species that require enriched media, 5% sterile sheep blood was added to blood agar base medium). After that, the media was poured to pre-labeled sterile petri-dishes aseptically and

allowed to congeal. Then, standard microbial strains were inoculated and spread on the respective selective media using inoculating wire loop following aseptic condition in Safety Cabinet (Bioair instruments, Eurolone® Company, Italy) and incubated accordingly.

The bacterial and fungal turbidity of each of species was prepared and standardized by following the guideline of Clinical and Laboratory Standard Institute (CLSI) (CLSI, 2012). The bacterial suspension in a nutrient broth (except of *Streptococcus* species as they were standardized in a test tube with brain heart infusion broth) and fungal suspension in a potato dextrose broth were prepared as follows. After preparing the broths in distilled water, 5 ml of the broth was transferred to test tubes and autoclaved (Astell Scientific Limited, England) and cooled. 3–5 well isolated colonies of the same morphological type of each bacterial and fungal strain were picked up by wire loop from fresh agar plates of bacterial and fungal culture and aseptically transferred into pre-labeled test tubes containing the sterile nutrient broth for bacteria and potato dextrose broth for fungus and then incubated for about six hours. The turbidity of the inoculum tube was adjusted visually by either adding colonies or by adding sterile normal saline solution to that of the already prepared 0.5 McFarland standard which is assumed to contain a bacterial concentration of $1x10^8$ CFU/ml and fungal concentration of 1-5 x 10^6 CFU/ml. The adjustment and comparison of turbidity of inoculum tube and that of 0.5 McFarland standard was performed by visually observing them with naked eye against a 0.5 McFarland turbidity equivalence standard with white background and contrasting black lines in the presence of adequate light.

3.3.3 Agar well diffusion

The antibacterial agar well diffusion assay was conducted by following the method described and used previously (Taye *et al*., 2011; Andualem *et al*., 2014). Bacterial broth culture was prepared to a density of 0.5 McFarland standardas stated above.The standardized broth suspensions were streaked on the sterile MHA plates or MHA with 5% v/v sheep blood (for *streptococcus* species to enrich the medium with nutrient) in 150 millimeter (mm) diameter sterile petri-dish, which was incubated at 37^0C for 24 hours to check for sterility. The bacteria were inoculated to the plates using a sterile swab in such a way ensure ever coverage of the plates and a uniform thick lawn of growth following incubation. To ensure even distribution of inoculum, the plate was rotated approximately 60^0 each time and finally rim of the agar was swabbed. Then, the seeded media were allowed to dry at room temperature for 30 minutes. On

each plate, wells were made with a 6 mm diameter sterilized cork borer and labeled with numbers corresponding to the extract placed (Taye *et al.,* 2011). The corresponding wells were filled with 100 µl of 800 mg/ml, 400 mg/ml and 200 mg/ml of the solutions of the crude extract (dissolved in water) and each of the solvent fractions dissolved in different solvents (aqueous fraction in water, n-butanol fraction in 5% DMSO in water, ethyl acetate fraction in 10% DMSO in water (**NB**: the examiners did not agree with the use of 10% of DMSO)). In addition, the commercial antibiotic discs of ampicillin 10 µg (for *streptococcus* species), ceftriaxone 30 µg (for *E. coli, S. aureus, P. aeruginosa and K. pneumoniae* species), and ciprofloxacin 5 µg (for *S. typhi* and *S. flexneri* species) were used as a positive control. The positive controls were selected based on the susceptibility of the bacterium used (CLSI, 2012). The solvents used to dissolve the crude extract and solvent fractions were used as a negative control. Then, the plates were left undisturbed for about 2 h at roomgive time to diffusion on the inoculated agar. The plates for the antibacterial study were incubated at 37^0C for 24 hours. The resulting diameters of zones of inhibition, including the diameter of the well, were measured using a ruler and reported in millimeter. The experiment was performed in three independent tests for each bacterium and the mean of zones of inhibition was calculated for each extract and the standard antibiotics.

The antifungal activities of the extracts were also determined using agar well diffusion method.The fungal broth cultures were standardized in the way stated above. The standardized fungal broth cultures were streaked onto the 150mm sterile PDA using sterile swab by rotating approximately 60^o each time to ensure even distribution of the inoculum. Petri-plates were left for 30 minutes at room temperature. After that, equidistant wells with a diameter of 6 mm were punched aseptically using sterile cork borer. Then, 100 µl volumes of both crude extract and solvent fractions at concentration of 200, 400 and 800 mg/ml were introduced in to the wells using micropipette. Positive controls (flucytosine 50µg for *C. albicans* and nystatin 100µg for *T. mentagrophytes*) and negative control (solvents used to dissolve extracts) were also added. Then, the plates were placed undisturbed at room temperature for 2 hours on prepared area to allow proper diffusion. The plates for antifungal study were then incubated at 35^oC for 24 hours in the case of *C. albicans* (Scorzoni *et al*., 2007) and 48 hours for *T. mentagrophytes*. The resulting diameters of zones of inhibition, including the diameter of the well, were measured using a ruler

and reported in millimeter. The experiment was performed in triplicates for both fungi and the mean of zones of inhibition was calculated for each extract and the standard antifungal agents.

3.3.4 Determination of the minimum inhibitory concentration (MIC) of the extract

The crude extract and solvent fractions that showed antimicrobial activity by agar well diffusion method were subjected to serial micro-broth dilution technique to determine their MIC using resazurin as a cell growth indicator (Gahlaut & Chhillar, 2013). Under aseptic conditions, 96 well micro-titer plates (Greiner Bio-One, Germany) were used for resazurin based micro-titer dilution assay.

