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Evaluation of antibacterial and antifungal activity, phytochemical content and acute oral toxicity of *Impatiens tinctoria* A. Rich root extracts

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SCHOOL OF GRADUATE STUDIES

This is to testify that the thesis prepared by Sileshi Degu: entitled "Evaluation of antibacterial and antifungal activity, phytochemical content and acute oral toxicity of *Impatiens tinctoria* A. Rich root extracts" and submitted in partial fulfillment of the requirements for the degree of Master in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology Specialty) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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List of abbreviations

AAU	Addis Ababa University
ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
EPHI	Ethiopian Public Health Institute
LD50	Median Lethal Oral Dose
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
Mg/ml	Milligram/milliliter
MIC	Minimum Inhibition Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
OECD	Organization of Economic Co-operation and Development
SD	Standard Deviation
TLC	Thin Layer Chromatography
TMMDR	Traditional and Modern Medicine Research Directorate
WHO	World Health Organization

Abstract

Background: Infectious diseases, caused by different microorganisms such as bacteria and fungi, are the major contributors of morbidity and mortality. The emergence of microorganisms' new resistance mechanism, and the emerging and re-emerging of new infectious disease aggravated the problem and have threatening our ability to treat infectious diseases. Therefore, researches for the development of new antimicrobials to combat resistant microbes and newly emerged infectious diseases are warranted. Hence, the best candidate is using plants for innovation of new drugs which are used in traditional medicine.

Objectives: To investigate the antimicrobial activities, acute oral toxicity and phytochemical screening of aqueous, ethanol and ethyl acetate root extracts of *Impatiens tinctoria* A. Rich.

Methods: The roots of *Impatiens tinctoria* A. Rich were collected from Gurage Zone around Butajira town and processed and extracted using solvents of ethyl acetate, ethanol and water. Agar well diffusion and agar dilution methods were used for screening the antimicrobial activity of the extracts and for determination of minimal inhibitory concentration, respectively. The minimum bactericidal and fungicidal concentration of the extracts was determined. Finally, the plant extracts were subjected to phytochemical screening and oral acute toxicity study. All these activities were carried out from January to October/2019.

Result: Gram positive bacteria were more susceptible to the extracts compared to gram negative bacteria whereas from tested fungi *T. rubrum* and *T. mentagrophytes* were more susceptible. The study screened the presence of at least 7 phytochemicals (alkaloids, flavonoids, anthraquinens, terpenoids, glycosides, quinones and saponins) and 6 compounds in the roots of the study plant. Ethyl acetate extract was more potent and broader spectrum of antimicrobial activities than ethanol and aqueous extracts. The LD50 was above 9600 mg/kg.

Conclusion: The roots of the study plant showed notable antibacterial and antifungal potency. However, further research should be undertaken to elucidate the involved phytochemicals and compounds present in the extracts and mechanism of action which clear out the road for developing new antimicrobial drugs.

Key words: Antibacterial, antifungal, acute toxicity, plant extract, *Impatiens tinctoria* A. Rich

1. Introduction

1.1 Background information

Infectious diseases are the world leading cause of premature deaths, killing almost 13.4 million people per year. The World Health Organization (WHO) forecasts 13 million deaths attributed to this cause in 2050 (1, 2). Infections due to a variety of bacterial and fungus etiologic agents become common and are taking the big share of the burden (1). Severe infections, including sepsis, meningitis, and pneumonia, are estimated to cause about a third of the 2.6 million neonatal deaths globally in which most of them are in less affluent regions of our planet (1). In Ethiopia, the top five leading causes of premature mortality in 2015 were lower respiratory infections, tuberculosis, diarrheal disease, ischemic heart disease, and Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome that indicates the dominant fatality of the infectious diseases (3).

There is also an alarming increase in the incidence of new and reemerging infectious diseases of which some of them don't have drugs that act against them (4,5). For instance, over the past 40 years a minimum of 50 emerging infectious agents have been identified across the globe; approximately 10% of them are bacterial agents (5). Additionally, drug resistance has been commonly reported from all over the world (6). For example, the development of resistance to methicillin has decreased the usefulness of this antibiotic in treating serious staphylococcal infections within the community and hospitalized patients (6). Presently, approximately 60,000 people in Europe and United States die each year due to serious infections caused by antimicrobial resistant bacteria (7). The problem is also high in Ethiopia as indicated by few studies (8-11).

In spite of such problems, medicinal plants have been used since ancient time to treat various diseases. They are bases for most of traditional healing practices in which around 4.3 billion people of the world's population use herbal medicines for some aspect of primary healthcare (12). Surveys carried out in developed countries like Germany and Canada tend to show that no less than 70% of their population have used herbal remedies at least once that reaches 80% when we come to the emerging world (13). Traditional remedies are the most important and sometimes

the only source of therapeutics for nearly 80% of the Ethiopian population and 95% of the preparations are of plant origin (14).

The leading effect of traditional healing practices on medicinal plants contribute the discovery of about 25% of modern medicines (like emetine, quinine and berberine), that is, descended from plants primarily used as traditional remedies (15). Medicinal plants are now more focused than ever due to the isolation of antimicrobial chemicals from them such as alkaloids, terpenoids, flavonoids, tannins and phenolic compounds (16-20). Therefore, using plants for innovation of new drugs by studying the potential antimicrobial activity of plant derived substances which are used in traditional medicine in different countries is a good alternative solution to treat infectious diseases (15).

The study plant *Impatiens tinctoria* A. Rich belongs to *Impatiens* genera and that of Balsaminaceae family is one of the species of over 1000 species belonging to this family (21). It is known by English vernacular name, Balsamine or by the vernacular name in Amharic, "Insosila". It is an upright perennial herb that grows to approximately 2 metres tall. It develops a large tuberous rootstock that lies at or just below the soil surface (figure 1) (22). It is native to East Africa and found many parts of Ethiopia (23). Flora biodiversity assessment studies recorded as *I. tinctoria* A. Rich is one of the most abundant herb species in Bonga Forest, Oromia Region of Ethiopia and in Mahoney / Maichew, Southern Tigray Region of Ethiopia has been found out as the dominant cash herb (24, 25).

Figure 1. Photograph of the *I.tinctoria* A. Rich A) parts above the ground B) Roots

In Ethiopia women chop or mash the inside of the tubers (roots) of *I. tinctoria* A. Rich in to a paste to dye the palms and nails of the hands and feet a dark reddish color (figure 2). It is considered a beauty treatment similar to that of henna. It also helps to control fungal infections like ringworm that cause athletes foot (tinea pedis), to toughen the skin and for abortion purpose (22, 26). The root decoction is also drunk against abdominal pains and as a purgative. The stem is chewed to treat mouth and throat diseases (27). Thus, using this information and other ethnobotanical studies (28-30) that support the medicinal value of the plant as a base line the root extracts of the study plant was evaluated for antibacterial and antifungal activities.

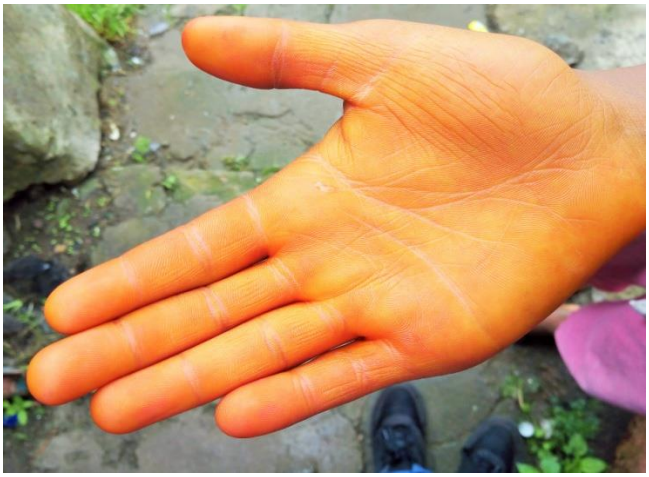

Figure 2. Photograph of dyed palms by a paste of *I.tinctoria* A. Rich roots

1.2. Statement of the problem

There is a major challenge to combat new emerged infections since there is no specific treatment against them (31). In the other way antimicrobial resistance becomes a global concern because new resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases, resulting in prolonged illness (increases the cost of health care with lengthier stays in hospitals), disability, and death. Medical procedures such as organ transplantation, cancer chemotherapy, diabetes management and major surgeries also become very high risky without effective antimicrobials (32).

In addition to these problems, antibiotics are sometimes associated with adverse effects on host which include hypersensitivity, depletion of beneficial gut and mucosal microorganisms, immune suppression and allergic reactions (33). Though some people are at greater risk than others (for example, people with chronic illnesses), no one can completely avoid the risk of these problems making it one of the world's most urgent public health problems (34). Moreover, in the last few decades drug development is becoming more and more inefficient mainly due to large development costs and low clinical trial drugs success rate (below 12%) (35).

Even though important drugs are developed from plants and their secondary metabolite constituents, only 15% of the angiosperms (flowering plants) have been chemically investigated for their medical potential (15,36). Cognizant of the fact that there are high burdens of infectious diseases from new and reemerging microbes, from drug resistant organisms and severe adverse reactions associated with the use of antibiotics have necessitated the continues development of new drugs with different mode of action from different potential sources such as medicinal plants.

1.3. Significance of the study

As a solution of stated problems scientific studies have to be conducted on the traditional medicinal plants to develop new, effective and safe antimicrobial drugs. Locally, Ethiopian women chop or mash the inside of the roots of *I. tinctoria* A. Rich in to a paste to dye the palms and nails of the hands and feet as a beauty treatment, to control fungal infections and to toughen the skin. In view of this, this study initiated to scientifically justify the antimicrobial potential of *I. tinctoria* A. Rich against selected bacteria and fungi pathogens. The acute oral toxicity evaluation was necessary to identify the range and concentration of dose that could be used and the possible clinical signs elicited by this medicinal plant. The preliminary phytochemical screening could be used as a starting point to quantify the identified phytochemical constituents and for further isolation of bio-active compounds. So, the study provides base line information for the development of alternative new drugs to the available conventional drugs.

2. Literature review

2.1. Infectious diseases and drug resistance

Infectious disease is still the leading causes of the majority of deaths though low and middle income countries suffer from a double burden from infectious and non-infectious diseases (1). Currently, there is warning of WHO that a number of emerging and re-emerging diseases including different bacterial and viral diseases have occurred in different regions (4, 5). Additionally, fungal infections are also increasing in alarming rate due to the growing population of immune compromised individuals, demographic changes and microbial adaptation (37).

In spite of the fact that more antimicrobial compounds were discovered and predicted that infectious diseases would be eliminated through the use of these antimicrobials, they did not become applicable as expected due to their resistance (16). Unfortunately, by now the increasing incidence of microorganisms becoming resistant to antibiotics has continuously become a scientific community concern as some pathogenic bacteria rapidly became resistant to many of the first effective drugs (6). For instance, the development of resistance to penicillin and vancomycin by S .*aureus*, pneumococci and enterococci by the production of a *B* -lactamase quickly decreased the usefulness of the antibiotics (6). This presents an enormous pressure and challenge to health professionals for infectious diseases treatment caused by these microorganisms (32).

According to a recent release from the WHO, *Escherichia coli (E. coli)* and *Klebsiella pneumoniae (K. pneumoniae)* resistant to cephalosporins and *S. aureus* resistant to methicillin (MRSA) have exceeded 50% of isolates found in multiple sites nationally (7). Bacterial resistance to third generation cephalosporins means that infections with these bacteria can only be treated using carbapenems, which are generally considered a last resort for severe infections. Notably, in some regions, the proportion of *K. pneumoniae* resistant to carbapenems has reached over 50% of isolates (36). Community acquired bacteria, such as *Streptococcus pneumonia*, non typhoidal *Salmonella, Shigella* species and *Neisseria gonorrhoeae*, have also displayed a rapid increase in antimicrobial resistance (36).

The high rates of morbidity and mortality caused by fungal infections are associated with the current limited antifungal arsenal and the high toxicity of the compounds. Additionally, identifying novel drug targets is challenging because there are many similarities between fungal and human cells (38). On the other way resistance to antibiotics in pathogenic fungi is a problem of special importance in the control of infections caused by these organisms (39). For instance study by Centers for Disease Control and Prevention (CDC) on *Candida* bloodstream isolates showed the increasing of resistant to the first and second line antifungal medications, such as fluconazole and the echinocandins (anidulafungin, caspofungin, and micafungin). About 7% of all *Candida* bloodstream isolates tested at CDC are resistant to fluconazole (40).

