ADDIS ABABA UNIVERSITY

INSTITUTE OF BIOTECHNOLOGY

Antimicrobial Properties of Endophytic and Rhizospheric Fungi Associated with Some Medicinal Plants

By: Fertuna Shemsedin

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Antimicrobial Properties of Endophytic and Rhizospheric Fungi Associated with Some Medicinal Plants

MSc. Thesis

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By

Fertuna Shemsedin

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ADDIS ABABA UNIVERSITY

INSTITUTE OF BIOTECHNOLOGY

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Demsachew Guadie (PhD) _____ _____

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Addis Simachew (PhD) _____ _____

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Antimicrobial Properties of Endophytic and Rhizospheric Fungi Associated with Some Medicinal Plants

Fertuna Shemsedin. MSc. Thesis

Addis Ababa University, September 2020

Abstract

*Nowadays the development of multidrug resistant human pathogenic microorganisms and the emergence of new diseases are the most challenging problems in public health care on a global scale. To overcome this problem it needs intensive searching for new sources of effective antimicrobial agent producing organisms. Therefore, the main objective of this study was to isolate and identify the antimicrobial properties of entophytic and rhizospheric fungi associated with some medicinal plants. A total of 150 plant parts and 50 soil samples were collected from five medicinal plants around Bale Zone, west Arsi Zone and Chancho Oromia Special Zone, Oromia Regional State of Ethiopia. All collected samples were processed following standard protocols. In vitro antimicrobial activities were tested against common resistant pathogenic organisms (*Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeroginosa *and* Candida albicans*). A total of 582 (316 endophytes and 266 rhizospheric) fungal isolates were obtained from the collected medicinal plants and soil samples. Accordingly, 78 (19.89%) isolates displayed antimicrobial activities against at least one target microorganism by fungal agar plug method. The ethyl acetate extracts of the crude metabolites of 18 isolates, showed antagonistic activity against at least one tested organisms with higher inhibition zone. Ethyl acetate extracts of isolate 30CRS showed highly significant (p≤0.001) inhibition zone against* E. coli *(30.33±0.57 mm),* E. faecalis *(25.33 ± 0.28 mm) and* S. aureus *(19.16± 0.28 mm) than positive control chloramphenicol whereas fungal isolate 37BRaL showed significantly (p≤0.001) higher inhibition zone against* S. aures *(19.16± 0.28 mm) and* C. albicans *(26.83 ± 0.76 mm). The mean MIC, 3.125 - 50mg/ml for gram positive bacteria, 6.25 - 50 mg/ml for gram negative bacteria and 12.5 – 50 mg/ml for yeast test organism. MBC 6.25-50mg/ml and MFC ranged from 12.5-50 mg/ml. The phytochemical screening of the fungal metabolite revealed the presence of flavonoids, alkaloids, glycosides, terpenoids, steroid, saponin, phenol, and tannin. A total of five potential fungi were examined by morphological characterization and Biolog identification, from this, isolates 30CRS and 37BRaL were identified as* P. simplicissimum and T. flavus var flavus, *also characterized for different Biolog carbon source utilization test using Biolog microbial identification system.*

Keywords/Phrases: Antibiotics, Bioactive compound, and Endophytic fungi

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ACRONYMS

ATCC	American Type Culture Collection Centre
DMSO	Dimethyl Sulfoxide
EBI	Ethiopian Biodiversity Institute
EtOAc	Ethyl Acetate
IZDs	Inhibition Zone Diameters
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory concentration
NC	NonClavicipitaceous

INTRODUCTION

Plants have served as a source of medicinal bioactive compounds against numerous forms of diseases for centuries. In recent years, microorganisms associated with plants rather than plants themselves have proved to offer materials and products with high therapeutic potential (Subbulakshmi *et al*., 2012). Endophytes are an endosymbiotic group of microorganisms often bacteria or fungi that colonize the intracellular locations of plants (Singh and Dubey, 2015). For these organisms, all or part of their life cycle occurs within their hosts, without causing any apparent symptoms of the disease. Endophytes can colonize in the stem, roots, leaf segments, fruit, buds, and seeds cells of plants (Stępniewska and Kuzniar, 2013). The population of endophytes in a plant species is highly variable and depends on various components such as host species, host developmental stage, and environmental condition (Dudeja and Giri, 2014).

Endophytes produce bioactive compounds of biotechnological interest for pharmaceutical industries (Joseph and Priya, 2011). For instance, many endophytic fungi produce secondary metabolites which are very attractive in terms of their activity and chemical structure. The secondary metabolite such as alkaloids, phenolic derivatives, terpenoids, and steroids plays an important role as a potential candidate of drug compounds (Pandey and Malviya, 2014).

Diverse microbial populations inhabit the rhizosphere region of many plants and principally comprise fungal and bacterial species. The organic materials from root provide the driving force for the development of active microbial biomass in the rhizosphere region compared to the bulk soil (Qureshi *et al*., 2011). The different compounds secreted by plant roots into the rhizosphere perform multiple functions after gaining residence in the soil. It serves as a source of energy and precursors of many metabolites production by associated microorganisms. Therefore, the natural products obtained from endophytic and rhizospheric fungi possess various properties such as antimicrobial, anticancer, antioxidants, and antidiabetic activities that are high medical importance. Therefore, the success of several antibiotics from the fungal microbial origin such as *Penicillium* spp, *Aspergillus* spp, *Phoma* spp, and *Phomopsis* spp have shifted the focus of drug discovery from plants to microorganisms (Balagurunathan and Radhakrishnan, 2007).

