Evaluation of *in-vitro* antibacterial and antifungal activities of crude extracts and solvent fractions of methanol extract of leaves of *Ricinus communis* Linn (Euphorbiaceae) against selected pathogens.

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This is to certify that the thesis prepared by Bedaso Kebede entitled "Evaluation of *in-vitro* antibacterial and antifungal activities of crude extracts and solvent fractions of methanol extract of leaves of *Ricinus communis* Linn (Euphorbiaceae) against selected pathogens." and submitted in partial fulfilment 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

Infectious disease impacts are reduced due to development of antimicrobial agents. However, the effectiveness of the antimicrobial agent is reduced over time because of the emergence of antimicrobial resistance. To overcome these problems scholars have been searching for alternative medicines, particularly focusing on traditionally used medicinal plants. *Ricinus communis* Linn is used as a traditional treatment for bovine mastitis, wound infection, and other medicinal purposes. Moreover, the antimicrobial activity of *Ricinus communis* Linn leaf at crude extract level has been confirmed against human originated pathogens in the previous studies. The objective of the present study was to further evaluate the antimicrobial activities of *Ricinus communis* Linn leaf extracts and fractions. *Ricinus communis* Linn leaves were macerated in absolute methanol and acetone solvents. The methanol crude extract was shown best antimicrobial activity and subjected to further fractionation via increasing polarity of solvents (n-hexane, chloroform, ethyl acetate, and aqueous). Test microorganisms included in the study were six laboratory reference bacteria (*E. coli, S. aureus, S. agalactiae, K. pneumoniae, P. aeruginosa,* and *S. Pyogenes*), two clinical isolate bacteria (*E. coli* and *S. aureus*), and one fungus (*Candida albicans*). The agar well diffusion method was employed to determine antimicrobial activity. The minimum inhibitory concentrations (MIC) and minimum bactericidal/fungicidal concentrations (MBC/MFC) were determined through broth microdilution. The results indicated that the best antimicrobial activity for ethylacetate fraction ranging from 14.67 mm (clinical *E. coli*) to 20.33 mm (*S. aureus*) at 400mg/ml however, n-hexane exhibited lowest antimicrobial activity. Among tested fractions, ethyl acetate fraction was produced lowest MIC values ranging from 1.5625mg/ml (*S. aureus*) to 16.67 mg/ml (*Candida albicans*). The ethyl acetate fraction showed bactericidal activity against all tested microorganisms. In conclusion, ethyl acetate fraction of crude methanol extract of *Ricinus communis* Linn leaf exhibited the best antimicrobial activity.

Keywords: - Antibacterial activity, Antifungal activity, *Ricinus communis* Linn, MIC, MBC

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List of abbreviations and Acronyms

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
CFU	Colony Forming Units
DMSO	Dimethyl Sulfoxide
MBC	Minimum Bactericidal Concentrations
MFC	Minimum Fungicidal Concentration
MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentration
MHA	Muller Hinton Agar
CLSI	Clinical Laboratory Standard Institute
SEM	Standard Error of Mean
SPSS	Statistical Package for Social Sciences

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1. INTRODUCTION

1.1. Infectious diseases

Infectious diseases are instigated due to invasion and multiplication of pathogenic microorganisms in a host body by evading the immune system (Kumar *et al.,* 2012). According to their occurrence in a particular area and host, infectious diseases are classified as emerging and re-emerging (Nii-Trebi, 2017). The emerging infectious diseases are consequences of novel infectious agents and previously identified pathogenic agents that developed an outbreak for the first time in the areas and cause threats in the regions. Re-emerging infectious diseases are those reappearing after their initial incidence lowered in humans or animals (Kuri-Morales *et al.,* 2015). The main factor for the occurrence of emerging and re-emerging infectious diseases are the emergence of antimicrobial resistance in addition to new infectious agents (Gupta *et al*., 2012). Aetiology of infectious diseases are bacteria, viruses, fungus, and parasites (WHO, 2001). Based on the capacity of host defences the aetiologies are classified as primary and opportunistic pathogens. The primary pathogens are pathogenic microbes that produce and spread disease among the normal and healthy hosts as a result of their intrinsic virulence. Whereas, opportunistic pathogens are microbes that live symbiotically in/on the host and produce diseases at a time host immunity is suppressed by several factors. For instance, normal flora of gastrointestinal and upper respiratory tract in humans and animals (Kumar *et al.,* 2012). The mode of transmission of infectious diseases is direct and indirect ways. The direct mode of spreading infectious diseases is via contact, droplet, transplacental and perinatal, while the indirect mode of spreading infectious diseases are due to contaminated vehicle, inoculation, airborne, food/waterborne and vector-borne (Figure 1) (WHO, 2001).

About 75% of emerging infectious diseases in humans have emerged from zoonotic diseases. *E. coli* widely exists in the form of commensal and pathogenic bacteria in both animals and humans (Rwego *et al*., 2008). *Pseudomonas aeruginosa* is an opportunistic pathogen and has been the most prevalent aetiology of nosocomial pneumonia in the USA (Emori and Gaynes, 1993; Milivojevic *et al*., 2018). *Klebsiella pneumoniae* cause a variety of infections in animal and human, and some of the infections were pneumonia, bloodstream infections, and pyogenic liver abscesses (Cheng *et al*., 2018). *Staphylococcus aureus* particularly occurs as commensal microbes which dwell on the skin, nose, and mucous membrane of healthy animal and human. Therefore, it causes multiple infectious diseases as an opportunistic pathogen and an example of bovine mastitis in the dairy

industry (Fluit, 2012; Lozano *et al.,* 2016). *Streptococcus pyogenes* is normal inhabitants of epithelial cell surfaces of throat and skin that caused local and systemic infections in animal and human, but more are reported from human (Vela *et al*., 2017). *Streptococcus agalactiae* is common in the occurrence of urinary tract infections, endocarditis, meningitis, pneumonia, and bovine mastitis and pronounced in those elderly and immunocompromised patients (Lyhs *et al*., 2016; Carvalho-Castro *et al*., 2017). *Candida albicans* is an opportunistic pathogen occur as commensal on the skin, gastrointestinal tract, and reproductive tract in human and animal. It causes dermatitis, vulvovaginitis, bovine mastitis, urinary tract infection, respiratory tract infection, and stomatitis (Jadhav and Pal, 2013; Romo and Kumamoto, 2020).

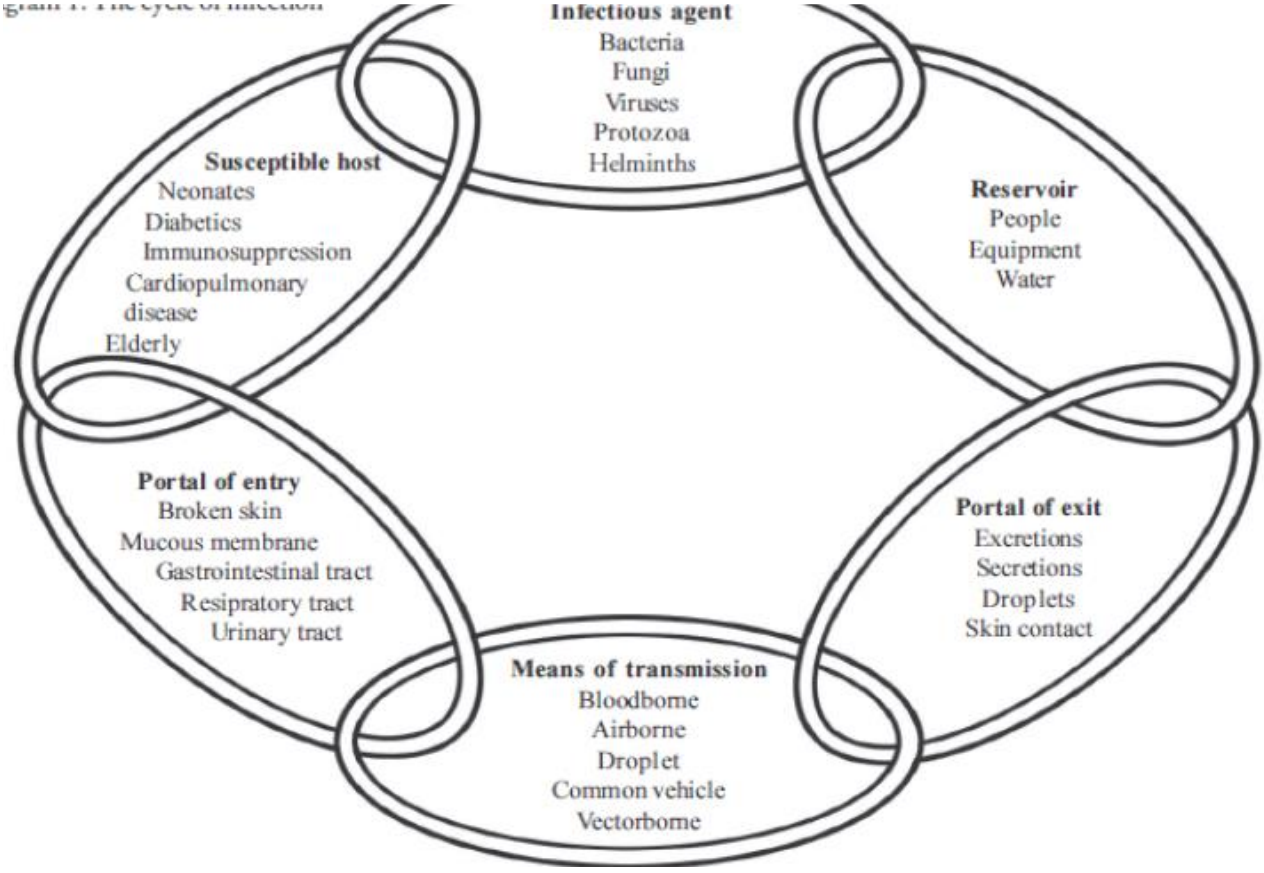

Figure 1: Diagrammatic view of the cycle of infection (WHO, 2001).

1.2. Management of infectious diseases

Infectious diseases in humans and animals are contained via administration of either traditional or modern drugs. The commencement of traditional treatment was from ancient times, where parts of medicinal plants were engaged in the treatment (Cowan, 1999). However, the discipline of chemotherapy for infectious diseases are emerged since the 20th century because of a deep understanding of the biochemical relationship between pathogens and drugs, selective toxicity, drug resistance development, and combined therapy role (Brooks *et al*., 2007; Kapoor *et al*., 2017). Modern antimicrobial discovery and development are brought by the dedication of scientists to minimize the impact of infectious diseases on human and animal (Bush, 2004). The scientists such as Paul Ehrlich and Alexander Fleming are contributed to the development of modern antimicrobial agents. Paul Ehrlich discovered sulfa drugs in 1935 which are produced a significant effect on the microorganisms, but safe for the host. Alexander Fleming discovered penicillin from mould variety called *Penicillium notatum* and awarded the Noble prize for his work (Sageman, 2015). The development of modern antimicrobial drugs has a considerable impact on the reduction of infectious diseases in humans and animals (Powers, 2004).

The modern antimicrobial agents are purposely used to fight against pathogenic microbes as curative or prophylactic therapy (Radostitis *et al*., 2006). Some of these antimicrobial agents include penicillin, cloxacillin, cephalosporin, gentamicin, trimethoprim, erythromycin, streptomycin, amoxicillin, enrofloxacin, cephalothin, ketoconazole, amphotericin, griseofulvin, tetracycline, ampicillin, oxytetracycline, and sulphonamide. These antimicrobial agents have their mechanism of actions (Riviere and Papich, 2018; Brunton *et al*., 2018).

1.2.1. Mechanisms of antimicrobial actions

1.2.1.1. Mechanisms of antibacterial actions

Based on their mechanisms of actions, antibacterial agents are categorized as cell wall synthesis inhibitor, cell membrane function inhibitor, protein synthesis inhibitor, metabolic pathway inhibitor and nucleic acid synthesis inhibitor. The drugs significantly damage cellular fragments of bacteria and expose them to harsh environment exerting bactericidal or bacteriostatic effect (Reygaert, 2018).

Cell wall synthesis inhibitors include glycopeptides (vancomycin), D-cycloserine, bacitracin, and β-lactam antibiotics which include penicillins, cephalosporins, carbapenems, and monobactams (Holten and Onusko, 2000). The β-lactam antibiotics target transpeptidase enzyme which is used for biosynthesis of peptidoglycan through transpeptidation reaction. The peptidoglycan layer in the cell wall of gram-positive bacteria is much thicker than that of gram-negative bacteria, which makes gram-positive bacteria more susceptible to β-lactam antibiotics (Brooks *et al*., 2007). The β-lactam ring structure imitates the structure of the D-alanyl-D-alanine portion of peptide which binds to penicillin-binding protein leading to production of new peptidoglycan (Vollmer *et al*., 2008; Dowling *et al*., 2017). Glycopeptides inhibit cell wall synthesis through binding to terminal D-alanyl-D-alanine residues of the nascent peptidoglycan and prevent cross-linking steps required for the synthesis of the stable cell wall. Generally, D-cycloserine and bacitracin are inhibited synthesis and transportation of peptidoglycan precursor, but β-lactams and glycopeptides inhibit crosslinking reaction and maturation (Tenover, 2006; Brooks *et al*., 2007; Kapoor *et al*., 2017).

Protein synthesis inhibitors include macrolides, aminoglycosides, tetracycline, and chloramphenicol. The macrolides inhibit translocation of peptidyl tRNA molecules from the acceptor site to the peptidyl donor site via binding to the 50S ribosomal subunit (Yoneyama and Katsumata, 2006; Kapoor *et al*., 2017). Aminoglycosides bind to the 30S ribosomal subunit leading to inhibiting transfer of amino acyl-tRNA to the peptidyl site and increasing the frequency of misreading of mRNA finally resulting in premature termination of the peptide chain (Kapoor *et al*., 2017; Dowling *et al*., 2017). Tetracycline binds to the 30S ribosomal subunit and inhibits the binding of amino acyl-tRNA to the acceptor site on the mRNA ribosomal complex (Chopra and Roberts, 2001; Yoneyama and Katsumata, 2006). Chloramphenicol binds reversibly to the 50S ribosomal subunit, preventing binding of amino acyl-tRNA to acceptor, and peptide bond formation is inhibited (Yoneyama and Katsumata, 2006; Kapoor *et al*., 2017).