The minimum inhibitory concentrations of extracts against bacterial species were determined as follows. The broth media were prepared in accordance with the manufactures' guideline; the crude extract and each solvent fraction with concentration of 400µg/ml; the bacterial suspension was standardized according to CLSI guideline (CLSI, 2012) to turbidity equivalent to 0.5 McFerland standard and diluted in the ratio of 1:20 in the respective broth; the resazurin solution was prepared by dissolving 0.01g of the resazurin powder in 100 mL of autoclaved distilled water. Then, all wells of micro-titer plate were filled with 100µl of Muller Hinton broth (except for *streptococcus* species filled with Brain Heart Infusion broth). After that, first row of micro-titer plate was filled with 100 µl of crude extract or solvent fractions solutions. Two fold serial dilution (throughout the row) was carried out by evenly mixing and transferring 100 µl test material from wells of first row to the subsequent wells along the next column of the same row using micropipette until 10^{th} column from which 100 µl of diluted extract was taken and discarded. Then, 20 µl of this suspension was added to every well in the micro-titer plate except the 12^{th} column which is reserved as sterility control and 7^{th} and 8^{th} row which are reserved as color contrast control. The 25 µl of resazurin solution prepared was added to each well in the micro-titer plate. Finally, to avoid the dehydration of bacterial culture, each plate was wrapped loosely with parafilm (American National Can ™, Greenwich) to ensure that bacteria did not become dehydrated and the plates were incubated in temperature controlled incubator at 37° C for 24 h (Bertanha *et al.*, 2013).

More or less, the procedures used for determination of the minimum inhibitory concentrations of extracts against fungal species were the same as the one for bacterial species. The differences

were on the media used for fungal species, which was potato dextrose broth and the time of incubation was 48 hours for *T. mentagrophytes*.

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

In this experiment, each micro-titer plate had a set of three controls. One of the controls was growth control which contained all solution except the plant extracts (11^{th} column). It was used to determine the viability and growth of bacteria or fungus in media. The other control was the color contrast control (7^{th} and 8^{th} row) which was filled with all solutions except the bacterial or fungal suspension. The color contrast control used to compare the color change in the rows with bacteria or fungus. The remaining control was sterility control which was the 12^{th} column contained media and resazurin solution. The sterility control was used to test the sterility of the plate, the media and resazurin solution.

3.3.5 Determination of minimum bactericidal and fungicidal concentration

The minimum bactericidal concentration (MBC) was determined by sub-culturing the contents of wells from the MIC results for individual bacterium to antimicrobial free agar as described in different studies (Yahya, 2011). In this technique, the contents of all wells containing a concentration of test material greater or equal to MIC value from each triplicate in the MIC determination test, was streaked using a sterile wire loop on MHA or MHA supplemented with 5% sheep blood (for streptococcus species) aseptically and incubated at 37ºC for 24 h. The lowest concentration of the extract which showed no bacterial growth after incubation was observed for each triplicate and noted as the MBC. The average value was taken for the MBC of test material against each bacterium.

The minimum fungicidal concentration (MFC) was determined by sub-culturing the contents of wells from the MIC results for each fungus to antimicrobial free agar. The content of the well with concentration of greater or equal to MIC was sub-cultured from each triplicate of MIC determined micro-titer plate to aseptically prepared potato dextrose agar in petri-dish. The lowest concentration with no fungal growth was observed for each triplicate and the average taken as MFC.

3.4 Preliminary phytochemical screening

The presence of phytochemicals like alkaloids, flavonoids, saponins, tannins, terpenoids and cardiac glycosides in crude and solvent fractions of *Justicia schimperiana* was evaluated using standard testing methods.

Test for alkaloids

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

Test for saponins

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

Test for polyphenols (phenolic compounds)

About 0.25 g of 80% methanol extract and each solvent fraction was treated with few drops of 5% neutral ferric chloride solution; the appearance of a greenish precipitate indicated the presence of phenols (Shetty *et al.,* 2016).

Test for flavonoids

Ten ml of ethyl acetate was added into a test tube having 0.25 g of 80% methanol extract and each solvent fraction, and heated on a water bath for 3 minutes. The mixture was cooled and filtered. Then, about 4 ml of the filtrate was taken and shaken with 1 ml of dilute ammonia

solution. The layers were allowed to separate and the yellow color in the ammonial layer indicated the presence of flavonoids (Shetty *et al.*, 2016).

Test for terpenoids

Two ml of chloroform was added into a test tube having 0.25 g of 80% methanol extract and each solvent fraction. Then, 3ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids (Shetty *et al.*, 2016).

Test for tannins

About 0.25 g of 80% methanol extract and each solvent fraction was boiled in 10 ml of water in a test tube and then filtered. Three drops of 0.1% ferric chloride were added to the filtrate. Presence of tannins was confirmed by the formation of brown greenish or blue-black color (Nwadiaro *et al.*, 2015).

Tests for cardiac glycosides

To 0.25 g of 80% methanol extract and each solvent fraction were diluted into 5 ml water, 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was under lied with 1 ml of concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the glacial acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer (Shetty *et al.*, 2016).

3.5 Statistical analysis

The experimental data are expressed as mean ± Standard Error of the Mean (SEM). Data were analyzed using the Statistical Package for the Social Sciences (SPSS), version 20.0 software. The statistical differences of the mean zone of inhibition of crude extract and solvent fractions for individual bacterium was carried out by employing one way analysis of variance (ANOVA) followed by Tukey Post Hoc Multiple Comparison test at a significance level of $P<0.05$. The MIC, MBC and MFC were analyzed using descriptive statistics using SPSS software. Moreover, the concentration dependent antimicrobial activities of the crude extract and solvent fractions for

each bacterial and fungal species were determined by linear regression analysis using the SPSS software.