Emerging antifungal resistance has been identified in species like *Candida auris* (*C. auris)*. Isolates of *C. auris* sent to CDC are almost all resistant to fluconazole, and up to one third are resistant to amphotericin B, usually reserved as a last resort treatment (41). .Multidrug resistant candida infections (those that are resistant to both fluconazole and an echinocandin) have very few remaining treatment options. The primary treatment option is amphotericin B, a drug that can be toxic for patients who are already very sick. Not surprisingly, there is growing evidence to suggest that patients who have drug resistant candidemia are less likely to survive than patients who have candidemia that can be treated by antifungal medications (42).

Although the most common antifungal resistance occurs in Candida species, resistance in other types of less common fungi, in *Aspergillus* (a mold) infections, is also a problem (43). Depending on location, up to 12% of *Aspergillus* infections are estimated to be resistant to antifungal medications (41, 44). Resistant *Aspergillus* infections can develop in people who have taken certain antifungal medications. However, resistant infections are also found in people who have not taken antifungal medications. Therefore, more research is needed about how *Aspergillus* becomes resistant and how to prevent people from getting resistant *Aspergillus* infections (45).

Presently, approximately 60,000 people in Europe and the United States die each year due to serious infections caused by antimicrobial resistant bacteria, primarily those acquired in health care settings (7). Studies also showed high level of drug resistance exists to commonly prescribed antibiotics in the African continent (46). For instance, a review on 12 published articles on antimicrobial drug resistance in East Africa found 50%-100% resistance to ampicillin

and cotrimoxazole infections, emerging resistance to gentamicin (20%-47%) and relatively high levels of resistance to ceftriaxone (46%-69%) among gram negative infections. Much of the resistance was reported to be in *Klebsiella* species and *E. coli*. Among gram-positive infections, extensive resistance was reported to ampicillin (100%), gentamicin and ceftriaxone (50%-100%), with MRSA prevalence ranging from 2.6%-4.0 %(47).

In Ethiopia, even though enough studies are not available especially on fungal microorganisms ,few studies such as *E. coli* from 62.55% to 27.51% and 1.4% of multi drug resistance tuberculosis has been reported (8,9). A resistance of 32% to one or more antifungal agents tested such as fluconazole, ketoconazole and itraconazole as well as 11% fluconazole resistant *Candida* isolates from Oropharyngeal sites has also reported in Ethiopia(10,11). Overall, resistance has increased the burden on and doubled the mortality in clinical practices. The development of some resistance is almost certainly an inevitable consequence of the clinical use of antimicrobial drugs (6, 7).

2.2. Antimicrobial activity of medicinal plants

The use of plants for treating disease is as old as human species that makes plants and plant-derived compounds to be a long history of clinical use, better patient tolerance and acceptance. This also led to the isolation of early drugs such as morphine, cocaine, codeine, digitoxin, quinine and pilocarpine, of which some are still in use (48). It is still genuinely urgent to discover and develop new therapeutic agents to fight diseases relentlessly. For this medicinal plants still enjoy significant position in the modern day drug industries due to the minor side effects as well as the synergistic action of plant's phytochemicals and compounds (49).

So many studies have also shown the antimicrobial efficacy of medicinal plants. For instance, the study on leaves extract of *Lawsonia intermis* L. (henna) showed the potency of the extract to inhibit the growth pattern of *A. niger, Fusarium oxysporun*, *Streptococcus species* and *S. aureus.* (50). Studies on many spices such as clove, oregano, thyme, cinnamon, and cumin also showed as these spices possessed significant antibacterial and antifungal activities against food spoilage bacteria like *Bacillus subtilis (B. subtilis)* and *Pseudomonas fluorescens*, pathogens like *S. aureus* and *Vibrio parahaemolyticus*, harmful fungi like *Aspergillus flavus (A. flavus)* and even antibiotic resistant microorganisms such as MRSA (51).

The method of extraction and the solvents used for extraction has a great influence on the antimicrobial activity of the plant materials. The study in India on aqueous and methanol extracts of 12 plants each belonging to different families were evaluated for antibacterial activity by agar disc diffusion and agar well diffusion method. The aqueous extracts were inactive but methanol extracts showed some degree of antibacterial activity against the tested bacterial strains. *Salmonella typhimurium (S. typhimurium)* was the most resistant bacteria while Bacillus cereus was the most susceptible bacteria. Amongst the plant species screened, methanol extract of Bauhinia variegata bark showed best antibacterial activity (52).

A study on root of *Zingiber officinale Roscoe* (ginger) extract in Bangladesh showed the potent antimicrobial activity of the ginger extract against all tested bacterial pathogens. Soybean oil extract of ginger showed highest zone of inhibition against *Salmonella* species and lowest zone of inhibition against *E .coli*. Ginger extract also showed lower zone of inhibition against *S. aureus* compared to the gram-negative bacteria. Soybean oil extract of ginger at boiling temperature has potential antimicrobial activity and could be used in food preparation to get the synergistic effect of soybean and ginger (53). Another study in Iraq- Baghdad on root of this herb resulted with marked inhibitory effect on S. *aureus* and *E. coli* with ethanol, methanol and hexane extracts, while aqueous and acetone extracts did not show inhibitory effect on microorganisms test while chloroform extract had weak inhibition with *S. aureus* but hadn't any inhibition with *E. coli* bacteria(54).

Another study was undertaken on the cold and hot water prepared extract of *Bergenia ciliate (B. ciliata*), *Jasminum officinale (J. officinale)* , and *Santalum album(S. album)* for their potential activity against five human bacterial pathogens including *S. aureus, B. subtilis, Pseudomonas aeruginosa(P. aeruginosa), Proteus vulgaris* and *E. coli* using agar well diffusion method. Among the three medicinal plants, *B. ciliata* extracts displayed potential activity against bacterial pathogens. Cold water extract of *B. ciliata* showed the highest activity against *B. subtilis*, which is comparable with a zone of inhibition exhibited by ceftriaxone and erythromycin. *J. officinale* and *S. album* extracts demonstrated variable antibacterial activity (55).

In Sudan, *in vitro* study on antimicrobial activity of Sudanese medicinal plants by using different extraction solvents showed that methanol was the best solvent for extracting antimicrobial substances from the tested plants .Methanol leave extracts of *Solanum nigrum* was very active

against all of the tested microorganisms (*S. aureus, B. subtilus, E. coli, Salmonella typhi, Candida albicans (C. albicans)* and *Aspergillus niger* (*A. niger*)) with high inhibition zone of *S. typhi* 25 mm. Chloroform extracts of all plants samples were found less active against all of the tested microorganisms as compared to methanol extracts (56). Study in Kenya found the extract of *Senna Didymobot*rya plant was active against both gram positive and gram negative bacterial as well as fungal isolates (57).

In Ethiopia, many studies have been conducted on the antimicrobial activity of medicinal plants. For instance, a study was undertaken on an Ethiopian endemic medicinal plant *Taverniera abyssinica* A. Rich roots commonly known under the local name of "Dingetegna". Roots of the medicinal plant were extracted by maceration method using three different extraction solvents to determine their antimicrobial activity against *S. aureus, Enterococcus faecalis (E. faecalis)* and *E. coli* and clinical isolate of *C. albicans* and *Aspergillus flavus(A. flavus)*. Extracts showed better antimicrobial activity against *S. aureus, E. faecalis* and *C. albicans* while *E. coli* and *A. flavus* were the most resistant microorganisms to this medicinal plant. Antimicrobial activity of the medicinal plant varies with extraction solvent and tested microorganisms (58).

Ephrem Abiy and Asefaw Berhe conduct a study on antibacterial effect of *Allium sativum* against clinical isolates of *S. aureus* and *E. coli* from patients attending Hawassa Referral Hospital, Ethiopia showed that standard *S. aureus* and *E. coli* were completely inhibited by 10 mg/ml and 15 mg/ml of agar media, respectively and their clinical isolates were completely inhibited by 25 mg/ml (59). Another study in Gurage and Silti Zones, south central Ethiopia found in vitro antimicrobial activity of *Asparagus africanus, Guizotia schimperi (G. schimperi), Lippia adoensis var. adoensis* and *Premna schimperi* were active against *C. albicans, E. faecalis* and *S. aureus* at a concentration of 512 μg/ml or lower. Strong antibacterial activity was observed for *G. schimperi* extract against 17 resistant and sensitive *Staphylococcus* strains, at a concentration comparable to standard antibiotics (60).

2.2.1. Plants phytochemical content and antimicrobial properties

Many scientists around the world are performing research on plants to be able to discover antimicrobial compounds. To date, many plant constituents are known to have diverse biological activities like antibacterial and antifungal activities (61). This huge variety of constituents

produced by plants used as natural protection against microbial and insect attack (62). Many laboratory based studies also showed phytochemicals screened from plants showed various antimicrobial activities. For instance, glycoalkaloid, monoterpene and flavoinoides showed antifungal activity against *T. rubrum (*16)**.**

A study on *T. amplexicaule* Roth to investigate biologically active phytochemicals identifies a mixture of terpenoids (beta-sitosterol, alpha-amyrin, lupeol, hexacosanoic acid, ceryl alcohol and hexacosane). These isolated terpenoids were screened for their possible bioactivities against selected pathogenic bacteria and resulted as hexacosane was more active against *E. coli* and hexacosanoic acid had greater activity against *A. flavus* (63). Another study on the stem bark of *Stereospermum zenkeri* has isolated two anthraquinones (zenkequinones A and B) together with other known phytochemicals. The antimicrobial activity of the isolate was evaluated against six multiresistant strains of pathogens. Zenkequinone B showed the best antibacterial activity against gram-negative *P.aeruginosa* (64).

The crude extracts of *Mahonia aquifolium* (Pursh) Nutt stem bark and its two main protoberberine alkaloids, berberine and jatrorrhizine, were tested for their *in vitro* antimicrobial activity. Twenty strains of coagulase negative staphylococci and 20 strains of *Propioni bacterium acnes* isolated from skin lesions of patients with a severe form of acne, and 20 strains of *Candida* species isolated from chronic vulvovaginal candidoses were tested for their susceptibility to crude extract and two isolated alkaloids. The MIC obtained in this study illustrates the varying degrees of antibacterial and antifungal activity of both the crude extract and isolated alkaloids (65). In Burkina Faso, alkaloid also isolated from Sida acuta which showed highest inhibition zone against gram-positive bacteria (66). Studies have also reported that saponins appear to suppress fermentation in continuous culture (62).

The study plant has no available laboratory based reports on its phytochemical content and antimicrobial potential. But, numerous studies have been conducted to investigate the feasibility of a medicinal use of members of the genus *Impatiens* because of the rich and varied composition of the genus. Most of the plants of this genus are rich sources of napthoquinones, flavonoids, glycosides and saponins (18, 19). More than one phytochemicals are identified in one species of the genus for instance a study on ethyl acetate extracts of *Impatiens bicolor* plant

phytochemical analysis revealed the presence of alkaloids, tannins, steroids, saponins and flavonoids. This study showed the extracts antimicrobial activity comparable with standard antibiotics (19). The bioactive constituents or plants extracts may be uses for treatment of various diseases and these would be used as a new formulation for the novel drugs discovery in pharmaceutical industries (18).

2.2.2. Antimicrobial activity of genus *Impatiens*

One study in Costa Rica *in vitro* antimicrobial activities of the ethanolic extract from whole plant of *I. balsamina*, *I. hawkeri* and *I. walleriana* was evaluated against *S. aureus*, *Staphlococcus epidermidis (S. epidermis)*, *Streptococcus pyogenees (S. pyogenes)*, *Streptococcus pneumoniae*, *E. coli*, *P. aeruginosa*, *C. albicans* and *A. niger* using microdilution assay for MIC assessment found promising sources of antimicrobial agents of the genus *Impatiens*. *I. balsamina* extract was active against all tested gram positive bacteria and *C. albicans* . This extract also showed the widest antimicrobial spectrum (67).

Studies on *Impatiens balsamina (I. balsamina)* have been reported to have various pharmacological activities such as antimicrobial effects (68). For example, studies on *I. balsamina* seed, leaf, root and stem extracts of the plant was examined for antimicrobial activity and it has been found to possess remarkable antibacterial and antifungal activities (69). Compounds of 2-methoxy-1,4-naphthoquinone and stigmasta-7,22-diene-3β-ol (spinasterol) were isolated from the pods and roots/stems/leaves *of I. balsamina* L, respectively. These compounds have antibacterial and antifungal activity (70).