Metabolic pathway inhibitors like sulfonamides inhibit dihydropteroate synthase enzyme which is important for the synthesis of folic acid precursor dihydropteroic acid using para-aminobenzoic acid (PABA) (Yoneyama and Katsumata, 2006; Capasso and Supuran, 2014). Nucleic acid synthesis inhibitors like quinolones inhibit DNA gyrase and topoisomerase IV which introduce negative supercoils to DNA of gram-negative and gram-positive bacteria, respectively (Piddock, 1998; Higgins *et al*., 2003). Cell membrane disruptors like polymyxins increase cell membrane

permeability that leads to leakage of cytosol content of bacteria (Tenover, 2006; Reygaert *et al.*, 2018; Brunton *et al*., 2018).

1.2.1.2. Mechanisms of antifungal action

Mechanisms of antifungal action include inhibition of cell wall synthesis, cell membrane synthesis, mitosis, and DNA/RNA synthesis (Sanglard, 2003). The azole family is the largest group of antifungal agents and comprises ketoconazole, fluconazole, miconazole, and itraconazole. The azole family blocks the biosynthesis of ergosterol the component of the cell membrane through inhibition of lanosterol 14-α-demethylase enzyme (Prasad *et al*., 2016; Robbins *et al*., 2017).

Echinocandin family is also comprised of caspofungin, micafungin, and anidulafungin. The antifungal agents inhibit fungal cell wall synthesis via non-competitive blockade of the (1,3)-β-D-glucan synthase enzymes (Robbins *et al*., 2017; Lee and Lee, 2018). Polyene family have nystatin and amphotericin β and they result in leakage of cytosolic components through aqueous pore formed by binding to the ergosterol and disrupting lipid component of the cell membrane (Prasad *et al*., 2016; Robbins *et al*., 2017).

Nucleoside analogs are comprised of pyrimidine analogs and include flucytosine. Flucytosine hinders DNA/RNA synthesis through inhibition of thymidylate synthase enzyme (Sanglard, 2003; Prasad *et al*., 2016). Allylamine and thiocarbamates hinder ergosterol synthesis through inhibition of the squalene-epoxidase enzyme (Prasad *et al*., 2016). Griseofulvin inhibit fungus mitosis through disruption of spindle and cytoplasmic microtubule production (Spampinato and Leonardi, 2013; Prasad *et al*., 2016; Scorzoni *et al*., 2017).

1.3. Challenges in the management of infectious diseases

Treatment failure and antimicrobial resistance are major challenges of infectious disease management. Treatment failure has complied with the inconsistency to health professional advice in the usage of antimicrobial agents (Pearson *et al*., 2018). Moreover, the major causes of treatment failures are wrong antimicrobial choice, delayed administration of antimicrobial, inadequate antimicrobial blood levels, inadequate penetration of the antimicrobial to the target-site, antimicrobial neutralization or antagonism, superinfection or unsuspected secondary bacterial infection, non-microbial infection and non-infectious source of illness (Bassetti *et al*., 2018). Not

only this, but a shortage of diagnostic facilities is also another factor contributing to treatment failure in the developing country and it leads to inappropriate therapy, overutilization of drugs, and inappropriate administration strategies (Beyene *et al.*, 2015; Ararsa and Bekele, 2015). Moreover, the clinical status of patients, foreign material presence, and location of the infection are factors that affected therapeutic success (Ghannoum and Rice, 1999).

The means to overcome treatment failure include proper selection of antimicrobials based on identification of pathogens and their susceptibility to the antimicrobials, potential toxicity of the drugs, pharmacokinetics, and pharmacodynamics of the drugs, the possibility of drug interaction, cost of a drug, combination of antimicrobial drugs and the convenience of administration routes (Hessen and Kaye, 2004).

Antimicrobial resistance leads to failure of production of the desirable effects used to be observed previously. This might result from inappropriate use of antimicrobial agents. Antimicrobial resistance could be natural and acquired (Mayer *et al*., 1995).

Natural antimicrobial resistance is also known as intrinsic or structural drug resistance. This could be due to absence of drug targets on microorganisms or antimicrobial is unreachable to the site of action. For instance, gram-negative bacteria are naturally resistant to vancomycin for it does not pass through the outer membrane (Nikaido, 2009).

Acquired antimicrobial resistance is developed due to alteration in the genetic features of microorganisms. It is also termed as chromosomal or extrachromosomal (plasmids, transposons, and integrons) resistance. The chromosomal resistance has resulted from the mutation of chromosomal genes by physical and chemical factors. While, extrachromosomal resistance occurs due to transfer of resistant genetic materials to other antimicrobial susceptible microorganisms via plasmids, transposons, and integrons (Jawetz *et al*., 1995). Acquired antimicrobial resistance has consisted of cross-resistance and multi-drug resistance (Manchanda *et al*.,2010).

Antimicrobial resistance has an incredible implication on the mortality, morbidity, and health costs in the human and animal. For instance, in 2014 the center for disease control (CDC) has been ranked antimicrobial resistance as a second significant health threat because 23,000 peoples have died from antimicrobial resistance infections annually (Sageman, 2015). Mechanisms of

antimicrobial resistance depend on the mechanism of action and chemical nature of the antimicrobial agents (Cesur and Demiroz, 2013).

1.3.1. Mechanisms of antimicrobial resistance

1.3.1.1. Mechanisms of antibacterial resistance

Mechanisms of antibacterial resistance include efflux pumps, enzymatic inactivation, target modifications, and increasing cell permeability. Mechanisms of β-lactam resistance could be efflux pump, enzymatic inactivation, and alteration of penicillin-binding protein (PBP) (Dowling *et al.,* 2017). The target of β-lactam drug is modified through overexpression and acquisition of foreign PBP and mutations are a common resistance mechanism to gram-positive bacteria (Mayer *et al*., 1995). While, enzymatic inactivation of antibacterial agents is done via overproduction of the β-lactamase enzyme and is a common mechanism of resistance for gram-negative bacteria (Blair *et al*., 2015).

Mechanisms of aminoglycoside resistances could be through loss of cell permeability, modification of the target ribosome, efflux pumps, and enzymatic inactivation. For instance, bacteria such as anaerobes and facultative aerobes (enterococci) are intrinsically prevented cell permeability to aminoglycoside agents (Cesur and Demiroz, 2013; Kapoor *et al*., 2017). The modification of the target rRNA via mutation and enzymatic methylation are contributed to the emergence of aminoglycoside resistance and is commonly occurred in gram-positive bacteria (Mayer *et al*., 1995). Overproduction of phosphotransferase, nucleotidyltransferase, and acetyltransferase enzymes are inactivated aminoglycoside agents (Alekshun and Levy, 2007; Dowling *et al*., 2017).

Mechanisms of chloramphenicol resistances include efflux pump and enzymatic inactivation. The chloramphenicol enzymatic inactivation is occurred by acetylation of hydroxyl groups of chloramphenicol via acetyltransferase enzyme (Kapoor *et al*., 2017). The mechanism of glycopeptide resistance is by a mutation in Staphylococci species and tolerance in pneumococci (Patel and Richter, 2015). Mechanisms of macrolide resistance include methylation of the ribosomes and efflux pumps (Alekshun and Levy, 2007). Quinolone mechanisms of resistance include an alteration of topoisomerase and reduction of intracellular accumulation of drugs (Alekshun and Levy, 2007).

Tetracycline mechanisms of resistance include ribosomal protection through inclusion of resistance genes within a broad host range of transferable genetic elements, inactivation via cytoplasmic enzymes and efflux pump (Dowling *et al*., 2017). Trimethoprim-sulfamethoxazole resistance is developed as a result of mutation, active efflux from the cell, and intrinsic resistance in *P. aeruginosa* (Cesur and Demiroz, 2013; Patel and Richter, 2015; Kapoor *et al*., 2017).

1.3.1.2. Mechanisms of antifungal resistance

Mechanisms of antifungal resistance include reduction of intracellular accumulation and alteration of target affinity (Prasad *et al*., 2016). Azole mechanisms of resistance involve reduction of intracellular accumulation of drugs, overexpression of lanosterol 14-α-demethylase enzyme, and alteration of sterol desaturase biosynthesis (Ghannoum and Rice, 1999; Robbins *et al*., 2017).

Mechanisms of echinocandin resistance is through point mutation of gene encoded for (1,3)-β-D-glucan synthase enzymes (Prasad *et al*., 2016). The mechanism of polyene resistance is through alteration of lipid concentration in cell membrane and ergosterol biosynthesis. Nucleoside analogs (Flucytosine) resistance is emerged due to deficiency of cytosine deaminase enzyme that reduced uptake of flucytosine (Spampinato and Leonardi, 2013; Prasad *et al*., 2016; Scorzoni *et al*., 2017).

1.3.2. Management of antimicrobial resistances

Antimicrobial resistances are a natural process that can be minimized but not avoided (WHO, 2019). The measures for management of antimicrobial resistance in humans and animals are immunization, avoidance of inappropriate and unnecessary use of antimicrobial drugs, development of novel efficacious compounds, and latest diagnostic technology (Uchil *et al*., 2014; WHO, 2019). Moreover, the transmission of the resistant organisms can be reduced by the protection of environmental hygiene and assessment of resistance trends in the microbial population (Liwa and Jaka, 2015).

Antibacterial and antifungal novel agents have been emerged to combat antimicrobial resistance (WHO, 2017). Tetracycline families are included phase 1 (KBP-7072, TP-271 and TP-6076) and phase 3 (eravacycline and omadacycline). Aminoglycoside family is comprised plazomicin (phase 3) (WHO, 2017). Topoisomerase inhibitors are encompassed delafloxacin (new drug application filed), alalevonadifloxacin (phase 1), finafloxacin (phase 2), and lascufloxacin (phase 3) (WHO,

2017; Andrei *et al*., 2018). Novel membrane-targeted antibiotics are included brilacidin and murepavidin (phase 2). Dihydrofolate reductase inhibitor is comprised iclaparim (phase 3) (WHO, 2017).

Orotomide class of antifungal agent is encompassed olorofim (F901318) phase two (Perfect, 2017). Echinocandin and polyene classes are included rezafungin (phase 3) and amphotericin B cochleate (phase 2), respectively (Gintjee *et al*., 2020). Tetrazole class is comprised VT-1161 (phase 3) and VT-1598 (phase 1) (Hoekstra *et al*., 2014). Glycosylphosphatidylinositol inhibitor fosmanogepix (phase 2) (Gintjee *et al*., 2020). Generally, all these novel antimicrobial agents are in the clinical pipeline.

1.4. Experimental plant

Ricinus communis Linn taxonomically belongs to the family of Euphorbiaceae and it is a sole species in the monotypic genus *Ricinus*. The vernacular names are "Qobboo" (in Afan Oromo), "Castor oil plant or castor bean" (in English), and "Gulo" (in Amharic). It grows in altitude ranging from 400-4500m above sea level in tropics and temperate regions of the world. The plant grows perennial as high as 5-10 meters with the 15cm thick and hollow trunk and leaves. It has a green or reddish colour, alternate, stipulate, long petiolate, and a membranous lobe of a leaf and fruit has thorny capsule covered a seed (Figure 2). It has been reproduced with mixed pollination of self-pollination (geitonogamy) and out-crossers by wind pollination (anemophily) or insect pollination (or entomophily) (Edwards *et al*., 1995; Neelam and Singh, 2015).

Ethnomedicinally *Ricinus communis* Linn is used in Ethiopia as a traditional treatment for human and animal diseases. A dried seed is chewed and swallowed to treat amoebiasis in Selale (Atnafu *et al*., 2018). Fresh leaves are crushed and mixed with water and cup of tea is taken consecutively for three days to treat rabies and fresh roots of *R. communis* and *J. schimpariana* are crushed and mixed in water and a cup of tea is taken for the liver ailments in Wollega (Megersa *et al*., 2013). Fresh fruits are crushed and mixed in marc of local beer (atella) and are given for the treatment of blackleg and actinomycosis in Wollega and Jimma (Bayecha *et al*., 2018). Seeds are pounded and mixed in water and is given orally for diarrhoea treatment in newborn, seed is powdered and mixed with water and formed ointment is applied on a wound, seeds are powdered and mixed in water and is drenched for sudden sickness and bloat, and bark is powdered and mixed with water and

formed ointment is applied and rubbed on the skin for treatment of skin rashes/ dermatitis in Gondar (Mengesha and Dessie, 2018). Seeds are ground and is applied to wound in Arsi (Gijan and Dalle, 2019). Root and seeds are crushed and mixed with a cup of water and is drunk for treatment of intestinal worm in Bale (Jima and Megersa, 2018). Fresh leaves are pounded and mixed with water and is administered orally to treat mastitis and poor mothering in southern Ethiopia (Romha *et al*., 2015).

The pharmacological activities of *Ricinus Communis* Linn include larvicidal activity (Elimam *et al*., 2009; Mandal, 2010; Wachira *et al*., 2014; Wafa *et al.*, 2014; Kehail *et al*., 2017; Aouinty *et al*., 2018), antioxidant activity (Ravishankar and Indira, 2012; Salib *et al*., 2014; Wafa *et al*., 2014), antinflammatory activity (Vieira *et al*., 2000; Saini *et al*., 2010), antidiabetic activity (Shokeen *et al*., 2008; Matthew *et al.*, 2012), anticancer activity (You *et al*., 2010; Ohish *et al*., 2014; Prakam and Gupta, 2014; Salib *et al*., 2014), analgesic activity (Ferraz *et al*., 1999; Taur *et al*., 2011), anthelmintic activity (Rana *et al*., 2013), anti-fertility activity (Isichei *et al*., 2000; Nath *et al*., 2015), Antiasthmatic activity (Dnyaneshwar and Patil, 2011), and antimicrobial activity (Naz and Bano, 2012; Abew *et al*., 2014; Suurbaar *et al.*, 2017).