4 Results

4.1 Activity against gram positive bacteria

According to agar well diffusion, the growth inhibition of the crude extract with the lowest tested concentration (200mg/ml) against gram positive bacteria was significantly smaller than the inhibition of the positive controls ($P<0.05$). Solvent fractions also inhibited significantly smaller area than positive control ($P<0.05$) except with the n-butanol fraction. On the other hand, the growth inhibition of the crude extract with the highest tested concentration (800mg/ml) against gram positive bacteria was comparable to the inhibition of positive controls. Similarly, the inhibition exerted by most of the tested fractions was comparable to that of positive controls (Table 1).

The crude extract of the plant inhibited the growth of gram positive bacteria in a concentration dependent manner with R^2 value ranging from 0.76 (for *S. pneumoniae*) to 0.89 (for *S. aureus*). Similarly, solvent fraction also inhibited the growth of tested gram positive bacteria in concentration dependent manner. For the aqueous fraction the R^2 value was ranging from 0.79 (for *S. pyogenes*) to 0.84 (for *S. pneumoniae*), for the n-butanol fraction, it was ranging from 0.69 (*S. pneumonia*) to 0.89 (*S. aureus*) and for ethyl acetate fraction, it was ranging from 0.81(*P. pneumoniae*) to 0.89 (*S. aureus*).

The growth inhibition of the crude extract against tested gram positive bacteria was slightly comparable to most of tested solvent fractions. But, the n-butanol fraction inhibited the largest area compared to the crude extract and the other tested solvent fractions (Table 1). And all the negative controls, which were the vehicles used to dissolve extracts, were not able show any detectable inhibition against gram positive bacteria.

Table 1: Zone of inhibition (mm) of the crude extract and the solvent fractions of the leaf of *Justicia schimperiana* against gram positive bacteria.

Category of test	Concentration	Bacterial species		
		S. aureus	*S. pneumonia*	*S. pyogenes*
80% methanol extract	200mg/ml	16.0 ± 0.6^{a3d3c1}	17.0 ± 0.6^{a3d2c1}	16.0 ± 6^{a3d2}
	400 mg/ml	18.7 ± 0.3^{a3b1}	21.3 ± 0.9^{a3b1}	18.0 ± 0.6^{a3d1}
	800 mg/ml	21.7 ± 0.3^{b3}	23.3 ± 0.3^{a3b2}	21.7 ± 0.7^{a2b2c1}
	Cef 10µg	23.3±0.7	NT	NT
	Amp 10µg	NT	31.3±0.7	27.7±0.9
Aqueous fraction	200mg/ml	16.7 ± 0.7^{a3d3}	17.0 ± 0.6^{a3d2}	18.7 ± 0.3^{a3d1}
	400mg/ml	20.7 ± 0.7^{b1}	18.7 ± 0.3^{a3}	20.7 ± 0.7^{a3}
	800mg/ml	23.0 ± 0.6^{b3}	20.7 ± 0.3^{a3b2}	22.3 ± 0.3^{a2b1}
	Cef 10µg	23.3±0.7	NT	NT
	Amp 10µg	NT	31.3±0.7	27.7±0.9
n-Butanol fraction	200mg/ml	22.0 ± 00^{d2}	28.0 ± 1.0^{d1}	24.0 ± 0.6^{a1d1}
	400mg/ml	23.7 ± 0.3^{d1}	30.3±0.9	25.3±0.7
	800mg/ml	26.0 ± 0.6^{a1b2c1}	32.3 ± 0.3^{b1}	27.3 ± 0.3^{b1}
	Cef 10µg	23.3±0.7	NT	NT
	Amp 10 µg	NT	31.3±0.7	27.7±0.9
Ethyl acetate fraction	200mg/ml	17.7 ± 0.3^{a3c1d2}	18.7 ± 0.3^{a3c1d3}	17.3 ± 0.3^{a3d1}
	400mg/ml	20.33 ± 0.3^{a1b1}	20.7 ± 0.3^{a3b1d1}	19.00 ± 0.6^{a3}
	800mg/ml	22.00 ± 0.6^{b2}	22.7 ± 0.3^{a3b3c1}	21.3 ± 0.7^{a3b1}
	Cef 10µg	23.3±0.7	NT	NT
	Amp 10µg	NT	31.3±0.7	27.7±0.9

Values are expressed as Mean ± S.E.M (n=3), analysis was performed with One-Way ANOVA followed by Tukey test;[a] compared to positive control; [b] compared to 200mg/ml; [c] compared to 400mg/ml; [d] compared to 800mg/ml; [1] $P< 0.05$, [2] $P<0.01$[3] $P<0.001$. The negative control has shown no antibacterial activity. The positive controls: Cef = ceftriaxone, Amp=ampicillin, NT= Not tested

4.2 Activity against gram negative bacteria

The growth inhibition of the crude extract of the plant against tested gram negative bacteria was smaller than its inhibition against gram positive bacteria. With lowest tested concentration of the crude extract and most solvent fractions against gram negative bacteria was significantly smaller than inhibition of positive control except the n-butanol fraction ($P<0.05$). With highest tested concentration of the crude extract the growth of *E. coli*, *K. pneumoniae* and *S. flexneri* was inhibited comparable area as the positive control. The growth inhibition of the crude extract against *P. aeruginosa* and *S. typhi* was significantly smaller than inhibition of the positive control. Similarly, the growth inhibition of the aqueous fraction and the ethyl acetate fraction was smaller for *P. aeruginosa* and *S. typhi*. The n-butanol fraction with highest tested concentration inhibited comparable area as the positive control.