Study carried out in India on antibacterial and antifungal activity of *Impatiens sulcata Wallich(I. sulcata Wallich)* extracts by disc diffusion assay against a set of bacterial and fungal strains showed the moderate antimicrobial activity of the plant. Petroleum ether, ethyl acetate and methanolic extracts of *I. sulcata* show potent activity against *K. pneumoniae*. The activity was higher against gram positive *S. aureus* compared to gram negative bacterial strains (*E. coli, Salmonella typhirium* and *K. pneumonia).* The ethyl acetate extract of *I. sulcata Wallich* showed excellent antifungal activity against all tested fungal strains of *Trichorderma viride*, *A. niger* and *Aspergillus fumigates*. The antibacterial and antifungal activity is related positively to the

presence of total phenolic contents of the extract as ethyl acetate extract demonstrated higher activity than methanolic and petroleum ether extract (71).

In the study of *in vitro* antimicrobial activity of the essential oil of *I. balsamina*, an endemic plant in Morocco, an important inhibiting activity on the five bacterial strains (*E. coli*, *Klebsiella*, *Citrobacter*, *Bacillus*, *S. aureus* and *Acinetobacter*) was found. The concentration of 1/250 v/v was sufficient to stop the growth of all the bacteria which was shown most vulnerable to this essential oil. The major components like the β-thujone, the camphor can be responsible for the differentiation of the antimicrobic activity (72).

An ethnobotanical study of traditional medicinal plants in Degadamot Wereda, Amhara Region of Northern Ethiopia reported that the chopped and crushed roots of *I. tinctoria* A. Rich being mixed with water are drunk once or twice for abortion purpose; and similarly, the chopped, crushed and boiled roots are drunk to treat arthritis(27). Another study conducted in Wayu Tuka District of Oromia Region reported that the powdered root given orally for treating livestock diseases called blackleg in the study area by the locals (28). The stem is also chewed to treat mouth and throat diseases (29). Locally, women apply the root paste on their skin, nail and palms with the assumption of controlling fungal infections like ringworm that cause athletes foot (tinea pedis) and it also toughens the skin (22).

3. OBJECTIVES

3.1. General objective

- To evaluate the *in vitro* antibacterial and antifungal activities, phytochemical content, and acute oral toxicity of the root extracts of *I. tinctoria* A. Rich

3.2. Specific objectives

- To determine the antibacterial and antifungal activities of *I. tinctoria* A. Rich root extracts on selected bacterial and fungal species by agar well diffusion method
- To establish the MIC and MBC/MFC value of *I. tinctoria* A. Rich root extracts against tested microorganisms
- To investigate the phytochemical constituents and the Thin Layer Chromatography(TLC) profile of *I. tinctoria* A. Rich root extracts
- To assess the acute oral toxicity of *I. tinctoria* A. Rich roots aqueous extract

4. Hypothesis

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HO: The root of *I. tinctoria* A. Rich, contains phytochemicals that have antimicrobial potency against fungi and bacteria, as has been used traditionally for treating fungal diseases and as recommended by ethnobotanical studies for the treatment of different illness.

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5. Materials and methods

5.1. Study settings

The study was conducted at Ethiopian Public Health Institute (EPHI), the primary body in Ethiopia for carrying out research in the areas of health and nutrition, located in Addis Ababa, Ethiopia. Traditional and Modern Medicine Drug Research Directorate (TMMRD), has a mission developing validated traditional medicine products through research on traditional medicine as well as modern medicine to promote the health of the public, is part of EPHI in which specifically the research was performed in this directorate microbiology laboratory.

5.2. Study design and period

In-vitro experimental study of antibacterial and antifungal activity of *Impatiens tinctoria* A. Rich roots by extracting the roots of the study plant with different solvents was carried out from January to October/2019.

5.3. Study Variables

5.3.1. Dependent variables

- Zone of inhibition
- MIC value
- MBC value

5.3.1. Independent variables

- Microorganism type
- Extract type (by solvent)
- Concentration of extract

5.4. Plant Material collection, preparation and extraction

5.4.1. Collection

The roots of *I. tinctoria* A. Rich were collected around Butajira town located in Southern central Ethiopia 130 km from Addis Ababa. The plant material was identified by a botanist, and a voucher specimen deposited in the herbarium of TMMRD.

5.4.2. Herbal material preparation

Fresh *I. tinctoria* A. Rich roots were washed with clean water and rinsed with distilled water, cut into pieces and dried at room temperature in shade and lastly milled to powder by using milling machine. Then, the powder was weighed using electronic weighting balance and packed in polyethylene bags to avoid entrance of air and any other contaminant and stored in closed container with proper labeling for further extraction processes.

5.4.3. Extraction

Different studies on medicinal plants showed as extraction solvents used for extraction has a great influence on the antimicrobial activity of the plant materials (32, 39-41). Therefore, the powder part of the study plant was extracted using three different solvents. 100 grams of the powdery sample was weighed into each of the three cleaned and dried 2000 ml reagent bottles and 1000 ml of each solvent (ethyl acetate, ethanol and water) was separately added to each of the bottle and shaken (100 revolution per minute) on a shaking orbit machine (VWR DS-500; The Lab World Group, Boston, MA, USA) for 24 hours at room temperature. The mixture was filtered through whatman no.1 filter paper. After filtration the ruminant was repeated for additional two cycles with fresh solvent. The ethyl acetate and ethanol filtrate was concentrated under reduced pressure at 40ºC by a rotary evaporator (R-200 Buchi, Switzerland). These concentrated filtrates were transferred to beakers and dried by keeping beakers in water bath by setting at 40 º C to avoid the remaining organic solvents while water filtrate dried by lyophilizer **(**freeze dryer**).** Finally, extracts were kept at 2-8°C (73).

5.4. Antibacterial and antifungal activity of the extracts

5.5.1 Microorganisms

Antibacterial and antifungal activity *I. tinctoria* A. Rich root extracts were evaluated on the following clinical strains or drug sensitive standard strains of American Type Culture Collection (ATCC) that were obtained from microbiology laboratory of TMMRD and National Clinical Bacteriology and Mycology Reference Laboratory of EPHI. I) Gram-positive bacteria strains: *S. aureus* (ATCC 25923), MRSA (clinical isolate), *S. epidermidis* (ATCC 12228), *S. pyogenes* (ATCC 19615), *Streptococcus agalactiae* (*S. agalactiae* (ATCC 12386)) and *E. faecalis* (ATCC

29212). II) Gram-negative bacteria strains: *E. coli* (ATCC 25922), *S. typhimurium* (ATCC 13311), *Shigella flexneri (S. flexneri* (ATCC 12022)),*Shigella sonnei (S. sonnei* (ATCC25931)), *P. aeruginosa* (ATCC 27853), *K. pneumoniae* (ATCC 700603) and *proteus mirabilis (P. mirabilis* (ATCC 35659)) and III) Yeast and mold fungals :*C. albicans*(clinical isolates),(*T. rubrum* (ATCC 28188)), *T. mentagrophytes* (ATCC 18748)) *A. niger* (ATCC 10535) and *A. flavus* (ATCC 13697).The microbial strains used in the study were kept on tryptic soy broth supplemented with 20% glycerol at -80°C in the TMMRD microbiology laboratory, EPHI.

5.5.2. Inoculum preparation

Inoculums preparation was according to recommended by Clinical and Laboratory Standards Institute (CLSI). All strains were grown in Petridishes containing agar medium specific to each microorganism as refreshment of each strain for actual test. Each bacterial strain was incubated for 18-24 hours at 37 °C and each fungus was incubated for 7 days at 25°C. Standardization was by taking 3-5 inoculums from a fresh, pure culture of the test organism and making a suspension with nutrient broth (for bacteria) and sabroud dextrose broth (for fungi). The absorbance of the prepared suspension was read by uv-visible spectrophotometer (Thermo Scientific Evolution 60s CAT 840210100) with a 1cm light path till obtained an absorbance reading of 0.08 to 0.1 at 625nm which is proportional to $1x10^8$ CFU/ml bacteria and $1x10^7$ spores/ml fungi. Then, these suspension diluted with appropriate broth in 1:10 to get $1x10^7$ CFU/ml and $1x10^6$ spore/ml bacteria and fungi, respectively that used to evaluate the antimicrobial activity of the extract parallel to positive and negative controls (74,75).

5.5.3. Screening antimicrobial activity of the extracts

Agar well diffusion technique was used for screening of antimicrobial activity of the plant extracts. A sterile cotton swab was dipped into the adjusted suspension. The swab rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This removes excess inoculums from the swab. The dried surface of a plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculums. As a final step, the rim of the agar swabbed (74).

The streaked plate punched aseptically with a sterile cork borer that has a diameter of 8mm to form a hole for placing extracts, positive and negative controls. A 100µl volume of the antimicrobial agent or extract solution at desired concentration (100mg/ml, 200mg/ml and 400mg/ml) was introduced into the well. Standard drugs as positive control and diluents as negative control were run simultaneously. Then, the plates incubated under suitable conditions depending upon the test microorganism (at 37°C up to 18-24hours for bacteria and at 25°C up to 7 days for fungi). The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested. The presence of inhibition zones were measured by ruler recorded and considered as indication for antimicrobial activity (75).

5.5.4. Determination of Minimum Inhibitory Concentration

Standard agar dilution method was employed to determine MIC value of the plant extracts against all screened 18 test organisms. Different concentrations of extracts starting from a highest concentration of 64 mg/ml to 0.0625 mg/ml were prepared by two-fold serial dilution. Then, 2ml of the prepared concentrations were added in to test tubes containing 18 ml of molten agar medium that cooled to 55^{o}C and mixed thoroughly followed by dispensing to the petridish. After dispensing the plates were allowed to solidify at room temperatures. The next procedure was putting inoculums by taking 2µl standardized suspension using micropipette. The inoculums suspension preparation and incubation time was similar to the screening agar well method. The MIC was considered as the lowest concentration which inhibits the growth of the respective microorganisms under suitable incubation conditions expressed in mg/ml (76).

5.5.5. Determination of Minimum Bactericidal/Fungicidal Concentration

Streaks were taken from MIC plates exhibiting invisible growth and sub cultured onto appropriate extract and standard drug free agar plate medium. The plates incubated under suitable conditions depending upon the test microorganism (at 37°C for 18-24hours for bacteria's and at 25°C up to 7 days for fungi). Then after examined for bacterial/fungal growth in corresponding to plant extract concentrations. The subculture showed growth of colonies indicates only bacteriostaticability; reduced number of colonies indicates a partial or slow bactericidal activity where as no growth indicates as the whole inoculums have been killed.

Therefore, MBC/MFC was taken as the highest diluted extract that showed absence of any growth (77).

Generally, during the above all antimicrobial assays positive controls, negative controls, sterility controls and growth controls were run parallel to the experimental tests. Discs of erythromycin (15µg) and vancomycin (30µg) for gram positive bacteria, ciprofloxaciline (5µg) for gram negative bacteria and amphotericin B (32µg) for fungi were used as positive control in well diffusion assays whereas by diluting the powdered form of these standard drugs was used for MIC value determination. Since ethanol and ethyl acetate extracts were dissolved by 5% tween 80 and aqueous extract was dissolved by distilled water, 5% tween 80 for ethanol and ethyl acetate extracts and distilled water for aqueous extract were used as negative control. All assays were performed in triplicate.

5.6. Phytochemical screening

The qualitative phytochemical investigation of each extract of the herbal material was performed to evaluate the presence of different classes of secondary metabolites such as alkaloids, flavonoids, phenols, saponins, tannins and terpenoids. The qualitative results were expressed as positive for the presence and negative for the absence of phytochemical/s. It was carried out using standard tests as described below.

Test for alkaloids (Wagner's Test): Extracts were dissolved individually in dilute HCl and filtered. Then**,** filtrates of each extract were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids (78).

Test for flavonoids (H_2SO_4 test): A fraction of the extracts was treated with concentrated H_2SO_4 and observed for the formation of orange color (78).

Test for phenols: About 0.5 g of plant extract was added to 1 ml of 10% $FeCl_3$ solution. A deep bluish green coloration was an indication for the presence of phenol (79).

Test for tannins: 0.5 g of plant extract was mixed with 2mL of water and heated on water bath. The mixture was filtered and 1mL of 10% $FeCl_3$ solution was added to the filtrate. A blue-black solution indicates the presence of tannin (80).

Test for free anthraquinones (Borntrager's Test): About 0.2 g of each extract was shaken with 10 ml of benzene and then filtered. Five ml of the 10% ammonia solution was then added to the filtrate and shaken. Appearance of a pink, red or violet color in the ammonia (lower) phase was taken as the presence of free anthraquinones (80).

Test for coumarins: To the 2ml of extract 10% NaOH was added and shaken well for 5minutes shows the yellow color that indicates the presence of coumarins (81).

Tests for quinones: To the 2ml of extract a concentrated H_2SO_4 added and shaken well for 5 minutes shows the red color that indicates the presence of quinones (81).