Toxicity study indicated that *Ricinus communis* Linn possesses a toxin compound ricin and ricinine. The toxic symptoms were comprised of abdominal pain, emesis, muscular pains, cramps in the limbs, dyspnea, circulatory collapse, dehydration, and dysfunction of kidney and liver. Deaths have occurred when the administered dose was above 340mg/Kg for intraperitoneal routes and 3g/Kg for an oral route (Bhaskaran *et al*., 2014; Abdul *et al*., 2018).

Figure 2: Aerial parts of *Ricinus communis* Linn (Photographed in October, 2019).

1.5. Rationales of Study

Infectious diseases are exacerbated due to the existence of zoonotic diseases and antimicrobial resistance (Rwego *et al*., 2008; Uchil *et al*., 2016). Several surveillances have been conducted on antimicrobial resistance. The augmentations of antimicrobial resistances have harmed both human and animal health, exposing to longer periods of hospitalization, and affect treatment costs (Kerro and Tareke, 2003; Getahun *et al*., 2008; Bedasa *et al*., 2018). Alternative medicines have been screening from a variety of plants for their pharmacological potential as secondary metabolites are less in drug adverse effects, resistances, and residues (Helander *et al*., 1998; Puupponen-Pimia *et al*., 2001; Zgurskaya *et al*., 2015; Felhi *et al*., 2017).

Ricinus communis Linn in Ethiopia is used in the treatment of blackleg and actinomycosis (Bayecha *et al*., 2018), diarrhoea, wound, and skin rashes/ dermatitis (Mengesha and Dessie, 2018; Gijan and Dalle, 2019), and bovine mastitis (Romha *et al*., 2015). The study on validation of antimicrobial activity of *Ricinus communis* Linn leaf crude extracts are conducted using different solvents in Pakistan and Ghana, and methanol crude extract is reported to have a promising antimicrobial potential (Naz and Bano, 2012; Suurbaar *et al*., 2017). Besides, in Ethiopia around

Gonder study on *Ricinus communis* Linn leaf has shown an antibacterial activity for an organic solvent solution, but the aqueous extract has shown less activity (Abew *et al*., 2014). However, methanol solvent was not used for extraction in Abew *et al*. (2014) study and none has been done on the antimicrobial activities of solvent fractions of *Ricinus communis* Linn leaf.

Antimicrobial activities of medicinal plants are not only determined by plant species. There are also other factors such as altitude, temperature, illumination, and moisture. These factors have regulated accumulation and metabolism of secondary metabolites in medicinal plants. Additionally, differences in the location of medicinal plants have contributed to presence of different active ingredients and their concentrations (Liu *et al*., 2016). Therefore, the current study is intended to evaluate antimicrobial activities of methanol and acetone crude extracts, and solvent fractions of methanol extract that exhibited the best antimicrobial activity.

2. OBJECTIVES

2.1. General objective

- To investigate the antimicrobial activity of *Ricinus communis* Linn leaf crude extracts and solvent fractions of methanol extract against selected bacterial and fungal pathogens.

2.2. Specific objectives

- To determine antibacterial and antifungal activities of *Ricinus communis* Linn leaf crude extracts and solvent fractions of methanol extract using agar well diffusion method against selected pathogens.
- To determine the minimum inhibitory concentration of *Ricinus communis* Linn leaf crude extracts and solvent fractions of methanol extract using broth microdilution technique against selected bacterial and fungal pathogens.
- To determine the minimum bactericidal and fungicidal concentration of *Ricinus communis* Linn leaf crude extracts and solvent fractions of methanol extract against selected bacterial and fungal pathogens.
- To determine qualitatively preliminary phytochemical constituents of *Ricinus communis* Linn leaf crude extracts and solvent fractions of methanol extract.

3. MATERIALS AND METHODS

3.1. Chemicals, Solvents, Standard drug and Media

Chloroform (Fisher Scientific Ltd., UK), Sulfuric acid (Fisher Scientific Ltd., UK), HCl (Fisher Scientific Ltd., UK), Ferric chloride (Sigma Aldrich Ltd., Germany), Mercuric chloride (Flinn Scientific, Canada), Potassium iodide (Flinn Scientific, Canada), iodine (Sigma Aldrich Ltd., Germany), 30% ammonium solution (Sigma Aldrich Ltd., Germany), glacial acetic acid (Sigma Aldrich Ltd., Germany), absolute methanol (Fisher Scientific Ltd., UK), acetone (Flinn Scientific, Canada), n-hexane (Fisher Scientific Ltd., UK), ethyl acetate (Fisher Scientific Ltd., UK), water, gentamicin 10µg (Sigma Aldrich Ltd., Germany), resazurin sodium salt (Sigma Aldrich Ltd., Germany), amphotericin (Azesto Impex Pvt. Ltd., India), dimethyl sulfoxide (Fisher Scientific Ltd., UK), tween 80 (Thermofisher scientific, USA), glycerol (Sigma Aldrich Ltd., Germany), mannitol salt agar (Hi Media Laboratories Pvt. Ltd., India), eosin methylene blue (Oxoid Ltd., England), cetrimide agar (Oxoid Ltd., England), brain heart infusion agar and broth (Sigma Aldrich Ltd., Germany), sabouraud dextrose agar and broth (Oxoid Ltd., England), blood agar base (Oxoid Ltd., England) supplemented 5% sheep blood, mueller hinton agar and broth (Sigma Aldrich Ltd., Germany), and nutrient broth (Hi Media Laboratories Pvt. Ltd., India) were used during the experiment. All chemicals, solvents, standard drug and media were analytical grade.

3.2. Plant collection and authentication

The experimental plant was collected from the Sululta district of special zone surrounding Finfinne city of Oromia regional state, Ethiopia which is located 25km far from capital city in October, 2019. It was authenticated by Mr. Melaku Wondafrash at the national herbarium of the college of natural and computational sciences, Addis Ababa University, and voucher number 002/BK specimen was kept for future reference in the national herbarium.

3.3. Plant preparation

The collected plant leaves were washed with the running water to remove unwanted and dirty substances. It was dried under shade at room temperature for 10 days. The dried leaves were pulverized to the coarse powder by mortar and pestle (Figure 3).

Figure 3: Dried Ricinus communis Linn leaves pulverized in pestle and mortar.

3.4. Processing of the plant

Plant processing was conducted at the Toxicology laboratory of Animal Products, Veterinary Drug and Animal Feed Quality Assessment Centre of Veterinary Drug and Animal Feed Administration and Control Authority.

3.4.1. Extraction of the plant

The extraction was performed according to Ogbiko *et al*. (2018). The powdered leaves of 200 grams were weighed on an analytical balance (Mettler Toledo, Switzerland) and macerated in 1000ml of absolute methanol and acetone in Erlenmeyer flask at the ratio of 1:5 after three days extract was collected and marc was re-macerated. Collection of the extract was carried out at the interval of three days, so leaves were macerated totally for 9 days with intermittent shaking on the rotary-shaker (VWR DS-500; The Lab World Group, Boston, MA, USA). The pool of collected extracts was first filtered through sieve mesh then followed by filtration via Whatman no.1 by using filtration apparatus or unit (Figure 4). A filtrate of extracts was concentrated in a rotary evaporator (Buchii model R-200, Switzerland) (Figure 5) at 40^{o}C temperature and 40 revolutions per minute (RPM) until solvents were completely removed and solid extracts were formed.

Figure 4: Filtration of Ricinus communis Linn crude extracts via Whatman no.1 paper by filtration apparatus.

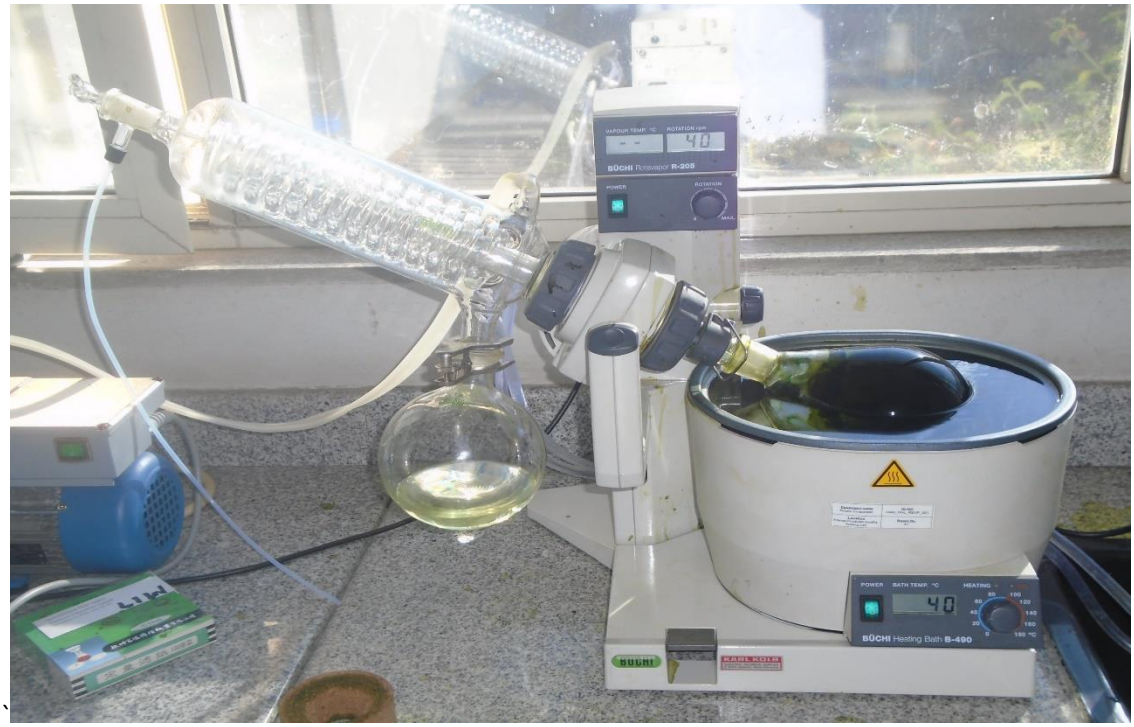

Figure 5: Concentration of Ricinus communis Linn crude extract filtrate in a rotary evaporator.

3.4.2. Crude extract solvent fractionation

The crude methanol extract exhibited better antimicrobial activity than crude acetone extract against test microorganisms. Based on this crude methanol extract was subjected to further solvent fractionation by increasing polarity including n-hexane, chloroform, ethyl acetate, and aqueous. Voukeng *et al.* (2017) methods were used for solvent fractionation with modification on the concentration of extract residue between fractionation intervals.

The methanol crude extract was not completely dissolved in water, as result for 90% methanol solvent was used instead. The methanol crude extract 60 grams were weighed on an analytical balance and subjected to dissolve completely in 100ml of 90% methanol (10ml water and 90ml methanol) in the beaker. The completely dissolved 100ml methanol crude extract was mixed with 100ml n-hexane for solvent partitioning in separatory funnel having a capacity of 250ml. The separatory funnel contained mixture was fixed to the standing stage pole and waited until a clear and separated layer formed between two solvents. Once a clear layer formed, the methanol part was taken first carefully to beaker and n-hexane partition to another container. This procedure was repeated three times, the n-hexane partition was collected together for future concentration. While the remaining crude methanol extract solution was subjected to evaporation in a rotary evaporator at 40^{o} C and 40 rpm to remove methanol solvent. Then, 90ml of water was added to the concentrated crude methanol extract to form a 100ml aqueous solution.

The 100ml aqueous solution of crude methanol extract was mixed with 100ml of chloroform in the separatory funnel. The separatory funnel was fixed on the standing stage pole and waited until a clear layer formed between the aqueous solution of crude methanol extract and chloroform. The chloroform portion was held lower layer and collected first in the container and aqueous portion in another container. It was replicated three times and the chloroform portion pooled in the container for later concentration. The remnant aqueous portion of crude methanol extract was concentrated on a rotary evaporator to remove the remaining chloroform.

The concentrated 100ml aqueous portion of crude methanol extract was mixed with 100ml ethyl acetate in the separatory funnel. The separatory funnel was fixed on the standing stage pole and waited till a clear layer appeared between aqueous fraction and ethyl acetate fraction (Figure 6). It was repeated three times, and aqueous fraction and ethyl acetate fraction were collected in different

containers. The aqueous fraction was lyophilized by lyophilizer (Operon, Korea vacuum limited, Korea), but n-hexane, chloroform, and ethyl acetate fractions were concentrated in a rotary evaporator.