The growth inhibition of the crude extract against gram negative bacteria was concentration dependent with R^2 value ranging from 0.81 (*P. aeruginosa*) to 0.84 (*K. pneumonia*). In the same way, the solvent fractions inhibition was also concentration dependent. And the n-buanol fraction inhibited the largest area against gram negative bacteria the same manner as gram positive bacteria. The negative control was not able to show any detectable inhibition against tested gram negative bacteria.

Table 2: Zone of inhibition (in mm) of the crude extract and solvent fractions of the leaf of *Justicia schimperiana* against gram negative bacteria.

Category of test	**Concentration and standard**	**Bacteria**				
		S. typhi	*K. pneumoniae*	*P.aeruginosa*	*E. coli*	*S. flexneri*
80% methanol extract	200mg/ml	12.7 ± 0.3^{a3d1}	15.0 ± 0.6^{a3d3}	9.0 ± 0.6^{a3d3c2}	15.3 ± 0.3^{a3c3d3}	21.7 ± 0.7^{a3d1}
	400mg/ml	15.0 ± 0.6^{a3}	16.7 ± 0.3^{a3d1}	11.7 ± 0.3^{a3b2}	19.7 ± 0.7^{a1b3d3}	23.3 ± 0.7^{a1}
	800mg/ml	17.0 ± 0.6^{a3b1}	18.7 ± 0.3^{b3c1}	13.3 ± 0.3^{a3b3}	23.0 ± 0.6^{b3c3}	26.7 ± 0.3^{b1}
	Cef 10µg	NT	20.3±0.3	24.3±0.3	24.7±0.33	NT
	Cipro 5µg	25.0±0.6	NT	NT	NT	28.3±0.7
Aqueous fraction	200mg/ml	10.7 ± 0.9^{a3d1}	16.0 ± 0.6^{a2d1}	8.3 ± 0.9^{a3d2}	12.0 ± 0.6^{a3d2c1}	19.3 ± 0.3^{a3d2}
	400mg/ml	12.7 ± 0.9^{a3}	17.0 ± 0.6^{a2}	11.3 ± 0.9^{a3}	14.7 ± 0.3^{a3b1}	23.0 ± 00^{a3}
	800mg/ml	14.7 ± 0.3^{a3b1}	19.0 ± 0.6^{b1}	12.7 ± 0.3^{a3b2}	16.0 ± 0.6^{a3b2}	25.0 ± 0.6^{a3b2}
	Cef 10µg	NT	20.3±0.3	24.3±0.3	24.7±0.3	NT
	Cipro 5µg	25.0±0.6	NT	NT	NT	28.3±0.7
n-butanol fraction	200mg/ml	18.0 ± 0.6^{a3d2}	18.3 ± 0.7^{d2}	16.3 ± 0.3^{a3d3}	20.7 ± 0.3^{a3c1d2}	25.3 ± 0.3^{d2}
	400mg/ml	20.7 ± 0.7^{a2}	19.0±0.0	18.7 ± 0.3^{a3d1}	22.7 ± 0.3^{a1b1}	27.3±0.9
	800mg/ml	23.0 ± 0.6^{b2}	21.0 ± 0.6^{b2}	22.3 ± 0.9^{b3c1}	23.3 ± 0.3^{b2}	30.0 ± 0.6^{b2}
	Cef 10µg	NT	20.3±0.3	24.3±0.3	24.7±0.3	NT
	Cipro 5µg	25.0±0.6	NT	NT	NT	28.3±0.7
Ethyl acetate fraction	200mg/ml	15.3 ± 0.3^{a3d2}	17.7 ± 0.7^{a2d1}	10.0 ± 0.6^{a3c1d3}	18.3 ± 0.7^{a3d2}	23.7 ± 0.7^{a2c1d2}
	400mg/ml	17.7 ± 0.7^{a3}	19.0±0.6	13.0 ± 0.6^{a3b1}	20.3 ± 0.3^{a2}	26.3 ± 0.3^{b1}
	800mg/ml	19.0 ± 0.6^{a3b2}	21.0 ± 0.6^{b1}	15.0 ± 0.6^{a3b3}	22.0 ± 0.6^{a1b2}	28.0 ± 0.6^{b2}
	Cef 10µg	NT	20.3±0.3	24.3±0.3	24.7±0.3	NT
	Cipro 5µg	25.0±0.6	NT	NT	NT	28.3±0.7

Values are expressed as Mean ± S.E.M (n=3), analysis was performed with One-Way ANOVA followed by Tukey test;[a] compared to positive control; [b] compared to 200mg/ml; [c] compared to 400mg/ml; [d] compared to 800mg/ml; [1] $P< 0.05$, [2] $P<0.01$ [3] $P<0.001$. The negative control has shown no antibacterial activity. The positive controls: cef = ceftriaxone, cip=ciprofloxacin. NT= Not tested, - = no activity

4.3 Activity against fungi species

According to agar well diffusion test, the inhibitions of the crude extract and solvent fractions with the lowest tested concentration were significantly smaller than the inhibitions of respective positive control except with the n-butanol fraction. The inhibition of highest tested concentration of the crude extract and the ethyl acetate fraction were comparable to the positive control inhibition ($p=0.05$). But, the inhibition of the tested highest concentration of the aqueous fractions was significantly smaller than inhibition of respective positive control ($p=0.05$) (Table 3). And the growth inhibition of the crude extract and the solvent fractions was concentration dependent with R^2 value for crude extract was 0.84(*C. albicans*) and 0.83(*T. mentagrophytes*).