Test for Saponins: To 1ml extract 20ml distilled water was added and shaken well in measuring cylinder for 15min. Formation of 1cm layer of foam indicates the presence of saponins (82).

Test for terpenoids (Salkowski test): The extract was mixed with 2ml of chloroform and concentrated H_2SO4 (3ml) is carefully added to form a layer. A reddish brown coloration of the interface shows positive result of the presence of terpenoids (82).

Test for glycosides: In 5ml extract, 2ml glacial acetic acid, one drop of 5% FeCl3 and conc. H_2SO4 were added. Appearance of brown ring indicates presence of glycosides (82).

5.7. TLC profiling

For TLC analysis TLC plates with Silica gel matrix on it with a layer thickness (200 μm), particle size (2-25 μm) and pore size (60 Å medium pore diameter) was used by cutting the 20×20cm plate sheet to 1×10cm strip. Plate markings were made with soft pencil. A half gram of the crude extracts of ethyl acetate, ethanol and water were dissolved in their respective solvent that is with 2 ml of ethyl acetate, ethanol and water, respectively. Capillary tube was used to spot a 2 drop of sample solution on the silica gel TLC plate at 1cm from the edged of the plate and the drop was allowed to dry (83).

The solvent systems used were hexane: ethyl acetate in a ratio of 7:5 for ethyl acetate and ethanol extract, dichloromethane: ethyl acetate in the ratio of 7:2 for water extract by adding a few drop of acetic acid to each prepared solvents. The chambers (for each solvent system) were allowed to saturate for at least 20 minutes before the TLC plates were placed. After the solvent

front reached 3/4th of the TLC height, the plates were removed from the chamber and allowed to dry. The plates were then observed in a UV fluorescent reader at 254 nm wavelengths. This TLC was done to provide a finger print of the crude extract by providing a rough number of components and nature in the extract indicated by the bands on the plate (83).

5.8. Oral acute toxicity

Acute toxicity study was performed according to the organization of economic co-operation and development (OECD) test Guidelines 420 (Acute Oral toxicity–Fixed dose procedure) with slight modification. Healthy young adult, nulliparous and non pregnant female albino mice was used. The testing animals were randomly selected from 8-12 weeks old mice, marked to permit individual identification, and kept in their cages for 5 days prior to dosing to allow for acclimatization to the laboratory conditions. The animals were fasted 3-4hours (food withdrawn but not water) prior to dosing after which the animals weighed to determine the fasted body weight. Each animal, at the commencement of its dosing, was 25-33gm weight (84).

The starting dose was 300 mg/kg which was increased by bi-fold till 9600mg/kg. Five animals were used for each dose. Treatment of animals at the next dose was delayed until assuring confident of survival of the previously dosed animals. The extract was calculated according to the body weight and dissolved in a consideration of the administered volume not exceed 1ml/100g of mice body weight. Then, the solvent alone for control groups and dilueted extract for treated groups were administered with oral guabage. After administration each mice was closely observed for the first 30 minutes, hourly during the first six hours, two hourly during the first 24 hours, and daily for a total of 14 days. All observations like changes in breathing, alertness, restlessness, diarrhea, behavioral pattern, mortality and consumption of food and water were systematically recorded. Moreover, the change in body weight was measured at initial day and at 7 and 14 post treatment days (84).

5.9. Data Quality Assurance

To ensure the quality of the data all the laboratory works were performed according to the SOPs and internationally accepted principles for testing of medicinal plants by the principal investigator with the help of experienced professionals. Each equipments, supplies, reagents and procedures were adequately controlled. The assessment of the antimicrobial activity, the TLC

profiling and phytochemical screening of the extracts was performed at least in triplicate with controls for insuring quality and reproducibility results. There was a continuous monitoring and supervision of the data extraction by advisors. The extracted data was examined for completeness and checked for consistency before entering it in to the excel spreadsheet.

5.10. Data analysis and interpretation

Data were entered into excel spreadsheet, exported to Minitab 16 software and analyzed. The statistical differences of the antimicrobial activity of crude extracts on each microorganism and the effect of each extracts on body weight of the albino mice were carried out by employing one way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. The experimental data was expressed as Mean ± Standard Deviation (SD). The result considered statistically significant at $P<0.05$.

5.11. Ethical considerations

Ethical clearance was sought from the Department of Research and Ethical Review Committee of Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University. Based on the ethical clearance, permission was obtained to conduct the study in the laboratory of TMMRD of EPHI as they were interested with this research title.

5.12. Operational definition of terms

Extract: is a byproduct of plant material extraction in which qualitatively analyzed for chemical composition (phytochemicals), biological activities (antimicrobial assay) and acute toxicity.

Zone of inhibition diameter: is the area showing no visible growth around the positive controls, negative controls and tested extracts that can be detected with the unaided eye. Zones are measured on the shortest diameter using a ruler including the diameter of the disc or well after incubation.

Minimum inhibition concentration: is the lowest concentration of extracts/antimicrobials which inhibits 100% the growth of the respective bacteria or fungi under suitable incubation conditions expressed in mg/ml.

Minimum bactericidal concentration/minimum fungicidal concentration: is the highest diluted extracts/antimicrobials that showed absence of any bacterial and fungal growth after sub culturing streaks taken from MIC plates expressed in mg/ml.

6. Results

6.1. Antimicrobial activity

6.1.1. Antimicrobial activity screening by well method

The antimicrobial activity of aqueous, ethanol and ethyl acetate extracts from the roots of *I.tinctoria* A. Rich were screened against selected microorganisms. A total of 18 microorganisms (13 bacteria and 5 fungi) by agar well diffusion assay were assessed at a concentration of 100, 200, 400mg/ml for each extract in triplicates.

6.1.1.1. Antibacterial activity against gram positive bacteria

The average zone of inhibition formed by all tested concentrations of ethanol and ethyl acetate extracts against *S. aureus* was better compared to the aqueous extract, significantly different at *P<0.05* (Table 1). Each extract has produced notable inhibition zone against MRSA at all concentrations. *S. epidermidis* produced the largest average zone of inhibition when compared to the other tested gram positive bacteria in which the inhibition zone against this bacterium ranges from 38.0±1.0 mm by 100 mg/ml aqueous extract to 43.7±1.5 mm by 400mg/ml ethyl acetate extract. Comparisons of the mean growth inhibition zones for *S. aureus* at 100, 200 and 400 mg/ml concentrations of the three tested extracts showed no significant differences ($P < 0.05$). It indicates absence of concentration dependent inhibition difference which was also observed on MRSA and *S. epidermidis.*

All extracts showed less inhibition zone against the two Streptococci bacteria (*S.pyogen* and *S.agalactiae*) when compared with other tested gram positive bacteria with each corresponding concentration. Erythromycin (15 µg) showed better inhibition diameter against the two tested streptococci than the three extracts at all concentrations that were significantly different at $p<0.05$. In extract type dependent manner, better inhibition against *S. pyogen* and *S. agalactiae* were obtained by ethyl acetate extract followed with ethanol extract. The least inhibition diameter 17±0.0 mm at 100 mg/ml aqueous extract against *S.agalactiae* was recorded when compared with all tested gram positive bacteria.

The three extracts showed better inhibition on 400 mg/ml than 100 mg/ml and 200 mg/ml concentrations to *E. faecalis* with a significant difference at $P<0.05$. The effect of vancomycin

(30 µg) on this bacterium revealed less inhibition to all extracts and doses, with a significant difference at P<0.05, except the aqueous extract at 100 mg/ml concentration. Similarly, erythromycin (15 µg) that produced 21.3±2.3 mm inhibition zone was greater than the inhibition of aqueous extract at 100mg/ml concentration and lesser than or equal to other extracts at all concentrations. The potency of almost all extracts on this bacterium was better than *S. pyogenes* and *S. agalactiae,* but lesser than *S. epidermidis, S. aureus* and MRSA.

Table 1. Inhibition zone diameter measurement (mm) of crude extracts of *I. tinctoria* A. Rich roots against gram positive bacteria.

Different solvent extracts (mg/ml)		Inhibition Zone Diameter (mm), including well diameter (8 mm)					
		S. aureus	MRSA	*S. epid*	*S. pyogen*	*S.agal*	*E. faecalis*
Aqueous	100	25.7±0.6^{d}	27.0±1.0^{d}	38.0±1.0^{b}	18.0±0.0^{f}	17.3±0.6^{h}	19.0±1.0^{de}
	200	27.3±0.6^{cd}	28.3±0.7^{cd}	41.0±1.0^{ab}	21.7±0.6^{e}	18.0±0.0^{gh}	22.3±0.6^{cd}
	400	29.3±1.2^{bc}	28.7±0.6^{cd}	41.7±1.5^{ab}	24.0±1.0^{cd}	21.3±0.6^{cde}	27.7±1.5^{ab}
Ethanol	100	35.0±1.0^{a}	29.7±0.7^{bcd}	41.0±1.7^{ab}	22.3±0.6^{de}	19.0± 0.0^{fgh}	21.3±1.5^{cde}
	200	35.7±0.6^{a}	30.3±0.6^{bcd}	42.0 ±1.0^{a}	23.3±0.6^{de}	19.7±1.5^{efg}	27.7±0.6^{ab}
	400	36.7±0.6^{a}	31.7±1.5^{bc}	43.0±1.0^{a}	24.0±1.0^{cd}	21.7±1.2^{cde}	28.7 ±1.2^{a}
Ethyl acetate	100	36.3±0.6^{a}	30.0±1.7^{bcd}	41.3±0.6^{ab}	25.7±0.6^{b}	22.3±0.6^{bcd}	24.3±0.6^{bc}
	200	36.0±1.0^{a}	33.0±2.7^{ab}	42.0±1.0^{a}	26.7±0.6^{b}	23.0±1.0^{bc}	27.7±0.6^{ab}
	400	37.0±1.0^{a}	35.7±1.2^{a}	43.7±1.5^{a}	26.0±1.0^{b}	24.0±1.0^{b}	29.7±1.5^{a}
Distilled water	-	8.0±0.0^{f}	8.0±0.0^{f}	8.0±0.0^{e}	8.0±0.0^{g}	8.0±0.0^{i}	8.0±0.0^{f}
5% tween 80	-	8.0±0.0^{f}	8.0±0.0^{f}	8.0±0.0^{e}	8.0±0.0^{g}	8.0±0.0^{i}	8.0±0.0^{f}
Vancomycin	30µg	21.3±1.5^{e}	21.3±1.5^{e}	21.7±2.1^{d}	22.0±1.0^{de}	20.7±0.6^{def}	18.7±1.2^{e}
Erythromycin	15µg	31.7±2.1^{b}	31.7±2.1^{bc}	32.3±2.1^{c}	30.3±1.5^{a}	31.0±1.0^{a}	21.3±2.3^{cde}

Key notes: Values are expressed as Mean ± SD (n=3), 8.0±0.0 = no inhibition (well diameter), "Means" that do not share a superscript letter are significantly different (only column wise) at P<0.05. *S. epid- S. epidermidis, S. agal- S. agalactiae*

6.1.1.2. Antibacterial activity against gram negative bacteria

A total of seven gram negative bacteria were used to assess the antibacterial activity of the extracts (Table 2). *S. flexneri, S. soni and P. mirabilus* showed the highest inhibition zone

among all gram negative bacteria at 400 mg/ml concentration of ethyl acetate extract with inhibition zone of 22.7±0.6 mm compared to other gram negative bacteria. *K. pneumoniae* measured the smallest inhibition zone of 17.3±1.2 mm at this concentration and extract type. However, the growth of this bacterium did not show any inhibition by 100 mg/ml aqueous extract.

The positive control, ciprofloxacin (5 µg/ml), showed significantly higher inhibition of the growth of all gram-negative bacteria compared to all tested extracts at all concentration ($P<0.05$). On the other hand, among the extract type, aqueous extract has showed significantly lower inhibition activity against the tested gram-negative bacteria ($P<0.05$). Moreover, no statistically significant different inhibitions were observed against *E. coli, S. typhimurium, P. aeroginosa, K. pneumoniae* and *P. mirabilus* at a concentration of 100 mg/ml of aqueous extract compared to the treatment of negative control (distilled water and 5% tween 80) ($P<0.05$). Surprisingly, except in this case and *K. pneumoniae*, the remaining bacteria did not show any significant different ($p<0.05$) susceptibility at most extract type and concentration.

Table 2. Inhibition zone diameter measurement (mm) of crude extracts of *I.tinctoria* A. Rich roots against gram negative bacteria.