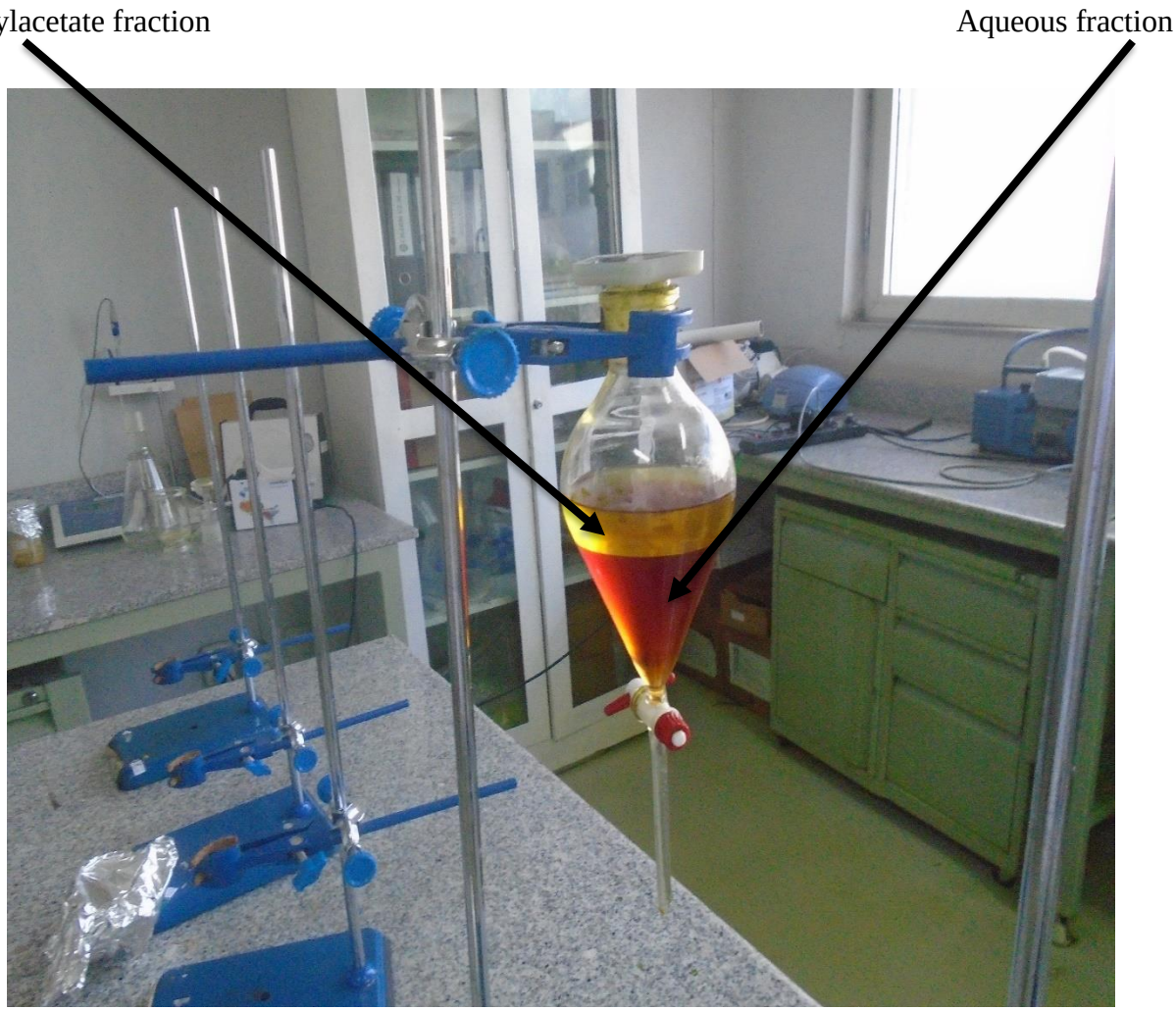

Figure 6: Solvent fractionation of crude methanol extract of Ricinus communis Linn leaf in the separatory funnel.

3.5. Antimicrobial activity

Antimicrobial activities of crude extracts or solvent fractions were conducted at Microbiology laboratory of Animal Products, Veterinary Drug and Animal Feed Quality Assessment Centre of Veterinary Drug and Animal Feed Administration and Control Authority.

3.5.1. Test microorganisms

Microorganisms selected for the experiment were standard strains including *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (ATCC 12386), *Streptococcus pyogenes* (ATCC 19615), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella* pneumoniae (ATCC 700603) brought from Ethiopian Public Health Institute, and clinical isolates *Staphylococcus aureus* and *Escherichia coli* obtained from Animal Products, Veterinary Drug and Animal Feed Quality Assessment Centre of Veterinary Drug and Animal Feed Administration and Control Authority, and *Candida albicans* (ATCC10231) brought from Ethiopian Biodiversity Institute. These microorganisms were selected because of their pathogenicity that causes a variety of diseases in humans and animals. Some of the diseases are pneumonia, liver abscesses, bovine mastitis, urinary tract infections, endocarditis, meningitis, and dermatitis (Fluit, 2012; Lozano *et al*., 2016; Carvalho-Castro *et al*., 2017; Milivojevic *et al*., 2018; Cheng *et al*., 2018; Romo and Kumamoto, 2020).

3.5.1.1.Verification of test microorganisms

The gram staining, selective media, haemolysin, and catalase test were conducted to confirm test microorganisms according to CLSI (2008) and Brown *et al.* (1965). The gram staining revealed gram-positive cocci in a cluster for *S. aureus*, gram-positive cocci in chains for *S. agalactiae,* and *S. Pyogenes*, and gram-negative rod shape for *E. coli*, *K. pneumoniae,* and *P. aeruginosa*.

Catalase test was positive for *S. aureus*, but negative for streptococcal species. The mannitol salt agar selective media of *S. aureus* was possessed 7.5% NaCl which inhibits the growth of other bacteria, mannitol, and phenol red indicator. The mannitol was fermented by *S. aureus* and resulted in acid production that was reacted with phenol red indicator to change indicator colour from red to yellow. The blood agar supplemented with 5% sheep blood was inoculated for confirmation of streptococcal species; a clear zone or β haemolytic and translucent colony appeared that characterize *S. pyogenes* and the narrow zone or slight β haemolytic and mucoid colony which characterize *S. agalactiae*.

Eosin methylene blue (EMB) agar is a selective media that inhibited the growth of gram-positive bacteria due to the presence of colour indicators eosin and methylene blue. The EMB was used to differentiate between lactose fermenter by producing colour change such as *E. coli* from non-

lactose fermented without colour change. EMB was inoculated with *E. coli* produced a dark center and green metallic sheen, but *K. pneumoniae* was produced mucoid blue-black colonies. *P. aeruginosa* was inoculated on selective media cetrimide agar produced a blue-green pigment because of pyocyanin and fluorescein production. The *Candida albicans* was inoculated on sabourauds dextrose agar supplemented with 5% sheep blood for the formation of hyphal elements radiating from colonies.

3.5.2. Standard drugs

Gentamicin 10µg disc was brought from Animal Products, Veterinary Drug, and Animal Feed Quality Assessment Centre of Ethiopian Veterinary Drug and Animal Feed Administration and Control Authority. Gentamicin was used as a positive control for testing antibacterial activity against gram-positive and gram-negative bacteria. Amphotericin-B was obtained from the Ethiopia Food and Drug Administration and Control Authority and used as a positive control for testing antifungal activity.

3.5.3. Media preparation

The media utilized for confirmation of test microorganisms and study experiments was prepared according to the manufacturer's instructions. The powder or coarse media weighed on an analytical balance to measure the desired weight that was dissolved in the desired volume of water in a flask. A dissolved media in the flask was heated over the heater to facilitate dissolution. After that, the media was autoclaved at 121^{o}C for 15 minutes. A media was removed from autoclave (Biobase Meihua, China) and put.in a 50^{o}C hot water bath to cool down. After media was cooled down to the 50^{o}C, 5% sheep blood was added to blood base agar and sabouraud dextrose agar then dispensed on the sterilized 90mm Petri dishes in a safety cabinet, but other media was dispensed without supplement. The sample of solidified agar media was placed in the incubator for 24h to determine media sterility and others were stored at +4^{o}C in the refrigerator till used. The broth media was prepared according to agar media, but 4ml and 9.5ml of broth media were added to the test tube for sterilization in an autoclave, while sample of broth media cooldown from autoclave placed in incubator for 24h to determine sterility. All antimicrobial activities were conducted in a biosafety cabinet (Biobase Meihua, China).

3.5.4. Agar well diffusion assay

The stock solution 400mg/ml was prepared from 1.6 grams of crude extracts or fractions dissolved in 4ml of sterile distilled water (ethyl acetate fraction and aqueous fraction), 2% DMSO (methanol and acetone crude extracts, chloroform fraction) and 10% Tween 80 (n-hexane fractions). The diluent was tested as a negative control for antimicrobial activity.

3.5.4.1.Antibacterial activity

The brain heart infusion (BHI) broth was prepared for streptococcal species and nutrient broth for other test bacteria. The 24h cultured 3-5 distinct colonies of bacteria based on their colony size were inoculated into 4ml broth media and incubated at 37^{o}C overnight. The nutrient or BHI broth was added to the overnight incubated bacterial suspension, and vortexed on a vortex mixer (Fisher Scientific. Ltd., England) for one minute to attained uniformly distribution. The vortexed bacterial suspension was adjusted to 0.5McFarland standard (Remel, Lenexa Kansas 66215, USA) (equivalent to 1-2x10^{8} CFU/ml) through contrasting against white paper black line striped and was used for experiment within 15 minutes (CLSI, 2015).

The 100µl of adjusted bacterial suspension was pipetted using a micropipette and applied on the surface of Mueller Hinton agar, and was swabbed at 60^{o} rotation to uniformly distribute bacteria throughout media surface using a cotton swab. The swabbed Mueller Hinton agar stands for 15 minutes to provide time for the attachment of bacteria on the media. After that, the sterilized cork borer of 6mm diameter was perforated with the swabbed media to create 6mm diameter wells. At the time of punching media for different test bacteria, cork borer was sterilized by immersing in alcohol and burning with bunsen burner flames (Umer *et al.*, 2013; Gonelimali *et al.*, 2018). The concentration of extracts for the experiment was determined based on a previous study on the plant (Abew *et al*., 2014). The created wells were filled with 50µl extracts or fractions at a concentration of 400mg/ml, 200mg/ml and 100mg/ml, and negative control, but positive control disc (gentamicin) was placed on the media surface. After all wells on Petri dishes were filled, and positive control was placed on Petri dishes, then Petri dishes were placed in the refrigerator at 4^{o}C for 2h to facilitate diffusion of extracts or fractions in the media. Subsequently, Petri dishes were incubated at 37^{o}C for 24h in the incubator (BioTechnics India). The inhibition zone diameter after

24h incubation was measured by a ruler in millimeter and recorded (Abew *et al*., 2014; Suurbaar *et al.*, 2017; Ohikhena *et al*., 2017). The experiment was done in triplicate.

3.5.4.2. Antifungal activity

The *Candida albicans* was cultured on sabouraud dextrose agar and incubated overnight. The overnight incubated yeast culture was inoculated into normal saline (0.85%). The inoculated normal saline was vortexed on a vortex mixer and adjusted to 0.5 McFarland standard (equivalent to 1-5x10^6cells/ml) by contrasting against white paper black line striped (EUCAST, 2003). The 100µl adjusted *Candida albicans* suspension was pipetted using a micropipette and applied on the surface of sabouraud dextrose agar and swabbed at 60^o rotation to uniformly distribute yeast throughout media surface using a cotton swab. The swabbed sabouraud dextrose agar stands for 15 minutes to provide time for the attachment of yeast on the media. After that, the sterilized 6mm diameter cork borer was perforated swabbed media to create a 6mm diameter of wells (Ohikhena *et al.,* 2017). The concentration of extracts for the experiment was determined based on a previous study on the plant (Suurbaar *et al*., 2017). The created wells were filled with the 50µl extracts or fractions at concentration 400mg/ml, 200mg/ml and 100mg/ml, negative, and positive control. The inoculated Petri dishes were placed in the refrigerator at 4^{o}C for 2h to facilitate diffusion of extracts or fractions in the media. Next to that, Petri dishes were incubated at 37^{o}C for 24h in the incubator. The inhibition zone diameter after 24h incubation was measured by a ruler in millimeter and recorded (Abew *et al*., 2014; Ohikhena *et al.,* 2017; Suurbaar *et al*., 2017). The experiment was done in triplicate.

3.6. Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration is the minimum concentration of extracts or fractions which have been inhibited the growth of microorganisms. The minimum inhibitory concentrations were determined using the broth microdilution technique for extracts or solvent fractions as their inhibition zones equal to or greater than 7mm in agar well diffusion techniques (Taye *et al*., 2011).

3.6.1. Determination of minimum inhibitory concentration for pathogenic bacteria

The 24h cultured 3-5 distinct bacterial colonies were inoculated into 4ml Mueller Hinton broth and incubated at 37^{o}C overnight. Overnight incubated bacterial suspension had been adjusted

(0.5McFarland standard) was diluted at a ratio of 1:20 with Mueller Hinton broth (0.5ml bacterial suspension was added to 9.5ml broth) and vortexed to have uniformly distributed bacterial suspension ($5x10^6$ CFU/ml). The UV radiated sterile microtiter plate (Greiner Bio-One, Germany) wells were filled with 100µl Mueller Hinton broth which commenced from well one to twelve (CLSI, 2015).

The serial double dilution technique was employed for extracts and fractions in broth filled wells. The serial double dilution was performed as 100µl extracts or fractions were added to the first well and thoroughly mixed for five times by rinsing using micropipette and 100µl of the mixture was transferred to the second well using a new micropipette tip and thoroughly mixed as above. A 100µl of the second well mixture was pipetted using a new micropipette tip and transferred to third well then was thoroughly mixed as above. The process was continued until the tenth well and 100µl mixture of the tenth well was pipetted and discarded to have an equal volume of fluid in wells (CLSI, 2015). The twofold serially diluted concentrations of extract for the experiment were determined from a previous study on the plant. The serially diluted concentrations used in the experiment were (200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml, 1.5625mg/ml, 0.78125mg/ml, and 0.3906mg/ml) (Abew *et al.*, 2014). The 100µl broth filled eleventh and twentieth wells were used as growth and sterility control, respectively. The 10µl diluted bacterial suspension (10% of 100µl well volume) was pipetted to wells from eleventh to first wells to reduce contamination to sterility control and attained final concentration of $5x10^5$CFU/ml bacteria in each well, but 10µl broth was pipetted to twelfth well. Finally, microtiter plates were sealed using parafilm and incubated at 37^{o}C for 24h (CLSI, 2015).

The incubated microtiter plate wells were filled with 0.01% resazurin sodium salt indicator from twelfth to first well and incubated for 2h at 37^{o}C. The resazurin sodium salt reaction with actively growing microorganisms produces colour changes which are important to determine the MIC of extracts or fractions based on colour changes. The blue or purple colour appears if the growth of microorganisms is inhibited, while pink or colourless change is observed for those actively growing cells which reduced resazurin sodium salt to resorufin. Resazurin sodium salt solution was prepared by dissolving 0.01gram in 100ml sterile distilled water and filtered through 0.2µ pore size filter paper and stored in a dark container at 4^{o}C refrigerator until use. (Ohikhena *et al.,* 2017; Blazic *et al*., 2019). The experiment was performed in triplicate.

3.6.2. Determination of minimum inhibitory concentration for pathogenic fungi

The 24h cultured three colonies of yeast were inoculated into sabouraud dextrose broth and incubated at 37ºC overnight. Overnight incubated yeast suspension had been adjusted (0.5McFarland standard) was diluted at a ratio of 1:20 with sabouraud dextrose broth (0.5ml yeast suspension was added to 9.5ml broth) and vortexed to have uniformly distributed yeast suspension ($0.5\text{-}2.5x10^5$ CFU/ml). The sterile microtiter plate wells were filled with 100µl sabouraud dextrose broth started from well one to twelve (EUCAST, 2003).