Table 3: Zone of inhibition (in mm) of the crude extract and solvent fractions of the leaf of *Justicia schmperiana* against fungal species.

Category of test	**Concentration**	**Fungus**	
		C. albicans	*T. mentagrophytes*
80% methanol extract	200mg/ml	14.7 ± 0.3^{a3d2}	14.0 ± 0.6^{a2d1}
	400mg/ml	16.3 ± 0.3^{a2}	16.0 ± 0.6^{a1}
	800mg/ml	18.0 ± 0.6^{b2}	17.3 ± 0.3^{b1}
	Keto 50µg	20.0±0.6	NT
	Nys 100 unit	NT	18.3±0.3
Aqueous fraction	200mg/ml	13.0 ± 0.6^{a3d1}	11.3 ± 0.7^{a3d1}
	400mg/ml	14.7 ± 0.3^{a3}	13.3 ± 0.6^{a3}
	800mg/ml	16.3 ± 0.3^{a2b1}	13.7 ± 0.3^{a3b1}
	Keto 50µg	20.0±0.6	NT
	Nys 100unit	NT	18.3±0.3
n-butanol fraction	200mg/ml	18.3 ± 0.3^{d2}	18.0 ± 0.6^{d2}
	400mg/ml	20.3 ± 0.9^{d1}	20.0±0.6
	800mg/ml	22.7 ± 0.3^{a1b2c1}	21.7 ± 0.3^{a2b2}
	Keto 50µg	20.0±0.6	NT
	Nys 100 unit	NT	18.3±0.3
Ethyl acetate fraction	200mg/ml	12.7 ± 0.3^{a3c1d3}	12.3 ± 0.3^{a3d3}
	400mg/ml	15.3 ± 0.3^{a3b1d1}	14.0 ± 0.6^{a3d1}
	800mg/ml	18.0 ± 0.6^{b3c1}	16.3 ± 0.7^{b3c1}
	Keto 50µg	20.0 ± 0.6	NT
	Nys 100 unit	NT	18.3±0.3

Values are expressed as Mean ± S.E.M (n=3), analysis was performed with One-Way ANOVA followed by Tukey test;[a] compared to positive control; [b] compared to 200mg/ml; [c] compared to 400mg/ml; [d] compared to 800mg/ml; [1] $P< 0.05$, [2] $P<0.01$ [3] $P<0.001$.The negative control has shown no antibacterial activity. The positive controls: Nys= nystatin, Keto= ketoconazole. NT= Not tested, - = no activity

4.4 Minimum inhibitory concentration of crude extract and solvent fractions against bacterial species

As presented in Table 4, the minimum inhibitory concentration of the crude extract against tested bacterial species were less than 10mg/ml for tested bacteria species other than *P. aeruginosa* and *S. typhi*. The smallest MIC of the crude extract was for *S. pneumonia,* which was 1.56mg/ml. The n-butanol fraction had inhibited the growth of bacteria in lowest concentration than crude extract and other solvent fractions. The MIC of the aqueous fraction was between 1.3mg/ml (for *S. pneumonia*) and 50mg/ml (for *P. aeruginosa* and *S. typhi*). The ethyl acetate extracts was less potent than the other extracts against most bacterial strains (The mean MIC ranges from 4.17mg/ml for *S. pneumoniae* to 100mg/ml for *P. aeruginosa*) (Table 4). Among the tested bacterial species, *P. aeruginosa* and *S. typhi* did not respond to lower concentration; they needed higher concentration of active extracts (12.5mg/ml to 100mg/ml for *P. aeruginosa* and 25mg/ml to 50mg/ml for *S. typhi*) to be inhibited. On the other hand, *S. pneumoniae* was inhibited with the lowest concentration than other bacterial species (Table 4).

Table 4: Minimum inhibitory concentration (mg/ml) of the crude extract and solvent fraction of leaf of *Justicia schimperiana* against bacterial species.

Bacteria	80% methanol extract	Aqueous fraction	n-butanol fraction	Ethyl acetate fraction
S. aureus	8.33±2.08	12.5±0.00	1.3±0.26	12.5±0.00
P. aeruginosa	25±0.00	50±0.00	12.5±0.00	100±0.00
E. coli	12.50±0.00	25±0.00	6.25±0.00	50±0.00
S. flexneri	4.17±1.04	4.17±1.04	0.78±0.00	5.21±1.04
S. typhi	25±0.00	50±0.00	25±0.00	50±0.00
K. pneumonia	5.2±1.04	5.21±1.04	4.17±1.04	6.25±0.00
S. pneumonia	1.56±0.00	1.3±0.26	0.52±0.13	4.17±1.04
S. pyogenes	4.17±1.04	2.08±0.52	1.3±0.26	6.25±0.00

Values are expressed as Mean ± S.E.M (n=3)

4.5 Minimum inhibitory concentration of crude extract and solvent fractions against fungal species

The MIC of crude extract against fungal species was 12.5 mg/ml for both tested fungal species. The aqueous fraction was less potent than the crude extract and the other solvent fractions. The fungal species also inhibited with lower concentration of the n-butanol like tested bacterial species (1.3mg/ml for *C. albicans* and 1.56mg/ml for *T. mentagrophytes*). On the other hand, the ethyl acetate fraction inhibited the fungal stains with lower concentration than aqueous fraction with average MIC of 5.2mg/ml *C. albicans* and 12.5mg/ml for *T. mentagrophytes* (Table 5).

Table 5: Minimum inhibitory concentration (mg/ml) of crude and solvent fraction of leaf of *Justicia schimperiana* against fungal species.