Extract Type	Conc.	Inhibition Zone Diameter (mm), including well diameter (8 mm)						
		E.coli	*S.typhim*	*S.flexneri*	*S.sonnei*	*P.aerog*	*K.pneum*	*P.mirabi*
Aqueous	100	8.7±0.6^{e}	9.0±0.0^{ef}	11.3 ±0.6^{h}	11.3±0.6^{g}	11.0±1.0^{f}	8.0±0.0^{f}	9.3±0.6^{g}
	200	11.7±0.6^{d}	11.7±0.6^{e}	13.3 ±0.6^{g}	13.0±1.0^{fg}	13.7±0.6^{ef}	9.0±0.0^{f}	11.7±0.6^{f}
	400	15.0±0.0^{c}	15.3±0.6^{d}	16.7 ±0.6^{f}	15.0±0.0^{ef}	16.0±1.0^{de}	12.0±1.0^{e}	14.7±0.6^{e}
Ethanol	100	16.3±0.6^{c}	16.7±0.6^{cd}	16.0± 1.0^{f}	14.7±0.6ef	17.7±1.2^{cd}	13.7±0.6^{de}	19.3±0.6^{cd}
	200	19.3±0.6^{b}	19.0±1.0^{bc}	17.3±0.6^{ef}	15.3±0.6^{ef}	19.3±1.5^{bc}	15.0±0.0^{cd}	21.3±0.6^{bc}
	400	20.7±0.6^{b}	20.7±1.5^{b}	18.7±0.6^{de}	17.3±1.2^{df}	19.7±1.2^{bc}	16.7±0.6^{bc}	23.3±0.6^{b}
Ethyl acetate	100	15.7±0.6^{c}	17.3±0.6^{cd}	20.0±0.0^{cd}	19.0±1.0^{cd}	17.3±1.2^{cd}	14.7±0.6^{cd}	18.7±0.6^{d}
	200	19.3±1.5^{b}	19.3±1.5^{bc}	21.3±0.6^{bc}	21.3±1.5^{bc}	19.3±1.5^{bc}	15.7±1.5^{bcd}	19.7±1.5^{cd}
	400	21.0±1.0^{b}	21.0±1.7^{b}	22.7±0.6^{b}	22.7±2.3b	21.7±0.6^{b}	17.3±1.2^{b}	22.7±1.5^{b}
DW	-	8.0±0.0^{e}	8.0±0.0^{f}	8.0±0.0^{i}	8.0±0.0h	8.0±0.0^{g}	8.0±0.0^{f}	8.0±0.0^{g}
5% T80	-	8.0±0.0^{e}	8.0±0.0^{f}	8.0±0.0^{i}	8.0±0.0^{h}	8.0±0.0^{g}	8.0±0.0^{f}	8.0±0.0^{g}
Cipro	5µg	30.7±0.6^{a}	33.7±0.6^{a}	32.3±0.6^{a}	31.0±0.0^{a}	28.7±0.6^{a}	23.3±0.6^{a}	32.3±0.6^{a}

Key notes: Values are expressed as Mean ± SD (n=3), 8.0±0.0= no inhibition (well diameter), Means that do not share a superscript letter are significantly different (only column wise) at $P<0.05$. Conc.-Concentration, Cipro- Ciprofloxacillin as positive control, DW- Distilled Water as negative control 1, *P.aero-P. aeruginosa*, *S.typhim-S. typhimurium*, T80-Tween 80 as negative control 2

6.1.1.3. Antifungal activity

Table 3 shows antifungal activity of the plant extracts against *C. albicans, T. rubrum, T. mentagrophytes, A. niger and A. flavus*. Ethyl acetate extract showed a higher inhibition of the growth of tested fungi when compared to aqueous and ethanol extracts ($P<0.05$). The inhibition activity of each extracts was dose dependent. There was no inhibition of growth of *C. albicans, A. niger* and *A. flavus* against the treatment of aqueous crude extract at 100mg/ml concentration. Among the extracts used aqueous extract has showed significantly lower inhibition of all tested fungi except *A. niger* ($P<0.05$).

The dermatophytes, *T. rubrum* and *T. mentagrophytes*, were more susceptible compared to the other fungal species at all tested extract types and concentrations. The minimum inhibition zones were recorded at 100mg/ml concentration of aqueous extracts with the value of 14.0±1.0 mm and 12.7±0.6 mm against *T. rubrum* and *T. mentagrophytes*, respectively. The maximum inhibition zones were recorded by ethyl acetate extract of 400mg/ml concentration with the inhibition diameter of 36.7±1.5 mm and 33.7±1.2 mm against *T. rubrum* and *T. mentagrophytes*, respectively. Similarly, higher inhibition zone against *C. albicans (*27.3±2.1mm), *A. niger* (27.3±0.6 mm) and *A. flavus* (28.7±0.6 mm) were observed at 400 mg/ml concentration of ethyl acetate extract.

Amphotericine B was used as a positive control at a concentration of 32 µg /ml. The anti fungal activity pattern of the drug showed the highest inhibition against the growth of *C. albicans* (25.3±0.6 mm) followed by *T. mentagrophytes* and *A. niger* with the inhibition diameter of 20.3±0.6 mm and 20.0±1.0 mm, respectively. The drug has also showed inhibition of the growth of *T. rubrum* and *A. flavus* with diameter of 15.7±0.6 mm and 13.3±0.0 mm, respectively. All extracts of the study plant at most tested concentration showed better inhibition against *T. rubrum* and *T. mentagrophytes* when compared to the positive control, significantly different at $p<0.05$. Generally, the antifungal activities of the ethyl acetate extract in terms of its zone of inhibition were better or in line with amphotericine B (32 µg /ml) against each fungal strain.

Table 3. Inhibition zone diameter measurement (mm) of crude extracts of *I. tinctoria* A. Rich roots against fungi.

Extracts Type	Conc. (mg/ml)	Inhibition Zone Diameter (mm), including well diameter (8 mm)				
		C. albicans	*T. rubrum*	*T .menta*	*A. niger*	*A. flavus*
Aqueous	100	8.0 ± 0.0^{f}	14.0 ± 1.0^{d}	12.7 ± 0.6^{g}	8.0 ± 0.0^{f}	8.0 ± 0.0^{h}
	200	$8.7\pm0.6e^{f}$	26.0 ± 1.0^{c}	16.7 ± 1.5^{f}	9.3 ± 0.6^{ef}	9.0 ± 1.0^{gh}
	400	$9.7\pm0.6de^{f}$	29.0 ± 1.0^{b}	26.7 ± 1.5^{cd}	11.3 ± 1.2^{de}	10.7 ± 0.6^{ef}
Ethanol	100	11.3 ± 0.6^{de}	29.0 ± 1.0^{b}	25.0 ± 1.0^{d}	9.7 ± 0.6^{ef}	10.0 ± 0.0^{fg}
	200	12.3 ± 0.6^{d}	31.3 ± 0.6^{b}	28.7 ± 1.2^{bc}	11.0 ± 1.0^{de}	11.3 ± 0.6^{ef}
	400	15.7 ± 0.6^{c}	35.7 ± 0.6^{a}	30.3 ± 0.6^{d}	13.0 ± 1.0^{d}	12.0 ± 0.0^{e}
Ethyl acetate	100	22.7 ± 1.5^{b}	28.7 ± 0.6^{bc}	29.0 ± 1.0^{bc}	22.3 ± 0.6^{b}	20.3 ± 0.6^{c}
	200	24.7 ± 1.2^{ab}	31.3 ± 1.5^{b}	31.3 ± 1.2^{ab}	24.3 ± 0.6^{b}	26.7 ± 0.6^{b}
	400	27.3 ± 2.1^{a}	36.7 ± 1.5^{a}	33.7 ± 1.2^{a}	27.3 ± 0.6^{a}	28.7 ± 0.6^{a}
Distilled water		8.0 ± 0.0^{f}	8.0 ± 0.0^{e}	8.0 ± 0.0^{h}	8.0 ± 0.0^{f}	8.0 ± 0.0^{h}
5% tween 80		8.0 ± 0.0^{f}	8.0 ± 0.0^{e}	8.0 ± 0.0^{h}	8.0 ± 0.0^{f}	8.0 ± 0.0^{h}
Amphotericin B	32 µg	25.3 ± 0.6^{ab}	13.3 ± 0.6^{d}	20.3 ± 0.6^{e}	20.0 ± 1.0^{c}	15.7 ± 0.6^{d}

Key notes: Values are expressed as Mean ± SD (n=3), 8.0±0.0 = no inhibition (well diameter), "Means" that do not share a superscript letter are significantly different (only column wise) at $P<0.05$. *Conc.-concentration, T. menta. - T.mentagrophytes*

6.1.2. Minimum Inhibitory Concentration of the extracts

Each tested microorganism was examined, starting from a high concentration of 64 mg/ml by descending with serial bi-fold dilution till 0.0625 mg/ml, to determine the MIC value. Based on the study the MIC value of the extracts was in agreement with its preliminary antimicrobial activities (on well method) against most of the microorganisms. Lower concentration of the extract was required for growth inhibition in most of the test microorganism that showed more susceptibility during the well method preliminary screening. The ethyl acetate extract of the plant was more potent against all organisms than ethanol and aqueous extracts. *S. aureus, S. epidermidis* and *T. mentagrophytes* inhibited at lower concentration by ethanol extract than aqueous extract. For other microorganisms the two extracts showed similar MIC value.

S.epidermidis was the most susceptible bacteria with MIC value of 0.7±0.3 mg/ml,1.0±0.0 mg/ml, 2.0±0.3 mg/ml for ethyl acetate, ethanol and aqueous extracts, respectively. The low antimicrobial activity was recorded by *E. faecalis* with the highest MIC value of 8.0±0.0 mg/ml by ethyl acetate extract and 16.0±0.0 mg/ml by both ethanol and aqueous extracts compared to other gram positive bacteria. From gram negative bacteria *S. typhimurium, S. sonnei* and *P. mirabilus* were most susceptible bacteria which have showed similar MIC value as *E. faecalis* at the three extracts. The lowest antimicrobial activities were recorded by *P. aeroginosa* and *K. pneumoniae* with MIC value of above 64 mg/ml for aqueous and ethanol extracts and 16mg/ml for ethyl acetate extracts.

Among the tested fungi, *T. rubrum* was the most susceptible with MIC value of 0.7±0.3 mg/ml, 1.0±0.0 mg/ml and 1.0±0.0 mg/ml by ethyl acetate, ethanol and aqueous extracts, respectively, followed by *T.mentagrophytes* which has showed MIC value of 1±0.0 mg/ml, 4±0.0 mg/ml and 8±0.0 mg/ml, respectively. *C. albicans, A. niger* and *A. flavus* were more resistant fungal species to the extracts with MIC value of greater than 64 mg/ml for both ethanol and aqueous extracts and for ethyl acetate extract the MIC value of 16±0.0 mg/ml and 32±0.0 mg/ml were recorded for *C.albicans* and *A. flavus*, respectively. The positive control drugs endowed better susceptibility than the extracts to all microorganisms.

6.1.3. Minimum Bactericidal/Fungicidal Concentration of the extracts

As displayed in Table 4, the MBC/MFC value was in agreement with its MIC, that is, the lower MIC value is the lower MBC/MFC value in most of the tested microorganisms. The ethyl acetate extract revealed best MBC value when compared with ethanol and aqueous extracts. *E. coli, K. puemoniae, P. aeroginosa, C. albicans, A. niger* and *A. flavus* MBC value were above 64 mg/ml by ethanol and aqueous extracts where as ≤64mg/ml in case of ethyl acetate extract. The minimum MBC value among all bacteria was 4.0±0.0mg/ml that was by ethyl acetate extract against *S. epidermidis*. *T. rubrum* MBC value was the lowest compared to all tested microorganisms with a concentration of 1±0.0 mg/ml, 2±0.0 mg/ml and 2.7±1.2 mg/ml to ethyl acetate, ethanol and aqueous extracts, respectively.