The serial double dilution technique was employed for extracts and fractions in broth filled wells commenced from first to tenth wells. The serial double dilution was performed as 100µl extracts or fractions were added to the first well and thoroughly mixed five times by rinsing using micropipette and 100µl of the mixture was transferred to the second well using a new micropipette tip and thoroughly mixed as above. A 100µl of the second well mixture was pipetted using a new micropipette tip and transferred to third well then was thoroughly mixed as above. The process was continued until the tenth well and 100µl mixture of the tenth well was pipetted and discarded to have an equal volume of fluid in wells (EUCAST, 2003). The twofold serially diluted concentrations of extract for the experiment were determined from a previous study on the plant. The serial double dilution concentrations used in the experiment were (200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml, 1.5625mg/ml, 0.78125mg/ml, and 0.3906mg/ml) (Suurbaar *et al*., 2017). The 100µl broth filled eleventh and twentieth wells were used as growth and sterility control, respectively. The 10µl diluted yeast suspension (10% of 100µl broth volume) was pipetted to wells from eleventh to first wells to reduce contamination on sterility control and attained final concentration of yeast suspension ($2.5x10^4$CFU/ml) in each well, but 10µl broth was pipetted to twelfth well. The filled microtiter plate wells were sealed by parafilm and incubated at 37ºC for 24h (EUCAST, 2003; CLSI, 2015).

The incubated microtiter plate wells were filled with 0.01% resazurin sodium salt indicator from twelfth to first well and incubated for 2h at 37ºC. The MIC of extracts and fractions were determined as blue or purple resazurin colour changed to pink or colourless (Ohikhena *et al.*, 2017; Blazic *et al*., 2019). The experiment was done in triplicate.

3.7. Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration is the minimum concentration of extracts or fractions that have been killed bacteria. It was determined through sub-culturing of 10µl content of microtiter plate well that showed greater or equal to the lowest minimum inhibitory concentration on the Mueller Hinton agar and incubated for 24h. After 24h incubation, the Petri dish was assessed for the presence of growth, and the minimum concentration of extracts or fractions with no visible growth was taken as minimum bactericidal concentration (Akinduti *et al*., 2019). The experiment was done in triplicate.

3.8. Determination of Minimum Fungicidal Concentration (MFC)

The minimum fungicidal concentration is the minimum concentration of extracts or fractions that have been killed fungi. It was determined through sub-culturing of 10µl content of microtiter plate well that showed greater or equal to the lowest minimum inhibitory concentration on the sabouraud dextrose agar and incubated for 24h. After 24h incubation, the Petri dish was assessed for the presence of growth, and the minimum concentration of extracts or fractions with no visible growth was taken as minimum fungicidal concentration (Akinduti *et al*., 2019). The experiment was done in triplicate.

3.9.Preliminary phytochemical screening

The phytochemical constituents screening was performed using different chemicals and reagents for the detection of secondary metabolites in extracts and solvent fractions.

Flavonoids

About 250mg of extracts or fractions were dissolved in 5ml of distilled water in the test tube. A few drops of 1% ammonia solution (diluted from 30%ammonia solution) was added into mixture in the test tube. The appearance of yellow colour has affirmed the presence of flavonoids (Shetty *et al*., 2016).

Terpenoids

About 250mg of extracts or fractions were dissolved in 5ml of distilled water in the test tube. The 2ml of chloroform was added into mixture in the test tube. Subsequently, 3ml of concentrated

sulfuric acid was gently added to form the layer. The appearance of reddish-brown coloured at the interface was indicated the presence of terpenoids (Shetty *et al*., 2016).

Phenolic compound

About 250mg of extracts or fractions were dissolved in 5ml of distilled water in the test tube. A few drops of 5% ferric chloride were added into mixture in the test tube. The appearance of deep blue-black colour throughout the mixture in the test tube has affirmed the presence of phenol (Shetty *et al*., 2016).

Alkaloids

Alkaloid screening was performed according to Santhi and Sengottuvel (2016). A 500mg of extracts or fractions were dissolved in 6ml of 1% hydrochloric acid on a water bath and filtered.

Mayer's test

Mayer's reagent was prepared by dissolving 1.358 grams of mercuric chloride in 60ml distilled water and 5 grams of potassium iodide was dissolved in 10ml water. Then both solutions were poured together and filled to 100ml by distilled water in a volumetric flask. A few drops of Mayer's reagent were added to 2ml filtrate in the test tube and the appearance of yellow cream precipitate has affirmed the presence of alkaloids.

Wagner's test

Wagner's reagent was prepared by dissolving 2grams of iodine and 6grams of potassium iodide in 100ml of distilled water. A few drops of Wagner's reagent were added to 2ml filtrate in the test tube and the appearance of brown or reddish-brown precipitate has affirmed the presence of alkaloids.

Saponins

About 500mg of extracts or fractions were dissolved in 5ml distilled water in the test tube and vigorously shaken. The appearance of persistent froth above mixture has affirmed the presence of saponins (Nwadiaro *et al*., 2015).

Steroids

About 100mg of extracts or fractions were dissolved in 2ml chloroform in the test tube. The sulfuric acid was gently added into the mixture in the test tube to form a layer. The appearance of a reddish-brown colour at the interface was affirmed by the presence of steroids (Nwadiaro *et al*., 2015).

Tannins

About 500mg of extracts or fractions had been mixed in 10ml distilled water in a test tube that was boiled and filtered. A few drops of 0.1% ferric chloride was added to 5ml filtrate in the test tube. The appearance of brownish-green or blue-black colour throughout the mixture in the test tube has affirmed the presence of tannins (Ayoola *et al*., 2008).

Cardiac glycosides

About 500mg of extracts or fractions were dissolved in 5ml distilled water in the test tube. Then 2ml of glacial acetic acid containing a drop of ferric chloride solution was added into mixture in the test tube. Subsequently, 1ml of concentrated sulfuric acid was gently added to form a layer. The appearance of the brown ring at the interface, formation of the greenish ring in the acetic acid layer above the brown ring, and violet ring appearance below brown ring was indicated the presence of cardiac glycosides (Ayoola *et al*., 2008).

Anthraquinones

About 500mg extracts or solvent fractions were dissolved in 5ml of chloroform in the test tube and filtered. Subsequently, 3ml of 10% ammonia solution was added into 3ml of filtrate and shaken. The appearance of pink-violet or red colour in the ammonia layer was indicated the presence of anthraquinones (Nwadiaro *et al*., 2015).

3.10. Data Quality Assurance

The operations and control of types of equipment, sterilization, disinfection, and waste disposal were performed according to standard operating procedures of the Animal Products, Veterinary Drug and Animal Feed Quality Assessment Centre of Veterinary Drug and Animal Feed Administration and Control Authority. The collected test organisms were verified, culture media

was tested for sterility via incubation overnight, whereas antimicrobial activity experiments were replicated three times to ensure reproducibility of the experiment. Moreover, the quality of the experiment ensured by using positive, negative, growth, and sterility controls. The solvents, chemicals, and media were used for study experiment were analytical grade.

3.11. Data Analysis

The data were entered into an excel spreadsheet for statistical analysis using Statistical Package for Social Science (SPSS) version 20. The descriptive statistics, one-way ANOVA, Tukey Post Hoc test, and linear regression R^2 were utilized for statistical analysis and inference. The descriptive statistics were employed for calculation of group mean of inhibition zone diameter as Mean±SEM. The one-way ANOVA was performed to determine the significant difference among group means. Whereas, Tukey Post Hoc test was followed by one-way ANOVA to determine the significant difference between each group mean. The linear regression R^2 was calculated to determine the concentration dependence of crude extracts and solvent fractions on antimicrobial activities against test microorganisms. Statistically significant differences were declared at a p-value of less than 0.05.

3.12. Ethical considerations

The study was reviewed and approved by the Research and Ethical Review Committee of Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University.

4. RESULTS

4.1. Antibacterial activity

4.1.1. Agar well diffusion assay

The agar well diffusion assay was employed in the determination of crude extracts and solvent fractions inhibition zone diameter. The inhibition zone diameter was observed for crude extracts, solvent fractions, and positive control, but not for negative control (Figure 7). The highest inhibition zone diameter was observed for crude methanol extract against gram-positive bacteria (17.33 mm against *S. pyogenes*) and gram-negative bacteria (14.67 mm against *P. aeruginosa*). However, the highest concentration of crude methanol extract was produced in the lowest inhibition zone diameter 12.67 mm against *K. pneumoniae*. The highest inhibition zone diameter was observed for crude acetone extract against gram-positive (14.33 mm against *S. pyogenes*) and gram-negative bacteria (13.33 mm against *E. coli*). Nevertheless, the lowest inhibition zone diameter of 11.67 mm against *K. pneumoniae* was produced with the highest concentration of crude acetone extract (Table 1 and 2). The highest inhibition zone diameter for ethyl acetate fraction was observed against gram-positive (20.33 mm against *S. aureus*) and gram-negative bacteria(16.67 mm against *P. aeruginosa*). Whereas, the lowest inhibition zone diameter with no antibacterial activity against *P. aeruginosa* was observed for n-hexane fraction. Additionally, ethyl acetate fraction produced the highest inhibition zone diameter than the crude extract of methanol and acetone extract as well as other solvent fractions (Table 1, 2, 3, and 4). The highest inhibition zone diameter of ethyl acetate fraction was produced against gram-positive (17.67 mm against *S. aureus*) and gram-negative (14.67 mm against *E. coli*) clinical isolate bacteria (Table 5).

The concentration-dependent R^2 predictive values were determined for crude extract methanol and acetone ranging from 0.790 (*E. coli*) to 0.893 (*S. agalactiae*), and 0.813 (*K. pneumoniae*) to 0.932 (*S. pyogenes*), respectively (Table 1 and 2). The determined concentration-dependent R^2 predictive values for solvent fractions, n-hexane fraction ranging from 0.723 (*S. pyogenes*) to 0.795 (*K. pneumoniae*), chloroform fraction ranging from 0.750 (*S. agalactiae* and *S. pyogenes*) to 0.875 (*K. pneumoniae* and *P. aeruginosa*), ethyl acetate fraction ranging from 0.864 (*S. pyogenes*) to 0.949 (*P. aeruginosa*), and aqueous fraction ranging from 0.764 (*E. coli*) to 0.916 (*S. pyogenes*) (Table 3 and 4).The calculated concentration-dependent R^2 predictive values ranging from 0.723 (*E. coli*)

in n-hexane and chloroform fractions to 0.875 (*E. coli*) in methanol and acetone crude extracts against clinical isolate bacteria (Table 5).

The observed mean inhibition zone diameters of each concentration of crude extracts and solvent fractions of methanol extract against tested microorganisms were significantly different ($P < 0.05$) from those of at least one respective concentration of crude extract and solvent fractions of methanol extract, and positive control. The mean inhibition zone diameter of each concentration of methanol extract exhibited against S. *aureus* was significantly different ($P<0.05$) from those of each respective standard drug and positive control. Mean inhibition zone diameter of each concentration of acetone extract observed against *E. coli*, S. *aureus*, and *S. pyogenes* were significantly different ($P<0.05$) from those of each respective standard drug and positive control (Table 1 and 2). The observed mean inhibition zone diameters of ethyl acetate fraction at the concentration of 100mg/ml (against *S. aureus*, *S. agalactiae*, *S. pyogenes*, *E. coli*, and *P.aeruginosa*), 200mg/ml (against *S. aureus*, *S. agalactiae*, *E. coli*, *K. pneumoniae*, and *P.aeruginosa*), and 400mg/ml (against *E. coli* and *K.* pneumonia) were significantly different ($P < 0.05$) from those of each respective standard drug and positive control. The observed mean inhibition zone diameters of the aqueous fraction at the concentrations of 100mg/ml and 200mg/ml (against *S. agalactiae*, and *S. pyogenes*) and 400mg/ml (against *S. aureus*, and *S. agalactiae*) were significantly different ($P < 0.05$) from those of each respective standard drug and positive control (Table 3 and 4). The observed mean inhibition zone diameters of methanol extract at the concentration of 400mg/ml (against Clin. *E. coli*), acetone extract at the concentration of 400mg/ml (against Clin. *S. aureus* and *E. coli*), ethyl acetate fraction at the concentration of 100mg/ml and 200mg/ml (against Clin. *S. aureus*) and aqueous fraction at the concentration of 400mg/ml (against Clin. *E. coli*) were significantly different ($P < 0.05$) from those of each respective standard drug and positive control (Table 5).

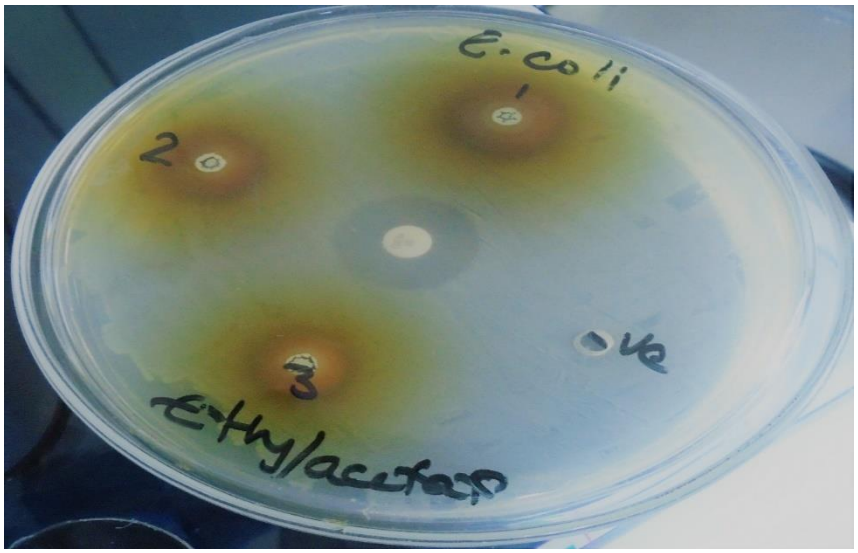

Figure 7: Inhibition zone diameter of *Ricinus communis* Linn leaf ethyl acetate fraction determined via agar well diffusion method.

Table 1: Mean inhibition zone diameter (mm) of *Ricinus communis* Linn leaf crude extracts against gram-positive bacteria.