Fungus	80% methanol extract	Aqueous fraction	n-butanol fraction	Ethyl acetate fraction
C. albicans	12.5±.00	25±.00	1.3±.26	5.2±1.04
T. mentagrophytes	12.5±.00	50±.00	1.56±.00	12.5±.00

Values are expressed as Mean ± S.E.M (n=3)

4.6 Minimum bactericidal concentration of crude extract and solvent fractions

Based on the MBC determination tests, the n-butanol extract MBC against tested bacteria was smallest compared to other extracts in the same way as MIC. The MBC of n-butanol fraction ranged from 1.04mg/ml for *S. pneumoniae* to 50mg/ml for *S. typhi*. The crude extract's highest MBC was 100mg/ml for *P. aeruginosa* and the lowest MBC was 4.17mg/ml for *S. pneumoniae*. The aqueous and the ethyl acetate fractions exhibited bactericidal activity with higher concentrations (Table 6).

Table 6: Minimum bactericidal concentration of the crude extract and solvent fraction of leaf of *Justicia schimperiana* against bacterial species.

Bacteria	80% methanol extract	Aqueous fraction	n-butanol fraction	Ethyl acetate fraction
S. aureus	12.50±0.00	25±0.00	2.08±0.52	100±0.00
P. aeruginosa	100±0.00	100±0.00	25±0.00	200±0.00
E. coli	25.00±0.00	50±0.00	12.5±0.00	100±0.0
S. flexneri	6.25±0.00	8.33±2.08	1.56±0.00	6.25±0.00
S. typhi	50±0.00	100±0.00	50±0.00	200±0.00
K. pneumoniae	8.33±2.08	12.5±0.00	6.25±0.00	12.5±0.00
S. pneumoniae	4.17±1.04	3.13±0.00	1.04±0.26	6.25±0.00
S. pyogenes	8.33±2.08	3.13±0.00	2.61±0.52	10.42±2.08

Values are expressed as Mean ± S.E.M (n=3), NT=Not tested

4.7 Minimum fungicidal concentration of crude extract and solvent fractions

The crude extract and the ethyl acetate fraction MFC against the tested fungal strains was 25mg/ml for *C. albicans* and 50mg/ml for *T. mentagrophytes*. For the aqueous fraction, fungal growth on sub cultured media was not observed with higher concentration than the crude and the other fractions. On the other hand, the n-butanol fraction MFC was smallest compared to other fractions (Table 7).

Table 7: Minimum fungicidal concentration of the crude extract and solvent fraction of leaf of *Justicia schimperiana* against fungal species.

Fungus	80% methanol fraction	Aqueous fraction	n-butanol fraction	Ethyl acetate fraction
C. albicans	25±.00	50±.00	2.6±.52	25±.00
T. mentagrophytes	50±.00	200±.00	3.13±.00	50±.00

Values are expressed as Mean ± S.E.M (n=3)

4.8 Phytochemical constituents of the crude extract and solvent fractions

As shown in Table 8, phytochemical analysis indicated the presence of alkaloids, saponins, polyphenols, terpenoids and flavonoids in crude extract and it's the n-butanol fraction. The aqueous fraction of the crude extract was positive for alkaloids, polyphenols and terpenoids among the tested phytochemicals. Alkaloids, polyphenols and flavonoids were also present in the ethyl acetate fraction of the crude extract.

Table 8: Phytochemical composition of the crude extract and solvent fractions of the leaf of *Justicia schimperiana*

Metabolites tested	**80% methanol extract**	**Solvent fractions**		
		Aqueous fraction	n-butanol fraction	Ethyl acetate fraction
Alkaloids	+	+	+	+
Saponins	+	-	+	-
Tannins	+	+	+	-
Polyphenols	+	+	+	+
Terpenoids	+	-	+	-
Flavonoids	+	+	+	+
Cardiac Glycosides	+-	-	-	-

+ Present, - absent

5 Discussion

The increased incidence of antimicrobial resistance and the dearth of new novel antimicrobial lead, indicate that there should be search for antimicrobial agents. The antibacterial activity of extracts of different parts of *Justicia schmperiana*has been reported in earlier studies (Murthy *et al.,* 1993). The current studywas undertaken to further investigate the antimicrobial activity (determination of zone of inhibition, MIC and MBC) of the 80% methanol leaf extract and different solvent fractions against a wider microbial species, including bacteria and fungi.

The 80% methanol extract of *Justicia schimpriana* inhibited the growth of both tested gram negative and gram positive bacteria while the negative control used in the study was not able to inhibit any detectable area. This difference in growth inhibition between the negative control and the crude extract indicate the presenceantibacterial constituents in the crude extract; agrees with previous finding on antibacterial activity of the leaves extract of the plant (Murthy *et al.,* 1993). The crude extract of *Justicia schimperiana* was positive for qualitative phytochemical screening test of alkaloid, tannins, saponin, flavonoids, phenols and terpenoids. There are reports on antibacterial activity of alkaloids against gram positive and negative bacteria (Singh & Verma, 2011). The remaining secondary metabolites (saponin, tannins, flavonoids, polyphenols and terpenoids) found in the crude extract of *Justicia schimperiana* also reported to have antibacterial activity against pathogenic bacterial species(Abegaz & Kebede, 1995;Soetan *et al.,* 2006; Jasmine *et al.,* 2007; Wink, 2015). So, the antibacterial activity of the crude extract of the leaf of *Justicia schimperiana* might be because of synergistic or additive effect of these secondary metabolites (Akharaiyi, 2011).

Solvent fractions of the 80% methanol extract of the plant inhibited the growth of most tested bacterial species with varied extent. The solvents used for dissolving solvent fractions were used as negative control and were not able to inhibit any detectable area. This indicates the presence of antibacterial constitutes in the tested solvent fraction. The extracts form leaves *Justicia schimperiana* in earlier studies also inhibited all tested bacterial species (Murthy *et al.,* 1993) which agrees with current finding.