Table 4. MIC and MBC/MFC value of tested microorganisms

Microorganisms MIC and MBC/MFC		Water extract	Ethanol extract	Ethyl acetate extract	Positive control
S.aureus	MIC	8.o±0.0	2±0.0	0.8±0.3	0.5±0.0
	MBC	32.0±0.0	16±0.0	8±0.0	1.0±0.0
MRSA	MIC	8.0±0.0	8±0.0	4±0.0	0.5±0.0
	MBC	32.0±0.0	32±0.0	8±0.0	4.0±0.0
S.epidermidis	MIC	2.0±0.0	1±0.0	0.7±0.3	0.25±0.0
	MBC	16.0±0.0	16±0.0	4±0.0	0.25±0.0
S.pyogenes	MIC	4.0±0.0	4±0.0	2±0.0	0.5±0.0
	MBC	16.0±0.0	16±0.0	8±0.0	4±0.0
S.agalactiae	MIC	4.0±0.0	4±0.0	2±0.0	0.5±0.0
	MBC	16.0±0.0	16±0.0	8±0.0	1±0.0
E.faecalis	MIC	16.0±0.0	16.0±0.0	8.0±0.0	0.5±0.0
	MBC	32.0±0.0	32±0.0	16±0.0	4±0.0
E.coli	MIC	32±0.0	32±0.0	8±0.0	0.5±0.0
	MBC	>64±0.0	>64±0.0	16±0.0	0.5±0.0
S.typhimurium	MIC	16±0.0	16±0.0	8±0.0	0.08±0.04
	MBC	32±0.0	32±0.0	16±0.0	0.5±0.0
S.flexneri	MIC	16±0.0	16±0.0	8±0.0	0.08±0.04
	MBC	32±0.0	32±0.0	16±0.0	0.5±0.0
S.sonnei	MIC	16±0.0	16±0.0	8±0.0	0.5±0.0
	MBC	32±0.0	32±0.0	16±0.0	0.5±0.0
P.aeroginosa	MIC	>64±0.0	>64±0.0	16±0.0	1.0±0.0
	MBC	>64±0.0	>64±0.0	32±0.0	2±0.0
K.pneumonia	MIC	>64±0.0	>64±0.0	16±0.0	1.0±0.0
	MBC	>64±0.0	>64±0.0	32±0.0	4±0.0
P.mirabilus	MIC	16±0.0	16±0.0	8±0.0	0.5±0.0
	MBC	32±0.0	32±0.0	16±0.0	0.5±0.0
C.albicans	MIC	>64±0.0	>64±0.0	32±0.0	32±0.0
	MFC	>64±0.0	>64±0.0	64±0.0	64±0.0
T.rubrum	MIC	1±0.0	1±0.0	0.7±0.3	2±0.0
	MFC	2.7±1.2	2±0.0	1±0.0	4±0.0
T.mentagrophytes	MIC	8±0.0	4±0.0	1±0.0	2.7±1.2
	MFC	16±0.0	8±0.0	2±0.0	4±0.0
A.niger	MIC	>64±0.0	>64±0.0	16±0.0	6.7±2.3
	MFC	>64±0.0	>64±0.0	16±0.0	8±0.0
A.flavus	MIC	>64±0.0	>64±0.0	32±0.0	16±0.0
	MFC	>64±0.0	>64±0.0	32±0.0	32±0.0

Key notes: Erythromycin, ciprofloxaciline and Amphotericin B are positive control drugs for gram positive bacteria, gram negative bacteria and fungi, respectively. The MIC and MBC/MFC values are expressed in mg/ml for extracts and in µg/ml for positive controls.

6.2. Phytochemical constituents of the crude extracts

The result of phytochemical screening test is shown in Table 5. According to the qualitative phytochemical screening study, around 7 secondary phytochemicals were detected at least in one of the three extracts. The ethyl acetate, ethanol and aqueous extracts of the roots of *I.tinctoria* A.Rich were found to be positive for 6, 5 and 4 tested secondary metabolites, respectively. Glycoicides, saponins and terpenoids were positive for all extracts whereas coumerines, phenols and tannins were negative for all extracts.

Table 5. Secondary metabolites detected with in aqueous, ethanol and ethyl acetate crude extracts of the root *I. tinctoria* A.Rich

Phytochemical constituent	Water extract	Ethanol extract	Ethyl acetate extract
Alkaloids	-	+	+
Free anthraquinens	+	-	-
Coumerines	-	-	-
Flavoinoids	-	-	+
Glycoicids	+	+	+
Phenols	-	-	-
Quinines	-	+	+
Saponin	+	+	+
Tannins	-	-	-
Terpenoids	+	+	+

Key notes: (+) positive, (-) negative

6.3. TLC profiling

TLC analysis of each extract was performed using various combinations of solvent systems till the analysis revealed a better separated constituents. The solvent system containing a mixture of hexane: ethyl acetate (7:5) produced a better separation for ethanol and ethyl acetate extracts. Though various solvent combinations with different proportions tested, the aqueous extract didn't give well separated constituents of compounds. Of the analyzed mixture of

dichloromethane: ethyl acetate (7:2) produced a little bit better separated components. The migrated constituents, formed by the solvent system, observed under UV-light at a wavelength of 254nm, revealed the presence of 6, 4 and 1 compounds with in ethanol, ethyl acetate and aqueous extracts, respectively (Figure 3).

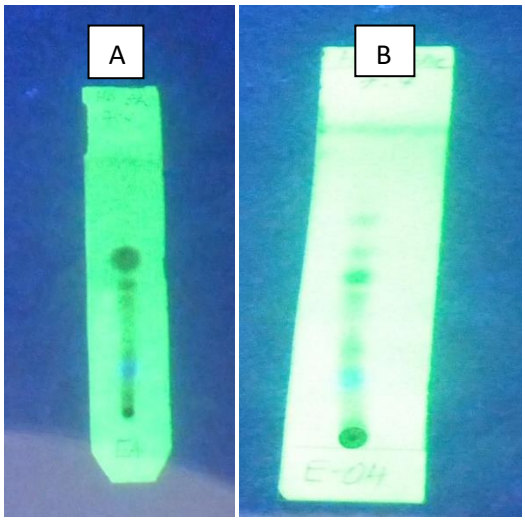

Figure 3. TLC fingerprint of A) Ethyl acetate extract and B) Ethanol extract

6.4. Acute oral toxicity study

6.4.1. Behavioral pattern and LD50

The results of an acute oral toxicity study showed that the aqueous extracts of the plant were appeared to be safe up to the dose of 9600 mg/kg. Testing parameters like restlessness, touch response, pain response, urination, skin color, fur erection, and food and water intake were assessed(Table 6). Drowsiness and erection of fur were observed at a dose of 4800 and 9600 mg/kg. Nevertheless, other groups did not show any sign of toxicity. Generally, the study revealed absence of signs of toxicity for most of the setted parameters and absence of mice death records up to the 14^{th} day. Therefore, LD50 of the extract might be considered to be greater than 9600 mg/kg.

Table 6. General appearance and behavioral observations of acute toxicity study for control and treated groups.

Observation	Dose of extracts in mg/kg						
	Control	300	600	1200	2400	4800	9600
Food intake	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Water intake	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Diarrhea	Not seen	Not seen	Not seen	Not seen	Not seen	Not seen	Not seen
Urination	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Breathing	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Skin color	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Drowsiness	Not seen	Not seen	Not seen	Not seen	Not seen	present	Present
Hypersensitivity	Not seen	Not seen	Not seen	Not seen	Not seen	Not seen	Not seen
Erection of fur	Not seen	Not seen	Not seen	Not seen	Not seen	present	Present
Sedation	Not seen	Not seen	Not seen	Not seen	Not seen	Not seen	Not seen
Death	Alive	Alive	Alive	Alive	Alive	Alive	Alive

Key notes: n=5 ,“present” means at least 1 out of the 5 mice showed the symptom, “not seen/normal” means no mice showed the symptom .

6.4.2. Body Weight

A weekly body weight has weighed on initial day, 7th and 14th days of the six groups as displayed on table 7. At these days all treated groups didn’t show any statistically significant difference changes in the body weight compared with control groups ($p<0.05$).

Table 7. The effect of aqueous extract of *I. tinctoria* A. Rich roots on body weight of mice at different days.

Group	Doses(mg/kg)	Weight(gm)		
		Initial day	7^{th} day	14^{th} day
I	Distilled water	29.4 ± 3.4^{a}	31.0 ± 4.2^{a}	31.2 ± 2.1^{a}
II	300	27.0 ± 2.3^{a}	30.1 ± 2.6^{a}	31.7 ± 3.2^{a}
III	600	27.4 ± 2.2^{a}	30.2 ± 1.9^{a}	30.1 ± 1.5^{a}
IV	1200	30.2 ± 2.3^{a}	30.2 ± 3.7^{a}	31.6 ± 2.5^{a}
v	2400	27.1 ± 1.4^{a}	31.8 ± 3.4^{a}	33.1 ± 3.8^{a}
vI	4800	27.0 ± 1.4^{a}	29.1 ± 1.8^{a}	32.3 ± 2.2^{a}
vII	9600	28.4 ± 2.1^{a}	29.5 ± 1.0^{a}	32.2 ± 1.7^{a}

Key notes: Values are expressed as Mean ± SD (n=5) one-way ANOVA followed by Tukey's multiple comparison tests ($P<0.05$), "Means" that do not share a superscript letter are significantly different (only column wise).

7. Discussion

7.1. Antimicrobial activity

Plants are important source of potentially useful structures for the development of new antimicrobial drugs. The first step towards this goal is the *in vitro* antimicrobial activity assay as many reports are available on the antibacterial and antifungal properties of plants (50, 51,56). The study plant, *I. tinctoria* A. Rich, is native to East Africa and found in many part of Ethiopia (14) that used as a beauty treatment like that of henna. It also used to toughen skin (26), to treat abdominal pain (27) and fungal infections like ring worms that cause tinea pedis (26). Its stem is chewed to treat mouth and throat diseases (27). There are also other previous ethnobotanical studies that support as it has different medicinal values (28-30). These were some of the provoking information to undertake the evaluation of antibacterial and antifungal activities on the roots of the herb by extracting with three solvents (ethyl acetate, ethanol and distilled water).

The well method assay showed various inhibition diameters of the tested microorganisms by all of the extracts with the exception of 100 mg/ml aqueous extract that did not show any inhibition for *C. albicans, A. niger* and *A. flavus.* The inhibition zone of the most susceptible bacteria (*S. aureus, S. epidermis* and MRSA) did not show any significant difference ($p<0.05$) at the tested concentration of 100 mg/ml, 200 mg/ml and 400 mg/ml for most tested extracts. Therefore, these bacteria have resulted with similar susceptibility at both the lowest and highest tested extract concentration in this method. These observations could possibly be explained as the effect of these concentrations might be the maximal efficacy portion on the dose-response curve where as the steepest portion might be below 100 mg/ml concentration with the assumption of the dose-response curve is sigmoidal curve. From this the minimum effective dose might be ≤100 mg/ml.

The remaining tested gram positive bacteria *S. pyogenes, S. agalactiae* and *E. faecalis* also did not show any significance difference inhibition at 200 mg/ml and 400mg/ml concentration for ethanol and ethyl acetate extracts which was a similar scenario with most of gram negative bacteria. However, most of the tested fungi inhibition by extracts showed statistically significant difference ($p<0.05$) between concentrations of 100mg/ml and 400mg/ml respected extracts in which higher concentration records longer inhibition zone than lower concentration, i.e., antimicrobial activity increased with the increase in the concentration of the extracts. In this case,

the extracts could contain antifungal constituents, whose concentration increased with dose and possibly increase the activity till it reaches its maximal efficacy that might be ≥400mg/ml.

The antibacterial activity of the extracts against MRSA resulted with a highest inhibition zone, lowest MIC and MBC value of 35.7±1.2mm, 4mg/ml and 8mg/ml, respectively. All extracts at all concentrations (100mg/ml, 200mg/ml and 400mg/ml) showed a better antibacterial activity than vancomycin (30μg) on the well method with a statistically significant difference at ($p<0.05$). This result indicates the promising effect of the study plant against MRSA that have exceeded 50% of isolates found in multiple sites throughout the world (7). This might be due to the ability of the extracts to inhibit penicillin-binding proteins of the bacteria that are involved in the synthesis of peptidoglycan which is impossible by the antibiotic methicillin. Therefore, it could be a good alternative as a natural product, as we are now in a situation where, in some cases, the glycopeptides antibiotic vancomycin, is the only option for antimicrobial therapy even its non susceptibility in *S. aureus* is on the increase (6, 85).

The well method zone of inhibition was in line with the MBC and MIC value concentration for most of the tested microorganisms except *S. pyogenes* and *S. agalactiae* that might suggest the consistency of the testing methods. The inconsistency of the two organisms might be due to the usage of 5% sheep blood muller-hinton agar. The sheep blood might in some extent decrease the looseness of the media that lead a weak diffusion of extracts than the pure muller-hinton agar that used for other bacteria. On the other way, these two bacteria might be susceptible for large molecules or hydrophobic molecules of the extracts constituents which did not diffuse easily as other studies support it (86). These might be the reasons that the two organism record better MIC and MBC value than those bacteria that had longer inhibition zone than them. For instance *S. agalactiae* and *E. faecalis* on 400mg/ml ethyl acetate extract showed inhibition zone of 24.0±1.0mm and 29.7±1.5mm,respectively(significantly different at $p<0.05$). This value was inversed as *S. agalactiae* records 2mg/ml and 8mg/ml where as *E. faecalis* records 8mg/ml and 16mg/ml of MIC and MBC value, respectively.