Crude extracts		***S. aureus***		***S. agalactiae***		***S. pyogenes***	
		Mean ± SEM	R^2	Mean ± SEM	R^2	Mean ± SEM	R^2
Methanol extract	100mg/ml	10.67 ± 0.333^{a3c1d3}		10.33 ± 0.333^{a3d3}		12.67 ± 0.333^{a3c2d3}	
	200mg/ml	12.67 ± 0.333^{a3b1d1}	0.876	12.00 ± 0.577^{a3d2}	0.893	15.67 ± 0.333^{b2d1}	0.831
	400mg/ml	15.00 ± 0.577^{a3b3c1}		14.67 ± 0.333^{a1b3c2}		17.33 ± 0.333^{b3c1}	
Gentamicin	10µg	20.67±0.333		16.67±0.333		16.67±0.333	
Acetone extract	100mg/ml	9.67 ± 0.333^{a3c1d3}		9.67 ± 0.333^{a3c1d2}		9.67 ± 0.333^{a3b1d3}	
	200mg/ml	11.33 ± 0.333^{a3b1d2}	0.916	12.00 ± 0.577^{a3b1}	0.809	11.67 ± 0.333^{a3b1d2}	0.932
	400mg/ml	13.67 ± 0.333^{a3b3c2}		$13.67\pm.333^{a2b2}$		14.33 ± 0.333^{a2b3c2}	
Gentamicin	10µg	20.67±0.333		17.00±0.577		16.67±.333	

Values expressed as Mean±SEM for n=3. The mean comparisons for different extracts and control (Gentamicin 10µg) were performed by one-way ANOVA followed by Tukey HSD Post Hoc multiple comparison test. Where, compared to [a]positive control, [b]100mg/ml, [c]200mg/ml and [d]400mg/ml. [1]P<0.05, [2]P<0.01, [3]P<0.001.

Table 2: Mean inhibition zone diameter (mm) of *Ricinus communis* Linn leaf crude extracts against gram-negative bacteria.

Crude extract		*E. coli*		*K. pneumoniae*		*P. aeruginosa*	
		Mean ± SEM	R^2	Mean ± SEM	R^2	Mean ± SEM	R^2
Methanol extract	100mg/ml	10.67 ± 0.333^{a3d2}		9.67 ± 0.333^{d2}		11.33 ± 0.333^{a3d3}	
	200mg/ml	12.00 ± 0.577^{a3}	0.790	10.67 ± 0.333^{d1}	0.875	12.67 ± 0.333^{a3d1}	0.889
	400mg/ml	13.67 ± 0.333^{a3b2}		$12.67\pm.333^{a2b2c1}$		14.67 ± 0.333^{a1b3c1}	
Gentamicin	10µg	19.67±0.333		10.00±0.577		16.67±0.333	
Acetone extract	100mg/ml	9.67 ± 0.333^{a3c1d3}		8.33 ± 0.333^{c1d2}		9.67 ± 0.333^{a3d2}	
	200mg/ml	11.33 ± 0.333^{a3b1d1}	0.893	10.33 ± 0.333^{b1}	0.813	10.67 ± 0.333^{a3d1}	0.843
	400mg/ml	13.33 ± 0.333^{a3b3c1}		11.67 ± 0.333^{b2}		12.33 ± 0.333^{a3b2c1}	
Gentamicin	10µg	19.33±0.333		10.00±0.577		16.33±0.333	

Values expressed as Mean±SEM for n=3. The mean comparisons for different extracts and control (Gentamicin 10µg) were performed by one-way ANOVA followed by Tukey HSD Post Hoc multiple comparison test. Where, compared to [a]positive control, [b]100mg/ml, [c]200mg/ml and [d]400mg/ml. [1]P<0.05, [2]P<0.01, [3]P<0.001.

Table 3: Mean inhibition zone diameter (mm) of *Ricinus communis* Linn leaf solvent fractions of methanol extract against gram-positive bacteria.

Solvent fractions		*S. aureus*		*S. agalactiae*		*S. pyogenes*	
		Mean ± SEM	R^2	Mean ± SEM	R^2	Mean ± SEM	R^2
n-hexane fraction	100mg/ml	7.33±0.333^{a3d2}	0.746	7.33±0.333^{a3d2}	0.764	7.33±0.333^{a3d1}	0.723
	200mg/ml	8.67±0.333^{a3}		8.33±0.333^{a3}		8.33±0.333^{a3}	
	400mg/ml	9.67±0.333^{a3b2}		10.00±0.577^{a3b2}		9.33±0.333^{a3b1}	
Gentamicin	10µg	20.33±0.333		17.33±0.333		16.67±0.333	
Chloroform fraction	100mg/ml	7.33±0.333^{a3c1d2}	0.750	7.67±0.333^{a3c1d2}	0.754	7.33±0.333^{a3c2d3}	0.750
	200mg/ml	9.67±0.333^{a3b1}		9.67±0.333^{a3b1}		9.67±0.333^{a3b2}	
	400mg/ml	10.67±0.333^{a3b2}		10.67±0.333^{a3b2}		10.67±0.333^{a3b3}	
Gentamicin	10µg	21.00±0.577		17.00±0.577		17.33±0.333	
Ethyl acetate fraction	100mg/ml	15.33±0.333^{a3c2d3}	0.928	12.67±0.333^{a3c1d3}	0.898	13.33±0.333^{a2c2d3}	0.864
	200mg/ml	17.67±0.333^{a2b2d2}		15.00±0.577^{a1b1d2}		16.33±0.333^{b3c1}	
	400mg/ml	20.33±0.333^{b3c2}		17.67±0.333^{b3c2}		18.33±0.333^{b3c1}	
Gentamicin	10µg	20.67±0.333		17.33±0.333		17.00±0.577	
Aqueous fraction	100mg/ml	12.67±0.333^{a3d2}	0.893	10.67±0.333^{a3c1d3}	0.893	13.67±0.333^{a3c1d3}	0.916
	200mg/ml	14.33±0.333^{a3d1}		12.33±0.333^{a3b1d1}		15.33±0.333^{a1b1d1}	
	400mg/ml	16.33±0.333^{a2b2d1}		14.33±0.333^{a2b3c1}		17.33±0.333^{b3c1}	
Gentamicin	10µg	20.67±0.667		17.33±0.333		17.33±0.333	

Values expressed as Mean±SEM for n=3. The mean comparisons for different crude methanol extract's solvent fractions and control (Gentamicin 10µg) were performed by one-way ANOVA followed by Tukey HSD Post Hoc multiple comparison test. Where, compared to [a]positive control, [b]100mg/ml, [c]200mg/ml, and [d]400mg/ml. [1]P<0.05, [2]P<0.01, [3]P<0.001.

Table 4: Mean inhibition zone diameter (mm) of *Ricinus communis* Linn leaf solvent fractions of methanol extract against gram-negative bacteria.

Solvent fractions		*E. coli*		*K. pneumoniae*		*P. aeruginosa*	
		Mean ± SEM	R^2	Mean ± SEM	R^2	Mean ± SEM	R^2
n-hexane fraction	100mg/ml	7.33±0.333^{a3d2}	0.764	7.33±0.333^{a2d2}	0.795	--	
	200mg/ml	8.33±0.333^{a3}		8.33±0.333		--	
	400mg/ml	10.00±0.577^{a3b2}		9.67±0.333^{b2}		--	
Gentamicin	10µg	19.67±0.333		9.67±0.333		17.33±0.333	
Chloroform fraction	100mg/ml	7.67±0.333^{a3c1d2}	0.816	8.67±0.333^{d2}	0.875	8.67±0.333^{a3d2}	0.875
	200mg/ml	9.33±0.333^{a3b1}		9.67±0.333^{d1}		9.67±0.333^{a3d1}	
	400mg/ml	10.67±0.333^{a3b2}		11.67±0.333^{b2c1}		11.67±0.333^{a3b2c1}	
Gentamicin	10µg	19.33±0.333		10.00±0.577		17.33±0.333	
Ethyl acetate fraction	100mg/ml	10.67±0.333^{a3c1d3}	0.898	10.67±0.333^{c1d3}	0.945	10.67±0.333^{a3c2d3}	0.949
	200mg/ml	13.00±0.577^{a3b1d2}		12.67±0.333^{a2b1d2}		13.33±0.333^{a2b2d2}	
	400mg/ml	15.67±0.333^{a2b3c2}		15.67±0.333^{a3b3d2}		16.67±0.333^{b3c2}	
Gentamicin	10µg	19.67±0.333		9.67±0.333		17.00±0.577	
Aqueous fraction	100mg/ml	10.33±0.333^{a3d2}	0.764	10.33±0.333^{d2}	0.795	10.67±0.333^{a3d2}	0.843
	200mg/ml	11.33±0.333^{a3}		11.33±0.333^{a1}		11.67±0.333^{a3}	
	400mg/ml	13.00±0.577^{a3b2}		12.67±0.333^{a2b2}		13.33±0.333^{a2b2}	
Gentamicin	10µg	19.67±0.333		9.67±0.333		17.00±0.577	

Values expressed as Mean±SEM for n=3. The mean comparisons for different crude methanol extract's solvent fractions and control (Gentamicin 10µg) were performed by one-way ANOVA followed by Tukey HSD Post Hoc multiple comparison test. Where, compared to [a]positive control, [b]100mg/ml, [c]200mg/ml and [d]400mg/ml. [1]P<0.05, [2]P<0.01, [3]P<0.001. No activity = --

Table 5: Mean inhibition zone diameter (mm) of *Ricinus communis* Linn leaf crude extracts and solvent fractions of methanol extract against clinical isolate bacteria.

Crude extracts and Solvent fractions		**Clinical *E. coli* isolate**		**Clinical *S. aureus* isolate**	
		Mean ± SEM	R^2	Mean ± SEM	R^2
Methanol extract	100mg/ml	7.67±0.333^{a3d2}	0.875	9.33±0.333^{a3d2}	0.735
	200mg/ml	8.67±0.333^{a3d1}		10.67±0.333^{a3}	
	400mg/ml	10.67±0.333^{a3b2c1}		12.00±0.577^{a3b2}	
Gentamicin	10µg	19.67±0.333		20.33±0.333	
Acetone extract	100mg/ml	7.67±0.333^{a3d2}	0.875	7.33±0.333^{a3d2}	0.860
	200mg/ml	8.67±0.333^{a3d1}		8.67±0.333^{a3d1}	
	400mg/ml	10.67±0.333^{a3b2c1}		11.00±0.577^{a3b2c1}	
Gentamicin	10µg	19.33±0.333		20.33±0.333	
n-hexane fraction	100mg/ml	7.33±0.333^{a3d1}	0.723	7.33±0.333^{a3d2}	0.795
	200mg/ml	8.33±0.333^{a3}		8.33±0.333^{a3}	
	400mg/ml	9.33±0.333^{a3b1}		9.67±0.333^{a3b2}	
Gentamicin	10µg	19.33±0.333		19.67±0.333	
Chloroform fraction	100mg/ml	7.33±0.333^{a3d1}	0.723	8.33±0.333^{a3d2}	0.795
	200mg/ml	8.33±0.333^{a3}		9.33±0.333^{a3}	
	400mg/ml	9.33±0.333^{a3b1}		10.67±0.333^{a3b2}	
Gentamicin	10µg	19.33±0.333		19.67±0.333	
Ethyl acetate fraction	100mg/ml	11.33±0.333^{a3d2}	0.804	13.00±0.577^{a3c1d3}	0.842
	200mg/ml	13.00±0.577^{a3}		15.67±0.333^{a2b2d1}	
	400mg/ml	14.67±0.333^{a2b2}		17.67±0.333^{b3c2}	

Gentamicin	10µg	19.00±0.577		19.33±0.333	
Aqueous fraction	100mg/ml	7.67±0.333[a3d2]		12.00±0.577[a3d2]	
	200mg/ml	8.67±0.333[a3d1]	0.843	14.00±0.577[a3]	0.766
	400mg/ml	10.33±0.333[a3b2d1]		15.67±0.333[a2b2]	
Gentamicin	10µg	19.67±0.333		19.67±0.333	

Values expressed as Mean±SEM for n=3. The mean comparisons for different extracts, crude methanol extract's solvent fractions, and control (Gentamicin 10µg) were performed by one-way ANOVA followed by Tukey HSD Post Hoc multiple comparison test. Where, compared to [a]positive control, [b]100mg/ml, [c]200mg/ml, and [d]400mg/ml. [1]P<0.05, [2]P<0.01, [3]P<0.001.

4.2. Antifungal activity

4.2.1. Agar well diffusion assay

Inhibition zone diameters of crude extracts and solvent fractions of methanol extract were determined using agar well diffusion assay. The assay showed inhibition zone diameter for crude extracts, solvent fractions, and positive control, but not for negative control. The aqueous fraction showed the highest inhibition zone diameter of 21 mm, and no inhibition zone diameter was detected for n-hexane and chloroform fractions against *C. albicans*. The determined concentration-dependent R^2 predictive values ranged from 0.735 for methanol crude extract to 0.928 for the aqueous fraction. The observed mean inhibition zone diameters of different concentrations of ethyl acetate and aqueous fraction were significantly different ($P < 0.05$) from those of each respective standard drug and positive control (Table 6).

Table 6: Mean inhibition zone diameter (mm) of *Ricinus communis* Linn leaf crude extracts and solvent fractions of methanol extract against fungi.