The inhibition of crude extract and solvent fractions (except n-butanol fraction) against *S. pneumoniae, S. pyogenes, S. typhi* and *P. aeruginosa* was significantly smaller than the inhibition of respective positive controls even with the highest tested concentration. While the inhibitions

of against *S. aureus, K. pneumoniae, E. coli* and *S. flexneri* were comparable to positive controls with concentration of 800mg/ml. By comparing the concentrations of extract used with positive controls, still positive controls were much more potent than the extracts. The presence of many components which have no antimicrobial activity in the plant extract could be the reason for the lower potency of the crude extracts and solvent fractions when compared to respective positive controls (Cowan, 1999).

Among all solvent fractions and crude extract, the n-butanol fraction showed larger zone of inhibition against all tested bacterial species. The n-butanol fraction with the highest tested concentration inhibited significantly greater area than respective positive controls used against *S. aureus, K. pneumomiae, E. coli* and *S. flexneri*. And also against the remaining tested bacterial species (*S. pneumoniae, S. pyogenes, S. typhi* and *P. aeruginosa*), the n-butanol fraction inhibited comparable area as the positive control. Moreover,the phytochemical test for secondary metabolites in n-butanol had shown the presence of alkaloids, saponins, tannins, flavonoids, phenols, and terpenoids. The presence of most of active phytochemical in n-butanol fraction with its greater antibacterial activity might indicate that most of active antibacterial constitutes are relatively polar which agree with previous finding reported that methanol extract has showed greater antibacterial activity (Murthy *et al.,* 1993). If furthert work is to be pursued on this plant for isolation of antimicrobialy active copmopounds, it would be wise to consider the n-butanol fraction.

The aqueous and the ethyl acetate fractions inhibited a slightly smaller area than the crude extract. This decrease in activity could be due to absence of some secondary metabolites, which are present in the crude extract. The phytochemical screening showed the presence of tannins, flavonoids and alkaloids in the aqueous fraction and flavonoids, alkaloid and polyphenol in the ethyl acetate fraction.

Among tested bacterial species *S. typhi* and *P. aeruginosa* showed lesser susceptibility to crude extract and solvent fraction of leaves of *Justicia schimperiana*. These could be because of 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 & Nandita, 2014; Tanya & Daniel, 2009).

Inhibition area of the crude extract and all solvent fractions increased with concentration, which implies that the antibacterial activity of the crude extract and solvent fractions were dose dependent; this proportional increase in inhibitory activity as the concentration of extract increase were 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 and solvent fractions. The mean zone of inhibitions of the crude extract and all solvent fractions were higher for gram positives than gram negatives at their comparable concentration with the exception of *S. flexneri*. These differences in susceptibility between gram positive and gram negative bacteria could be because of their difference in their cell wall composition (Nikaido & Vaara, 1985). 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 extract and solvent fractions 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*., 2001).

There appears to be negative correlation between the minimum inhibitory concentrations of the crude extract and the zone of inhibition obtained with the agar well diffusion test. This is true for the n-butanol fraction, where the lowest concentration inhibited bacterial growth and the fraction also exhibited maximal inhibition.

The minimum bactericidal concentration test showed that the MBCs for bacterial species of all tested plant extracts were directly proportional to their MIC value. The bacterial species inhibited in lower concentration of plant extracts also show no growth in subculture media with relatively lower concentration. To classify an antibacterial agent as bactericidal the MBC/MIC ratio should be between 1:1 and 4:1 (French, 2006). According to this, the crude extract and most solvents fraction were bactericidal except ethyl acetate fraction whose MBC/MIC ratio was 8:1 for *S. aureus*.

According to the current study, the 80% methanol extract of *Justicia schimperiana* was also active against tested fungal species. This finding agrees with previous report on anticandidial activity of powdered root of*Justicia schimperiana* which inhibited the growth of *C. albicans* in

agar media (Kothai, 2012). The phytochemicals found in 80% methanol extract of *Justicia schimperiana* could be the reason for its antifungal activity. Among the phytochemicals found in crude extract of *Justicia schimperiana*: saponins, tannis, flavonoids, terpenoids, alkaloids and phenolics had been reported to have antifungal activity (Tim & Andrew, 2005; Joyce *et al.*, 2007; Singh, *et al.*, 2007; Anjana *et al.*, 2010; Carlos *et al.*, 2014; Wink, 2015).

Solvent fractions of crude extract of leaf of *Justicia schmperiana*were also able to inhibit detectable area against the tested fungal species. The solvents of all solvent fractions, which have been used as negative control, were not able to inhibit any detectable area. This indicates the presence antifungal constitutes in most of tested solvent fractions. The n-butanol fractions zone inhibition was greater than the zone inhibition of the crude extracts and other solvent fractions. The composition and concentration of phytochemicals present in n-butanol fraction of crude extract of *Justicia schimperiana* could be the reason for its greater antifungal activity. All the phytochemicals found in crude extract are also found in n-butanol fraction.The ethyl acetate fraction showed comparable antifungal activity to aqueous fraction. The antifungal activity of ethyl acetate fraction could be due to presence of active phytochemicals (flavonoids, alkaloid and phenol) (Tim & Andrew, 2005; Singh, *et al.*, 2007; Carlos *et al.*, 2014).