As observed from the inhibition zone, MIC and MBC value of the extracts the study plant also showed antibacterial activity against gram negative bacteria in extraction solvent dependent manner. Of the extracts ethyl acetate extract showed better antibacterial activity against all gram negative bacteria. For example, *K.peumoniae* and *P.aeroginosa* had >64mg/ml of both MIC and

MBC on water and ethanol extracts where as ethyl acetate extract had 16mg/ml MIC and 32mg/ml MBC which was a great difference in between. This notable better efficacy of ethyl acetate extract supported by other previous studies on plant extracts (52, 53, 87). Thus, of the extracts ethyl acetate extracts might has a better penetration ability of the outer membrane of gram negative bacteria and disturbing cellular function, metabolism, and loss of cellular constituents, leading their inhibition and death of the bacteria.

It has been found that the gram positive bacteria were more susceptible to the extracts compared to gram negative bacteria. Many other studies on different medicinal plants also revealed as gram positive bacteria tend to be more sensitive to the antimicrobial properties of plant extracts than gram negative bacteria (66, 67, 71, 88). These could be due to gram negative bacteria have an outer membrane that is composed of high density lipopolysaccharides that serves as a barrier to many environmental exposures including antibiotics (89).

At 400mg/ml concentration *C. albicans*, *A. niger* and *A. flavus* inhibited with a diameter of 9.7±0.6mm, 15.7±0.6mm and 27.3±2.1mm by aqueous, ethanol and ethyl acetate extracts, respectively. The 100mg/ml of aqueous extract did not show any antifungal activity against these fungi. These results were too low compared to *T.rubrum* and *T. mentagrpophytes* that showed a lowest inhibition zone of 14.0±1.0 and 12.7±0.6mm by 100mg/ml aqueous extracts and a highest inhibition zone of 36.7±1.5mm and 33.7±1.2mm by 400mg/ml of ethyl acetate extracts, respectively. MBC and MIC values also support these results and showed the more susceptibility of *T.rubrum* and *T. mentagrpophytes* not only compared to the above three tested fungi's but also to all tested bacteria. Therefore, the application of the paste of the study plant roots by Ethiopian females as a treatment of tinea (22) which is caused by primarily by *T.rubrum* followed by *T. mentagrophytes* and other dermatophytes (90, 91) supported by this findings as the extracts of *I.tinctoria* A.Rich endowed good antifungal effect against these most prevalent Trichophytons (*T.rubrum* and *T.mentagrophytes) (92)* than the other tested fungi.

In addition, this study confirms as the roots of *I. tinctoria* A. Rich had also a promising antibacterial activity especially against *S. aureus* and *S. epidermis* which are commonly found in the skin even though the traditional application is to control fungal infections and to toughen the skin (22, 26). Hence, locally dying of skin, applying on cloths and different materials might prevent infection transmission of Staphylococci (S. *aureus*, MRSA and *S. epidermidis*), the most

abundant skin-colonizing (biofilm forming) bacteria and the most important causes of community associated and hospital acquired skin infections (93-95), and dermatophytes.

Of the screened 10 phytochemicals seven were positive in which some were positive for the three extracts or two extracts and others for one extract of *I.tinctoria* A.Rich roots. There for, the phytochemical composition of the three extracts differ by type and those similar type phytochemicals that found in different extracts might also differ by concentration. Different studies support the extraction solvents used for extraction has a great influence on the antimicrobial activity of the plant materials against different microorganisms (52, 71) and also on the type and concentration of phytochemicals screened (96-97). The solvents used in the extraction of the plant materials might not only contribute for the difference in kind of these secondary metabolites among the three extracts but might also contribute for the difference in concentration.

The study revealed the presence of terpenoids, saponins and glycoicides with in the three extracts of *I. tinctoria* A. Rich roots. Previously, numerous phytochemicals such as alkaloids, anthraquinens, flavonoids, terpenoids, glycosides, quinones and saponins have also revealed from many medicinal plants that had health benefits such as antimicrobial effect through different mechanisms (19, 80,98). For instance, a study on the isolate of terpenoides from the medicinal plant *Trichodesma amplexicaule* Roth found the presence of antibacterial and antifungal activity of this phytochemical (63). On other study anthraquinones isolated from the plant *Stereospermum zenkeri* showed best antimicrobial activity against *P.aeruginosa* (64). Therefore, the isolated phytochemicals from the root extracts of the study plant might help for the presence of antimicrobial activity as different studies revealed their antibacterial and antifungal potency.

Alkaloid was the other phytochemical screened from ethyl acetate and ethanol extracts but not in aqueous extract. The presence of this phytochemical in the ethyl acetate and ethanol extract could contribute for their better antimicrobial activities than the aqueous extract. This idea supported by a study in Slovakia on antibacterial and antifungal effect of Mahonia aquifolium crude extract and its major isolated alkaloids berberine and jatrorrhizine on 20 bacterial and fungal tested strains revealed that the isolated alkaloids berbeine showed more susceptibility on most the microorganisms (65). Another study in Burkina Faso on antibacterial activity of

alkaloids from *Sida acuta* medicinal plant revealed a good antimicrobial activity of alkaloides against different bacteria especially to gram positive bacteria (66). In addition to the above phytochemicals only ethyl acetate extract was positive for flavoinodes that might have a great contribution for its antimicrobial activity as different studies support the antimicrobial effect of this phytochemical (81,99).

The study detected greater number of phytochemicals in ethyl acetate extract than ethanol and aqueous extracts. Of the three extracts the ethyl acetate extract has stronger and broader spectrum of antimicrobial activities as observed from mean inhibition diameter measurements and the consistent MIC and MBC values. This might be due to the synergistic effect of the secondary metabolites as many studies assure synergistic effect of secondary metabolites increase the antimicrobial potency of plant extracts (54,100-101). Additionally, the bioactive phytochemicals that only found in ethyl acetate extract might have relatively more antimicrobial activity than others. On the other way not only by type but also the concentration of the phytochemicals present in the ethyl acetate extract might be greater than ethanol and aqueous extracts that could increase the antimicrobial activity of ethyl acetate extract than ethanol and aqueous extracts.

7.2. Acute toxicity

The evaluation of the toxic characteristics is usually a preliminary step in screening medicinal plants for pharmacological activity. But, there is a lack of scientific validation on the toxicity and adverse effects of medicinal plants. Therefore, scientific knowledge towards acute oral toxicity study is much needed since it helps to identify the dose that could be used subsequently and to reveal the possible clinical signs elicited by these medicinal plants under investigation. In addition, in order to increase the confidence on medicinal plants or preparations safety to human being the data of toxicity studies should be obtained (102).

The oral acute toxicity study of the tested plant extracts was carried out on albino mice at a single dose of 300, 600,1200,2400,4800 and 9600mg/kg body weight and was continuously monitored for first 4 hours, followed for a period of 14 days daily for any toxic effect after the treatment period. Mortality and major changes in behavior were not observed in all groups. However, drowsiness and erection of fur were observed in each mouse of treated groups of 4800

and 9600mg/kg body weight. These signs were disappeared, almost among all of the mice that showed the symptom, after the 4^{th} hour of administration of the extracts. The extract seems to be safe at a dose level of 9600 mg/kg, and the LD_{50} is considered be >9600 mg/kg. According to Hodge and sterner toxicity classification the root extract of *I. tinctoria* A. Rich is classified at least as practically non toxic herbal medicine as LD_{50} between 5000 to 15000mg/kg is practically non toxic according to this classification (103).

The body weight of each mouse was carefully weighed at first day, 7th day and on the day of sacrifice. The body weights of tested animals of both control and treated groups were increased progressively throughout the study period though it was not statistically significant changes ($p<0.05$). The body weight changes serve as a sensitive indication of general health status of animals (104). Therefore, the normal increment in body weight and the zero death report could give confidence to state roots of *I. tinctoria* A. Rich did not interfere with the normal metabolism of animals.

8. Strength and Limitation

8.1. Strength

- The study investigated the antimicrobial activity of the roots of the study plant against 18 bacteria and fungi (wide spectrums of microorganisms) by taking representatives from gram negative bacteria, gram positive bacteria, yeasts and molds.

8.2. Limitation

- Maceration speed and temperature of 40°C during concentration of macerated filtrate are influential factors that might cause degradation of active compounds.
- Antimicrobial activity of the study plant by extracting using non polar solvents was not performed due to low yield of non polar solvents extraction that enforced us to stop antimicrobial activity since there were no enough plant materials.
- The study plant collection site was changed from Mahoney to Butajira due to instability around Mahoney during sample collection.

9. Conclusion and recommendation

9.1. Conclusion

According to this study, the plant extracts showed varying antimicrobial activities in which gram positive bacteria, *T.rubrum* and *T.mentagrophytes* were more susceptible at all corresponding extract types and concentrations compared to other tested microorganisms. Even though traditionally women apply medically with the assumption to control fungal infections and to toughen the skin, this study provides scientific basis as the root of *I.tinctoria* A.Rich had not only antifungal activity but also had a promising antibacterial activity especially against *S.aureus* and *S.epidermidis* which are commonly found on the skin. The antimicrobial activity of the study plant showed notable extraction solvent dependent manner in which ethyl acetate extract showed more antimicrobial activity in most of the tested microorganisms compared to the aqueous and ethanol extracts. This might be due to the type and concentration of phytochemicals present in each extract. on the other way, the acute oral toxicity study of the aqueous extracts of the plant were appeared to be safe up to the maximum tested dose that classify *I.tinctoria* A.Rich at least with in practically non toxic category. Therefore, the antimicrobial potential and practically non toxicness of the study plant can take the attention of scientific communities for the development of new, effective and safe antimicrobial drugs by further studying the plant in different directions.

9.2. Recommendation

- The antimicrobial profile of the study plant on this study support applying of *I.tinctoria* A.Rich roots paste topically for control of certain fungal infections as well, beyond the traditional assumption, for some bacterial infections though further studies are needed.
- *In vivo* antimicrobial studies of the extracts should be conducted to offer conclusive insights about the roots of the study plant.
- Further deep assessment should be undertaken to identify the bioactive phytochemicals and compounds with their respective possible mechanism of action.
- Furthermore, chronic toxicological studies should be done to increase the confidence on the study plant extracts safety.

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Annexes

1. Standard operating procedures

1. 1.SOP of antimicrobial activity

1.1.1. SOP of screening of antimicrobial effect of extracts by well diffusion method

Purpose: This procedure provides instructions to perform antibacterial hole diffusion assay on natural product.

Principle: Reservoir containing the plant extract to be tested is brought into contact with an inoculated medium (e.g. agar) and after incubation; the diameter of clear zone around the reservoir is measured as antimicrobial activity of plant extract.

Materials:

Supplies and reagents	**Equipments**
Organic solvent (solublizer)	Biosafety Cabinet
Alcohol	Incubator
Detergent	Spectrophotometer
Glove	Balance
Petridishes	Autoclave
Pipette (2, 5, 10 mm)	Sterile cork borers
Pipette tips (0-5, 10-200, 1000µl)	Pipette filler
Test tube	Micro pipette (0-5, 10-200 µl)
Erlenmeyer Flasks	Vortex
Measuring Cylinder	Colony counter
Test organism	Refrigerator
Nutrient broth (Muller-Hinton broth)	Bunsen burner
Muller-Hinton agar	Thermometer
Standard antibiotics	Deep Freezer
Dimethyl Sulfoxide	Borer
Distle water	

Test sample (crude extract or synthetic compound)	
Applicator stick	
PH meter	

Sample: Adequate plant extract that is stable for 3 months with storage of at 4-50c temperature.

Quality control: Quality control materials such as standard antibiotics and solublizers should be run with the experiment. Positive control and negative control will run per experiment.

Procedure and interpretation

Step	Action
1	All the test organisms present in the deep freezer will be refreshed by growing with the appropriate media.
2	Take a loop full of microorganism and standardize by reading the absorbance by spectrophotometer till 0.08-0.1 OD range at 625nm wave length
3	Take inoculum by a sterile cotton swab and rotate several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab.
4	Streak the swab over the entire sterile agar surface
5	Perform the assay in triplicate
6	Punch required numbers of holes using a sterile cork borer ensuring proper distribution of holes (10 cm from the edge of Petri dish and 20 cm from each other holes) in the periphery and one in the center. Agar plugs are removed.
7	Pour 100 µl in to holes of the test sample dissolved in appropriate solvent into appropriately labeled cups (these are marked at the

	back of the cup before filling) using a 10-200 sized micro-pipette. Use standard drug in comparison.
8	Place the plates at room temperature for 2 hr, to allow diffusion of the sample.
9	Incubate the plate face upwards at 37^{O}C for 18-24 hr(for bacteria) at 25 ^{O}C for 7 days(for fungus)
10	The diameter of zones of inhibition is measured to the nearest mm (the cup size also being noted) as positive for antimicrobial activity. No inhibition zone as no any antimicrobial activity.