Test extract and fraction		***Candida albicans***	
		Mean ± SEM	R^2
Methanol extract	100mg/ml	7.33 ± 0.333^{a3d2}	0.735
	200mg/ml	8.67 ± 0.333^{a3}	
	400mg/ml	10.00 ± 0.577^{a3b2}	
Amphotericin	20µg/ml	23.33±0.333	
Acetone extract	100mg/ml	7.33 ± 0.333^{a3d2}	0.746
	200mg/ml	8.67 ± 0.333^{a3}	
	400mg/ml	9.67 ± 0.333^{a3b2}	
Amphotericin	20µg/ml	22.67±0.333	
n-hexane fraction	100mg/ml	--	
	200mg/ml	--	
	400mg/ml	--	

Amphotericin	20µg/ml	23.33±0.333	
Chloroform fraction	100mg/ml	--	
	200mg/ml	--	
	400mg/ml	--	
Amphotericin	20µg/ml	23.33±0.333	
Ethyl acetate fraction	100mg/ml	11.33±0.333^{a3b1d3}	0.890
	200mg/ml	13.33±0.333^{a3b1d1}	
	400mg/ml	15.33±0.333^{a3b3c1}	
Amphotericin	20µg/ml	22.67±0.333	
Aqueous fraction	100mg/ml	14.67±0.333^{a3c1d3}	0.928
	200mg/ml	17.00±0.577^{a3b1d2}	
	400mg/ml	21.00±0.577^{a1b3c2}	
Amphotericin	20µg/ml	23.33±0.333	

Values expressed as Mean±SEM for n=3. The mean comparisons for different extracts, crude methanol extract's fractions, and control (Amphotericin 20µg/ml) were performed by one-way ANOVA followed by Tukey HSD Post Hoc multiple comparison test. Where, compared to [a]positive control, [b]100mg/ml, [c]200mg/ml, and [d]400mg/ml. [1]P<0.05, [2]P<0.01, [3]P<0.001. No activity = --

4.3. Determination of minimum inhibitory concentration.

The minimum inhibitory concentration was determined by the broth microdilution technique which has been used microtiter plates and incorporated resazurin sodium salt as a growth indicator (Figure 8).

MIC end point

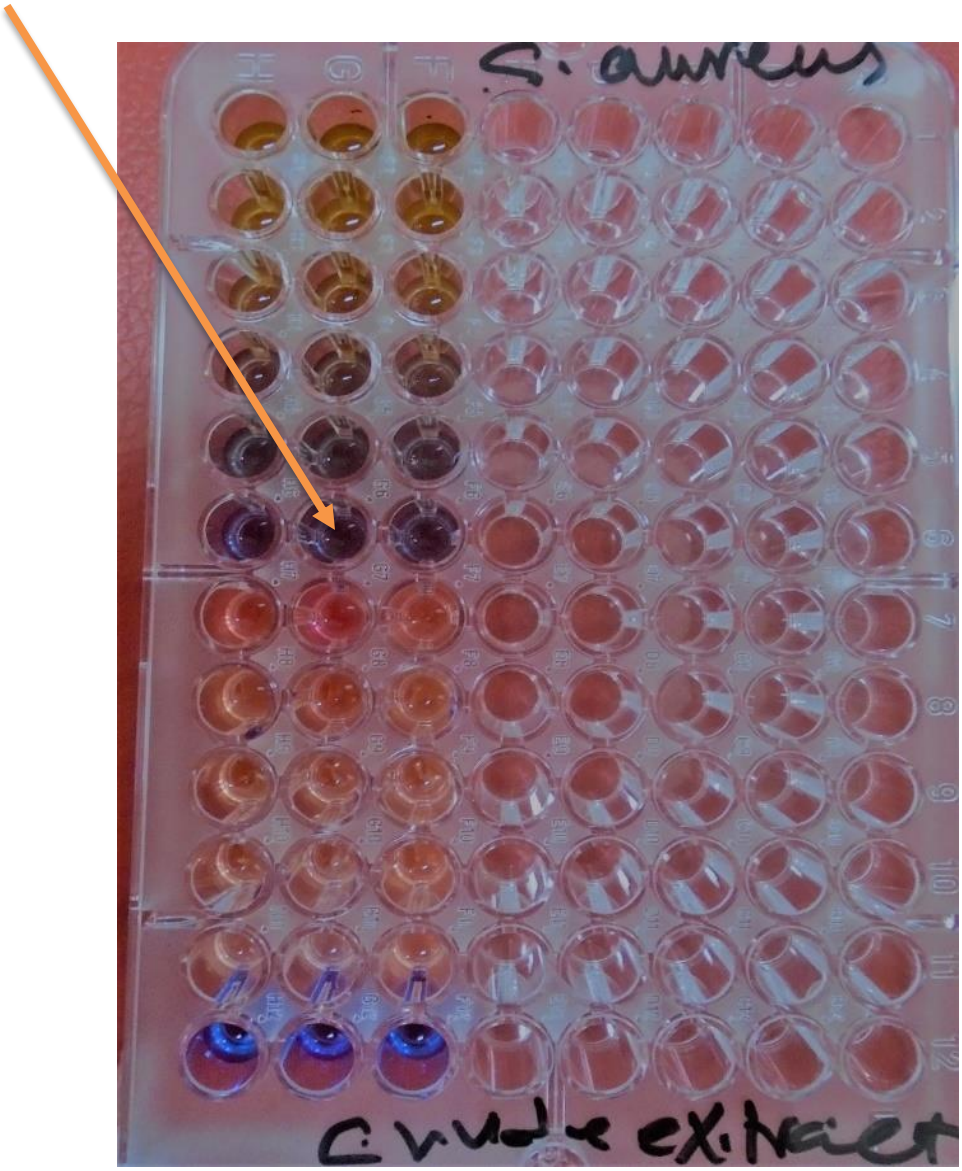

Figure 8: The microtiter plate displayed MIC for methanol crude extract in the presence of resazurin sodium salt.

4.3.1. Determination of minimum inhibitory concentration of crude extracts and solvent fractions of methanol extract against pathogenic bacteria.

The minimum inhibitory concentration of methanol crude extract ranging from 6.25mg/ml (*S. aureus*) to 25mg/ml (*E. coli*, *K. pneumoniae*, *P. aeruginosa*) and acetone crude extract ranging from 8.33mg/ml (*S. pyogenes*) to 100mg/ml (*K. pneumoniae*). Also, minimum inhibitory concentration ethylacetate fraction ranging from 1.5625mg/ml (*S. aureus*) to 12.5mg/ml (*P. aeruginosa*) and for aqueous fraction ranging from 6.25mg/ml (*S. aureus* and *S. pyogenes*) to 66.67mg/ml (*K. pneumoniae*) (Table 7 and 8). The minimum inhibitory concentration for clinical isolate bacteria ranging from 3.125mg/ml of ethyl acetate fraction (*S. aureus*) to 100mg/ml of n-hexane and chloroform fractions (*S. aureus* and *E. coli*) (Table 9).

4.4. Determination of minimum bactericidal concentration of crude extracts and solvent fractions of methanol extract.

The minimum bactericidal concentration (MBC) was determined as there has been no visible growth observed at the lowest concentration of extracts or solvent fractions (Figure 9). The MBC of methanol extract ranging from 100mg/ml (gram-positive bacteria) to 200mg/ml (*P. aeruginosa*) and was not detected in *E. coli* and *K. pneumoniae*. The minimum bactericidal concentration of acetone extract was 200mg/ml in gram-positive bacteria, but not detected in gram-negative bacteria. The minimum bactericidal concentration of n-hexane and chloroform fractions were not detected. However, minimum bactericidal concentration ethylacetate fraction ranging from 25mg/ml (*S. aureus* and *S. agalactiae*) to 200mg/ml (*E. coli* and *K. pneumoniae*), and in clinical isolate bacteria ranging from 88.33mg/ml (*S. aureus*) to 200mg/ml (*E. coli*) (Table 7, 8 and 9).

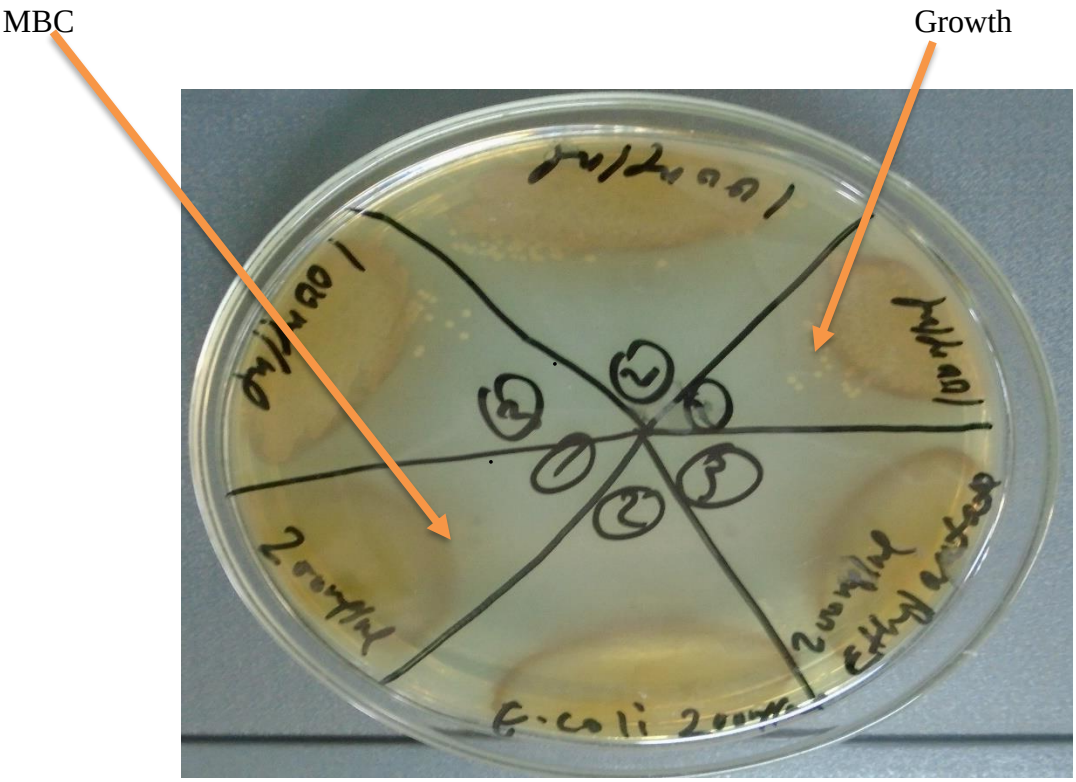

Figure 9: The minimum bactericidal concentration of ethyl acetate fraction determined from minimum inhibitory concentration by subculturing on Mueller Hinton agar.

Table 7: MIC and MBC of *Ricinus communis* Linn leaf crude extracts and solvent fractions of methanol extract against gram-positive bacteria.

Test extract and fraction	Activities	*S. aureus*	*S. agalactiae*	*S. pyogenes*
		Mean±SEM (mg/ml)	Mean±SEM (mg/ml)	Mean±SEM (mg/ml)
Methanol extract	MIC	6.25±0.000	12.500±0.000	6.25±3.1250
	MBC	100.00±0.000	100.00±0.000	100.00±0.000
Acetone extract	MIC	18.75±6.25	16.67±4.167	8.33±2.083
	MBC	200.00±0.000	200.00±0.000	200.00±0.000
n-hexane fraction	MIC	100.00±0.00	100.00±0.00	100.00±0.00
	MBC	ND	ND	ND
Chloroform fraction	MIC	83.33±16.667	16.67±4.167	16.67±4.167
	MBC	ND	ND	ND
Ethylacetate fraction	MIC	1.5625±0.00	4.17±1.0417	3.125±0.000
	MBC	25.00±0.000	25.00±0.000	50.00±0.000
Aqueous fraction	MIC	6.25±0.00	12.50±0.000	6.250±0.000
	MBC	200.00±0.000	200.00±.000	200.00±.000

Mean value expressed as Mean±SEM (n=3), ND = not detected

Table 8: MIC and MBC of *Ricinus communis* Linn leaf crude extracts and solvent fractions of methanol extract against gram-negative bacteria.

Test extract and fraction	**Activities**	***E. coli***	***K. pneumoniae***	***P. aeruginosa***
		Mean±SEM (mg/ml)	Mean±SEM (mg/ml)	Mean±SEM (mg/ml)
Methanol extract	MIC	25.00±0.000	25.00±0.000	25.00±0.000
	MBC	ND	ND	200.00±0.000
Acetone extract	MIC	66.67±16.667	100.00±0.000	66.67±16.667
	MBC	ND	ND	ND
n-hexane fraction	MIC	100.00±0.000	100.00±0.000	NT
	MBC	ND	ND	NT
Chloroform fraction	MIC	83.33±16.667	83.33±16.667	50.00±0.000
	MBC	ND	ND	ND
Ethylacetate fraction	MIC	4.17±1.0417	6.250±0.000	12.50±0.000
	MBC	200.00±0.000	200.00±0.000	50.00±0.000
Aqueous fraction	MIC	50.00±0.000	66.67±16.667	25.00±0.000
	MBC	ND	ND	200.00±0.000

Mean value expressed as Mean±SEM (n=3) ND = not detected, NT = not tested

Table 9: MIC and MBC of *Ricinus communis* Linn leaf crude extracts and solvent fractions of methanol extract against clinical isolate bacteria.

Test extract and fraction	Activities	Clinical *E. coli* isolates	Clinical *S. aureus* isolates
		Mean±SEM (mg/ml)	Mean±SEM (mg/ml)
Methanol extract	MIC	25.00±0.000	12.50±0.000
	MBC	ND	ND
Acetone extract	MIC	25.00±0.000	12.50±0.000
	MBC	ND	ND
n-hexane fraction	MIC	100.00±0.000	100.00±0.000
	MBC	ND	ND
Chloroform fraction	MIC	100.00±0.000	100.00±0.000
	MBC	ND	ND
Ethylacetate fraction	MIC	6.250±0.000	3.125±0.000
	MBC	200.00±0.000	83.33±16.667
Aqueous fraction	MIC	66.67±16.667	25.00±0.000
	MBC	ND	ND

Mean value expressed as Mean±SEM (n=3), ND = not detected

4.5. Determination of minimum inhibitory and fungicidal concentration of crude extracts and solvent fractions of methanol extract against *Candida albicans*.

The minimum fungistatic concentration of crude extracts and solvent fractions ranging from 3.125mg/ml of the aqueous fraction to 66.67mg/ml of methanol crude extract against *C. albicans*. The minimum fungicidal concentration of crude extracts and solvent fractions ranging from 50mg/ml of the aqueous fraction to 200mg/ml of ethyl acetate fraction. However, the minimum bactericidal concentration of methanol and acetone crude extracts were not detected against *C. albicans* (Table 10).

Table 10: MIC and MFC of *Ricinus communis* Linn leaf crude extracts and solvent fractions of methanol extract against fungi.