The minimum inhibitory concentrations of the crude extract and most of the solvent fractions go in agreement with its inhibition in agar well diffusion test for most extracts. This is true for the n-butanol fraction which inhibited the growth of test fungi with lowest concentration and exhibited the highest inhibition in agar well diffusion test. However, the ethyl acetate fraction has shown greater potency than solvent fractions other than n-butanol unlike the agar well test result. The diffusion in media prepared by water as solvent could be factor for its small inhibition (Sanchez *et al*., 2010).

The minimum fungicidal concentration showed that the MFCs of the crude extract and the solvent fraction against tested fungal species were directly proportional to their respective MICs value. Antifungal agent considered as fungicidal when the ratio of MFC/MIC ranges between 1:1 and 2:1 (Ige *et al*., 2015). According to this, the crude extract and the aqueous fraction for *C. albicans* and n-butanol fraction for both tested fungal species were fungicidal. The crude extract and the aqueous fraction for *T. mentagrophytes* and the ethyl acetate fraction were not fungicidal because their respective MFC/MIC ratio was above 2:1.

The medicinal potential of most plants majorly depends on the presence of active phytochemical compounds called secondary metabolites (Das *et al.,* 2010). The phytochemical screening test of the 80% methanol extract and solvent fractions of *Justicia schimperiana* had revealed the presence of the following secondary metabolites: alkaloid, tannins, saponin, flavonoids, polyphenols, and terpenoids. Even though, the mechanism of the antimicrobial activity of the crude extract and solvent fractions is not known, there is possible speculation which is based on the secondary metabolites present in the extracts.

The antimicrobial activity of most extracts of *Justicia schimperiana* could be contributed by alkaloids they possess. Alkaloids are reported to have antimicrobial activity against different pathogenic microorganisms (Singh *et al.,* 2011; Charles*et al.*,1987). Possible mechanism of action of extracts having alkaloids could be disruption of cell membranes or inhibition of the protein synthesis of bacteria (Sathyabama & Kingsley, 2013).

Tannins are the other compounds that have been found in extracts of *Justicia schimperiana* and reported in different studies to have antimicrobial activities (Wink, 2005). The antimicrobial mechanism of tannins might be because of its membrane damaging effects and inhibition of metabolic pathways of bacteria like oxidative phosphorylation which could lead to death of the microorganism (Funatogawa *et al*., 2004; Scalbert, 1991).

The phenolic compounds are among secondary metabolites found in the plant extract and reported to have antimicrobial activity (Thippeswamy *et al*., 2013). The mechanisms thought to be responsible for *Justicia schimperiana* extracts with phenolic compounds could be; phenolic toxicity to microorganisms including enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason & Wasserman, 1987).

Saponins are among secondary metabolites found in the crude extract and some solvent fractions of *Justicia schimperiana*. There are reports on antimicrobial activity of saponins (Soetan *et al*., 2006; Mattana *et al*., 2010). Saponins could contribute for antimicrobial activity of current plant extracts. The antimicrobial mechanism of saponins is not well understood.

Flavonoids are hydroxylated aromatic substances which are known to be synthesized by plants in response to microbial infection (Dixon *et al*., 1983). So, it not surprising that they have been

found *in-vitro* to be effective antimicrobial substances against a wide array of microorganisms (Batista, 1994; Borris, 1996). Their activity is probably due to their ability to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al*., 1996). The antimicrobial activity of extracts especially against gram positive bacteria could be due to the presence of flavonoids because they mainly act on cell wall as mentioned above.

Terpenoids are other phytochemicals found in the extract of *Justicia schimperiana* which have antimicrobial activity. Terpenoids were reported to have antimicrobial activity against bacteria (Habtemariam *et al*., 1993), fungus (Ayafor *et al.*, 1994), virus (Hasegawa *et al.*, 1994) and protozoa (Ghoshal *et al.,* 1996). The mechanism of action of terpenoids is not fully understood but, it is speculated to involve membrane disruption by the lipophilic compounds (Brehm-Stecher and Johnson, 2003).

Generally, there are different speculations in antimicrobial mechanism of medicinal plant extracts, based on the phytochemical they contain. Even though the mechanism are not well understood, the extracts of the studyplant has antimicrobial property with different degrees of activity against tested bacterial and fungal species; which confirm the potential of the extracts of leaves of *Justicia schimperiana*for isolation of effective compounds in the treatment of infections caused by the tested bacteria and fungi. This effect of extracts of the leaf of *Justicia schimperiana* might be attributed to either the individual class of compounds present in the crude and the active solvent fractions or to the synergistic effect that each class of compound exert to give the observed antimicrobial activity findings.

6 Conclusion

The present study revealed that the crude (80% methanol) extract of leaves of *Justicia schimperiana*have antimicrobial activities against selected pathogenic bacteria and fungus. The aqueous, n-butanol and ethyl acetate solvent fractions also showed antibacterial and antifungal activity with varying antimicrobial spectrum. The n-butanol fraction has shown the highest antimicrobial activity. The antimicrobial activities of the plant might be due to the presence of bioactive secondary metabolites (alkaloids, terpenoids, polyphenols, saponins and flavonoids), which can act either individually or synergistically.

7 Suggestions for future work

- The acute, sub-chronic and chronic toxicological studies should be done for the safety of the extracts of the plant.
- Further studies should be conducted on solvent fractions especially n-butanol fraction, to isolate, purify and identify bioactive principle(s) responsible for antibacterial and antifungal activities of the plant.
- *In-vivo* antimicrobial studies of the crude and active solvent fractions should be conducted to confirm *in-vivo* antibacterial and antifungal effectiveness of the plant.
- Mechanistic studies for the responsible antimicrobial agent of the study plant have to be conducted for the antibacterial and antifungal activity.
- The antimicrobial activities of the plant should also be tested on other microbial species which were not addressed by this study.

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