Clinical utility: For the search of novel antibacterial and antifungal drugs from natural products (74,75).

1.1.2. SOP of Minimum Inhibition Concentration

Purpose: This procedure provides instructions how to perform antibacterial and antifungal agar broth dilution assay of natural products.

Principle; A fixed amount of extract mixture is mixed with nutrient broth or Muller-Hinton broth containing bacterium. Colony growth is taken as an indication of microbial density. When no growth takes place, the medium remains clear; when sample is inactive against the germ tested and there is growth.

Materials: Appropriate materials in SOP 1.1 will used.
Sample: The plant extracts stored at 2-8 ^{O}C which is stable for 3 months.

Quality control: Quality control materials such as standard antibiotics and solublizers should be run with the experiment.

Procedure:

Step	Action
1	Prepare the extracts by diluting in water (in case of water-soluble samples) or by organic solvents like 5% tween 80 serially in two fold.
2	Take 2ml of the prepared concentrations and add in to test tubes containing 18 ml of molten agar medium (55^{o}C).
3	Adequately mix and pour into petridishes
4	Make dry the agar
5	Take a loop full of microorganism and standardize by reading the absorbance by spectrophotometer till 0.08-0.1 OD range at 625nm wave length
6	Take 2 microlitre prepared suspension by a micropipette and drop on the prepared extract containing agar surface.
7	Perform the assay in triplicate
8	Place the plates at room temperature for 2 hr, to allow diffusion of the sample.
9	Incubate the plate at 37^{O}C for 18- 24 hr(for bacteria) at 25 ^{O}C for 7 days(for fungus)
10	After incubation observe the last petridish with no visible growth of the microorganism is taken to represent the MIC of the test sample which is expressed in mg/ml.

Clinical utility: For the determination of Minimum Inhibitory Concentration of Natural products which will help the search for novel antibacterial drugs from natural products (76).

1.1.3. SOP for Minimum Bactericidal/Fungicidal Concentration of the extracts

Purpose: The MBC/MFC assay is performed as an adjunct to the MIC and is used to determine the concentration of the extract that is lethal to the target bacteria or fungus in vitro.

Principle: This is checking of the lethality effect of the extract by sub culturing all plates not showing visible growth in the MIC test. The inhibition zone in the MIC will be sub cultured and microcidality of the extract will be checked by the absence of growth and if there is growth it assures the inhibition ability of the extract but not lethal.

Materials: use appropriate materials on 1.1

Sample: Adequate plant extract that is stable for 3 months with storage of at 2-8 ^{o}C temperature.

Quality control: Quality control materials such as standard antibiotics and solublizers should be run with the experiment. Positive control and negative control will run per experiment.

Procedure and interpretation:

1. Inoculums will be taken from each MIC agar plate without visible growth and streak onto appropriate media.
2. Record the dilution of the subculture MIC plates on each plate and incubate as described at MIC part.
3. Then, after incubation, examine the MBC plates for colony growth or lack of growth for each subcultures. No growth indicates that the extract is bactericidal/ fungicidal at that dilution. Growth indicates that the extract is micro-static but not microcidal at that dilution.

Clinical utility: For the determination of Minimum Bactericidal Concentration of natural products which will help the search for novel antibacterial drugs from natural products (77).

1.2. Acute oral toxicity test

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

Materials and Reagents: - Diluting solution

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

Sample:

- Sample type: -Plant extracts
- Amount required:-1ml/100g of body weight

- Transport and Storage:- At room temperature
- Stability:- non- applicable

Principle: It is based on the principle in which mice were administered orally with different dosages of tested extracts and mortality, signs of toxicity, food and water consumption and change in body weight was observed for 14 days. The information obtained from the observation is useful in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity, and, occasionally, revealing delayed toxicity.

Procedure:

Step	Action
1	Use Albino mice of either sex, weighting 20-35g.
2	Acclimatize the animals with the working environment.
3	Divide the mice's randomly in to groups based on sex and body weight (five mice in each group).
4	Withdraw food but not water for 3-4 hours prior to the experiment
5	Administer the diluting solution for one group as a negative control.
6	For the other groups administer the plant extract orally via gavage as required dose (Graded dose).
7	Observe sign of toxicity and mortality for the first four hours and in two hours interval for 24 hours, and daily till 14 days.

Procedure Note:

- ❖ The use of vehicle control groups should be considered.
- ❖ Animals should be observed for 14 days after administration. All mortalities, clinical signs, time of onset, duration, and reversibility of toxicity should be recorded.

Calculation: - Determine LD50

Result Interpretation

- Response data and dose level for each animal (i.e. animals showing signs of toxicity including mortality, nature, severity and duration of effects);

- Individual weights of animals at the day of dosing, in weekly intervals thereafter, and at time of death or sacrifice**.**
- Date and time of death if prior to scheduled sacrifice.
- Time course of onset of signs of toxicity and whether these were reversible for

Clinical Utility

The information obtained from these studies is useful in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity, and occasionally, revealing delayed toxicity (84).

2. Photographs captured during the study

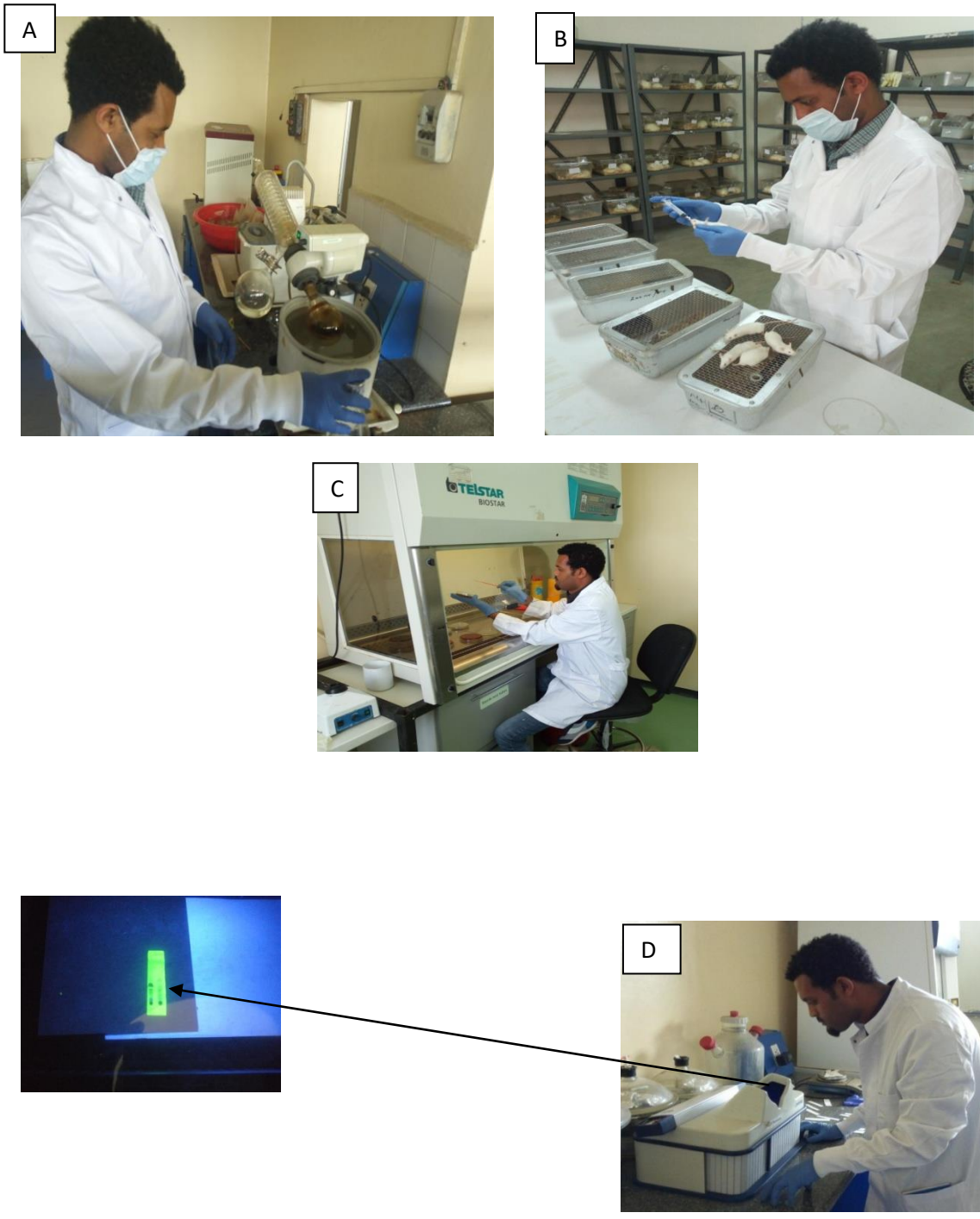
2.1. The plant materials in different forms

Roots of *I.tinctoria* A. Rich

Milled roots

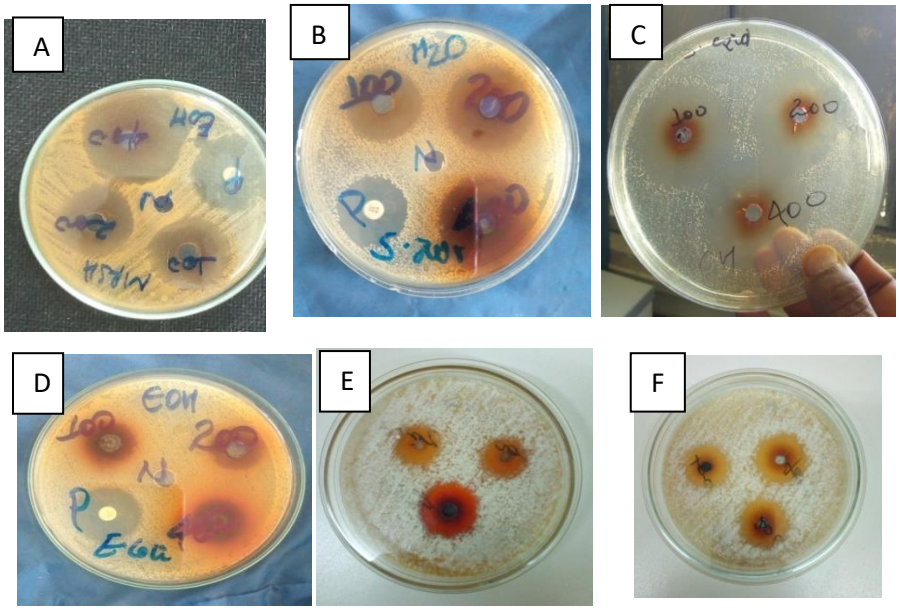
Final extracts

2.2. Photographs that show some of the procedures during the experiment



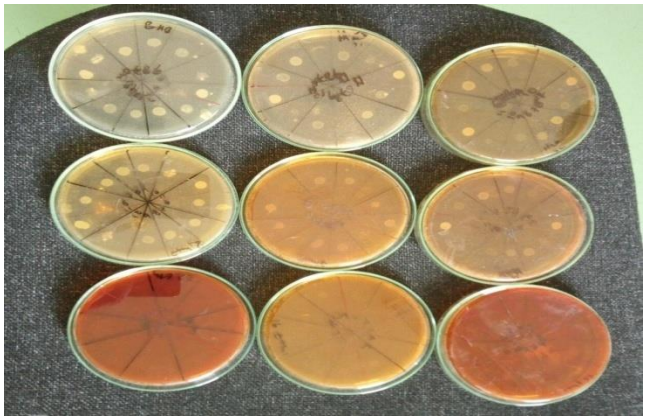
Activities during concentrating the macerated filtrate by Rota vapor (A), extracts administration to mice for acute toxicity study (B), antimicrobial assessment(C), TLC reading (D)

2.3. Some of the photographs that show the inhibition ability of the extracts against tested microorganisms



Inhibition zone on MRSA (A), *S.aureus* (B), *S.epidermis*(C), *E.coli* (D), *T.mentagrophytes* (E), *T.rubrum* (F)

2.5. Photographs of some bacteria MIC plate after incubation



Sample of MIC plates of some bacteria

All tested fungi's ethyl acetate extract (row **A**), ethanol extract (row **B**), aqueous extract (row **C**). For all extracts the concentration is starting from 64mg/ml to 1mg/ml (from right to left).

Declaration

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

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Date of submission_____/_____/_____

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

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