Test extract and fraction	**Activities**	***Candida albicans***
		Mean±SEM (mg/ml)
Methanol extract	MIC	66.67±16.667
	MFC	ND
Acetone extract	MIC	41.67±8.333
	MFC	ND
n-hexane fraction	MIC	ND
	MFC	ND
Chloroform fraction	MIC	ND
	MFC	ND
Ethyl acetate fraction	MIC	16.67±4.167
	MFC	200.00±0.000
Aqueous fraction	MIC	3.125±0.000
	MFC	50.00±0.000

Mean value expressed as Mean±SEM (n=3), ND, = not detected

4.6. Physical characteristics and preliminary screening of phytochemical constituents of *Ricinus communis* Linn leaf

The physical characteristics of *Ricinus communis* Linn leaf crude extracts and solvent fractions were dark green and reddish-brown, sticky solid in consistency, and percent of the yield was ranging from 7.5% to 41.67%. The phytochemical screening investigated alkaloids, flavonoids, terpenoids, tannins, cardiac glycosides, steroids, anthraquinones, saponins, and phenols in crude methanol extract and ethyl acetate fraction of *Ricinus communis* Linn leaf (Table 11 and 12).

Table 11: Percentage yield and physical characteristics of crude extracts and solvent fractions of methanol extract of *Ricinus communis* Linn leaf.

Test extract and fraction	Consistency	Colour	Weight of leaf powder or extracts	Weight of extracts or fractions	Percentage of yield
Methanol extract	Sticky solid	Dark green	200g	40g	20%
Acetone extract	Sticky solid	Dark green	200g	15g	7.5%
n-hexane fraction	Semisolid	Dark green	60g	10g	16.67%
Chloroform fraction	Solid	Dark green	60g	15g	25%
Ethyl acetate fraction	Sticky solid	Reddish-brown	60g	5g	8.33%
Aqueous fraction	Sticky solid	Reddish brown	60g	25g	41.67%

Table 12: Phytochemical constituents of crude extracts and solvent fractions of methanol extract of *Ricinus communis* Linn leaf.

Secondary metabolites	**Crude extract**		**Solvent fractions**			
	Methanol	Acetone	n-hexane	Chloroform	Ethylacetate	Aqueous residue
Flavonoids	+	+	–	+	+	+
Alkaloids	+	+	+	+	+	+
Saponins	+	–	–	+	+	+
Cardiac glycosides	+	+	–	–	+	+
Terpenoids	+	+	–	–	+	+
Tannins	+	+	+	+	+	+
Steroids	+	–	–	–	+	+
Phenols	+	+	+	+	+	+
Anthraquinones	+	–	–	–	+	–

-, absence, +, presence

5. DISCUSSION

The current study aimed to investigate antimicrobial activities of crude extracts and solvent fractions of methanol extract of *Ricinus communis* Linn leaf against pathogenic bacteria and *Candida albicans*. However, antibacterial activity had been done by a previous study from Gonder, Ethiopia, but this study did not include antifungal activity and methanol solvent for extraction (Abew *et al*., 2014). Furthermore, the previous findings have been reported that methanol solvent extract exhibited best antimicrobial activities from Ghana and Pakistan (Nazi and Bano, 2012; Suurbaar *et al.*, 2017). Both methanol and acetone crude extracts were also assessed for their antimicrobial activities to select the one which exhibited better antimicrobial activity for further solvent fractionation. There was a difference in the antimicrobial activities of the two extracts for presence and concentration of secondary metabolites could be affected by the type of solvent used for extraction (Liu et al., 2016).

The current study indicated that ethyl acetate fraction exhibited the highest antimicrobial activities in all tested microorganisms. Crude extracts were tested for their effects against gram-positive and gram-negative bacteria for their antimicrobial activities. Methanol crude extract revealed higher antimicrobial activity than acetone crude extract at the same concentrations. This finding agrees with that of the previous studies of Chandrasekaran and Venkatesalu (2004), Naz and Bano (2012), and Suurbaar *et al*. (2017). It is payable to the capability of methanol dissolving more secondary metabolites (Chandrasekaran and Venkatesalu, 2004). Methanol and acetone crude extracts exhibited greater antibacterial activities against gram-positive bacteria than gram-negative bacteria in a concentration-dependent manner. This could be because of differences in cell surface structure between gram-positive and negative bacteria. The outer membrane of gram-negative bacteria possesses lipopolysaccharides and lipoproteins. The lipopolysaccharides are amphipathic compounds that comprised hydrophilic polysaccharide at the core that makes up a more rigid outer membrane which slows down the diffusion of hydrophobic compounds through gram-negative bacteria cell membranes and acts as a barrier of permeability (Helander *et al*., 1998; Puupponen-Pimia *et al*., 2001; Zgurskaya *et al*., 2015).

The solvent fractions of methanol extract exhibited antimicrobial activity in a concentration-dependent manner except for n-hexane fraction that showed no antibacterial activity against *P. aeruginosa*. Hexane and chloroform fractions revealed the lowest antibacterial activity and no

antifungal activity. This could be due to variations in the concentration of secondary metabolites present in the solvent fractions (Osuagwu and Emi, 2013; Palmer-Young *et al*., 2017). Ethyl acetate fraction revealed highest antibacterial activity than crude extracts and solvent fractions which is in agreement with the finding of the previous study done by Voukeng *et al.* (2017). The aqueous fraction exhibited the highest antifungal activity followed by ethyl acetate fraction perhaps due to the capability of ethyl acetate solvent in concentrating a greater number of secondary metabolites from partitioning of methanol crude extract and interaction of these phytochemical constituents. Secondary metabolites with antifungal activity are concentrated more in aqueous solvent of methanol crude extract (Osuagwu and Emi, 2013; Palmer-Young *et al*., 2017; Sisay *et al*., 2019).

Mean of inhibition zone diameter of crude extracts and solvent fractions were significantly ($P < 0.05$) lower than the mean of inhibition zone diameter of positive control. The reason could be crude extracts and solvent fractions possessed both pharmacologically active and non-active substances whereas, control positive possessed purified and concentrated active ingredient (Ezekiel *et al*., 2009). Contrary to this, mean of inhibition zone diameter of crude methanol extract and aqueous fraction against *S. pyogenes*, crude acetone extract and chloroform fraction against *K. pneumoniae*, and ethyl acetate fraction against all tested gram-positive bacteria, *P. aeruginosa* and clinical *S. aureus* at 400mg/ml displayed inhibition zone comparable to the positive control. Furthermore, the mean inhibition zone diameter of crude methanol extract, ethyl acetate fraction, and aqueous fraction against *K. pneumoniae* at 400mg/ml was significantly ($P < 0.05$) higher than the mean of inhibition zone diameter of positive control. Mean of inhibition zone diameter of crude extracts and solvent fractions against clinical isolate *E. coli* and *S. aureus* were slightly lower than that of laboratory strains *E. coli* and *S. aureus* which is in agreement with the finding of Molla *et al.* (2016).The resistance mechanisms such as efflux pumps, β-lactamase production, and biofilm formation could have hindered the effectiveness of antibacterial in clinical isolates than laboratory strains (Patel and Richter, 2015; Kapoor *et al*., 2017).

The broth microdilution technique revealed the lowest minimum inhibitory concentration for ethyl acetate fraction against pathogenic bacteria whereas, aqueous fraction against yeast. The experiment indicated that the minimum inhibitory concentration of the broth microdilution technique was inversely proportional to the inhibition zone of the agar well diffusion technique.

This is an indication of the reproducibility of an experiment (Scorzoni *et al*., 2007). The ethyl acetate fraction also exhibited minimum bactericidal and fungicidal concentration against all tested microorganisms. Apart from this, n-hexane and chloroform fractions were devoid of bactericidal and fungicidal activity. This could be due to the concentration of higher number secondary metabolites in ethyl acetate fraction than crude extract and solvent fractions despite the detection of phytochemical constituents (Palmer-Young *et al*., 2017; Sisay *et al*., 2019).

The maceration technique was performed for the extraction of *Ricinus communis* Linn leaf and yielded a higher percent for methanol crude extract than acetone crude extract. The solvent fractionation yielded a higher percent for aqueous fraction than other solvent fractions. The phytochemical constituents screening revealed the presence of flavonoids, alkaloids, saponins, cardiac glycosides, terpenoids, tannins, steroids, phenols, and anthraquinones in methanol crude extract and ethyl acetate fraction whereas, anthraquinones were not detected in the aqueous fraction. Saponins, steroids, and anthraquinones were absent in acetone crude extract, but only alkaloids, tannins, and phenols were presented in n-hexane fraction. The capacity of methanol solvent in extracting more percent of extract yield and phytochemical constituents is in agreement with the findings of Felhi *et al.* (2017) from Tunisia and Truong *et al*. (2019) from Vietnam. The variation in types and concentration of phytochemical constituents and percent of extract yield is because of the difference in substance solubility among solvents. The difference in solubility of a substance might be based on the physical and chemical properties of solvents and phytochemical constituents. Types, quantity, and interactions of secondary metabolites present in crude extracts and solvent fractions are determinants of antimicrobial activities (Cowan, 1999; Shafique *et al.*, 2011; Felhi *et al.*, 2017; Palmer-Young *et al*., 2017).

The proposed mechanism of actions of secondary metabolites has been described by previous studies. They indicate various mechanisms of actions that are vital in additive or synergistic effects for antimicrobial activity. Flavonoids are a phenolic substance which is ubiquitously found in larger plants or photosynthesizing cell and are reported to have antimicrobial activity according to various scientific reports (Taleb-Contini *et al*., 2003; Cushnie and Lamb, 2005; Xie *et al*., 2015; Adamczak *et al*., 2020). The ways flavonoids reveal antimicrobial activities is through inhibition of nucleic acid synthesis (Ulanowska *et al*., 2006), disruption of cytoplasmic membrane function (Cushnie and Lamb, 2005), inhibition of energy metabolism (Eumkeb and Chukrathok, 2013), and

inhibition of porin on the cell membrane (Xie *et al*., 2015). Tannins are polyphenol compounds widely distributed in different parts of plants and have antimicrobial activities (Doss *et al*., 2009; Masietta *et al*., 2019). Mechanisms of antimicrobial activity of tannins are inhibition of biofilm formation (Scalbert, 1991), inhibition of extracellular microbial enzymes (Trentin *et al*., 2013), complexation of metal ions (Barbieri *et al*., 2017), and deprivation of substrates (Maisetta *et al*., 2019). The specialized tissues of plants produce and secrete multicyclic structure terpenoids which are possessed antimicrobial activities (Jasmine *et al*., 2011; Barbieri *et al*., 2017; Guimaraes *et al*., 2019). Terpenoids act through inactivation of enzymes (Oz et al., 2015), and rupturing of the cell membrane (Guimaraes *et al*., 2019). Alkaloids are nitrogen-containing organic base originated from plants, fungi, bacteria, and animals. It is possessed antimicrobial activities (Barbieri *et al*., 2017; Gurrapu and Mamidala, 2017; Othman *et al*., 2019). The antimicrobial activities of alkaloids are through inhibition of bacterial cytokinesis (Beuria *et al*., 2005), disruption of bacterial biofilms formation (Barbieri *et al*., 2017), and rupture of the bacterial cytoplasmic membrane (Alhanout *et al*., 2010). Phenolics are ubiquitously distributed in all parts of plants and possessed antimicrobial activities (Puupponen-Pimia *et al*., 2001; Maddox *et al*., 2010; Barbeiri *et al*., 2017; Othman *et al*., 2019). Phenolic compounds are exhibited antimicrobial activities via disruption of the cell membrane (Barbeiri *et al*., 2017), and bind to adhesins and cell wall proteins on the surface of microbial and rendering them unavailable (Cowan, 1999). Saponins are glycoside distributed in all parts of plants and possessed antimicrobial activities (Soetan *et al*., 2006; Tagousop *et al*., 2018; Dong *et al*., 2020). The saponin mechanisms of actions are through degradation of the cell wall, disruption of cell membrane integrity, and interruption of the biofilm system (Dong *et al*., 2020). Anthraquinones are coloured quinones compounds that are widely distributed in parts of plants and possessed antimicrobial properties (Locatelli *et al*., 2011; Wei *et al*., 2014; Duraipandiyan *et al*., 2016; Kemegne *et al*., 2017). Anthraquinones mechanisms of antimicrobial activities are through disruption of the cell wall and cell membrane of microbes (Wei *et al*., 2014), and inhibition of nucleic acid synthesis (Ankita and Richa, 2013). Steroids have antimicrobial properties (Taleb-Contini *et al*., 2003; Raquel and Epand, 2007). Mechanisms of actions of steroids were not clear, but Raquel and Epand (2007) suggested that the mechanism of action to be associated with membrane lipids where it removes lipids from liposomes.

6. LIMITATION OF STUDY

The current study limitation was small number of tested microorganisms.

7. CONCLUSION

Ricinus communis Linn leaf was subjected to different solvents for extraction where methanol solvent yielded more crude extract. The crude methanol extract constituted all screened secondary metabolites and exhibited the best antimicrobial properties against all tested microorganisms in a concentration-dependent manner. The crude methanol extract exposed to different solvents for solvent fractionation. Among those solvent fractions, ethyl acetate fraction constituted all screened secondary metabolites and revealed the most pronounced antimicrobial activity than crude extracts and other solvent fractions, but aqueous fraction exhibited higher anticandidal activity. The current study supports the claim for the use of *Ricinus communis* Linn leaf as traditional medicine for treatment of infectious diseases caused by bacterial and fungal pathogens.

8. RECOMMENDATIONS

Based on the current study findings the following points were forwarded.

- Studies should be conducted on ethyl acetate fraction to further isolate, purify, and identify bioactive principle(s) responsible for antibacterial and antifungal activities of the plant.
- Further study should be conducted on antimicrobial activities of the plant on other bacterial and fungal pathogens apart from currently tested microorganisms
- Mechanistic studies on isolated, purified and identified bioactive principle(s) of ethyl acetate fraction against bacterial and fungal pathogens.
- *In vivo* antimicrobial study should be conducted to confirm the *in vitro* antimicrobial activities of crude extracts and solvent fractions of plant against selected bacterial and fungal pathogens.

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