Antimicrobial agents are synthetic from microorganisms, plants, and animal products and used to treat microbial diseases (Alkhyat and Al-Maqtari, 2014). However, nowadays the development of multidrug-resistant human pathogenic microorganisms and the emergency of new diseases are the most challenging problems in public health care on a global scale and a major challenge in Ethiopia (Alemayehu Reta *et al*., 2019). Development of resistant pathogenic bacteria against commonly used antibiotics due to misused and overused in developing countries like Ethiopia in hospital setting (Feleke Moges *et al*., 2014). Furthermore, lack and the high cost of new generation drugs have escalated infection-related morbidity, mortality, losses in productivity, and economy (Andargachew Mulu *et al*., 2006; Borkotoky, 2013). Infections caused by resistant bacteria also adversely affect treatment outcomes, treatment costs, disease spread, and prolonged illness (Feleke Moges *et al*., 2014). As already known, methicillin-resistant *S. aureus* (MRSA), extended-spectrum ß lactamase (ESBL) *E.coli*, vancomycin resistant *S. aureus* and *Enterococcus* associated morbidity and mortality are global problems (Alabi *et al*., 2013). Therefore, these problems have encouraged the need to search for drugs with better efficacy against drug-resistant pathogenic microorganisms as well as for the better treatment of newly emerging diseases (Liang *et al*., 2012). Worldwide, there is a renewed interest in search of novel bioactive compounds having high effectiveness, low toxicity, and negligible environmental impacts.

On the other hand, indiscriminate exploitation of medicinal plants for the extraction of antimicrobial agents of plant origin and limitations of plant resources due to various factors like a requirement of land for cultivation, environmental competence of plants and seasonal specificity encouraged the use of the microbial source for the production of antimicrobial agents. Currently, some medicinal plants are being studied worldwide for their ability to host endophytic and rhizospheric fungi having antimicrobial potential (Liang *et al*., 2012). The result of these studies indicated that medicinal plant-associated fungi are potential sources for new drug discovery (Chioma *et al.,* 2016). Ethiopia is rich in plant biodiversity, more than 7500 higher plant species, of which 20 % have medicinal value have been described; many of these plants are being processed and widely used in a rural community in the different part of the country. But they are poorly studied for their endophytic and rhizospheric fungal population and function (Nitin *et al*., 2019)

Thus, this research aimed to isolate medicinal plant-associated fungi from five selected medicinal plants of Ethiopia (*Solanum incanum*, *Aloe vera*, *Rumex abyssinicus*, *Rumex nervosus*, and *Myrsine africana*) and to evaluate their antimicrobial activities. Moreover, the identification of potential fungal isolates with antimicrobial activity to species level was also the focus of this research.

1.1. Objectives of the Study

1.1.1. General objective

1. The General objective of this study was to explore antimicrobial potential of endophytic and rhizospheric fungi associated with some medicinal plants of Ethiopia.

1.1.2. Specific objectives

The specific objectives of the current study were:

1. To isolate antimicrobial producing endophytic and rhizospheric fungi from different selected medicinal plants samples (*Solanuum incanum, Aloe vera, Rumex abyysinicus, Rumex nervosus* and *Mysrine africana*) and to evaluate antimicrobial property of the fungal isolates by primary and secondary screening

2. To evaluate the Minimum Inhibitory Concentration (MIC) of the fungal extracts using different fractional concentrations, Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) and determine the active biomolecule profile of representative isolates using a qualitative approach

3. To determine the optimum fermentation conditions of the potent fungal isolates for biomolecule production and identify the promising fungal species using morphotyping and BioLog System

2. LITERATURE REVIEW

2.1. Medicinal plant in Ethiopia

According to the World Health Organization (WHO,1991) medicinal plants is any plant in which one or more of its organs contain substances that can be used for therapeutic purposes or which are precursors chemo-pharmaceutical and semi-synthesis (Olalde, 2005). Many medicines widely in use today incorporate ingredients from plants. Traditional medicinal plants have greatly contributed to the development of modern medicines. In many developing countries such as Ethiopia, traditional medicinal plants are still commonly used in daily life and play important roles as complements to underdeveloped health care services, 80% of human and 90% of livestock populations depend on traditional medicines derived from medicinal plants for their primary health care (Alves and Rosa, 2007). Therefore, medicinal plants as a bioresource are used since antiquity for the treatment of various infectious diseases. The diverse active components of the plants contain various classes such as alkaloids, terpenoids, saponins, flavonoids, coumarins, and phenol. The composition of biologically active compounds of medicinal plants varies widely depending on the plant species, soil type, and on their association with microbes (Worku Abebe, 2016). This diversity has been exploited by niche pharmaceutical companies and institutions as a source of anticancer, antimicrobial, and antipsychotic drugs, either approved or under clinical development (Taylor, 2013). Plant species such as (*Solanum incanum, Aloe vera, Rumex abyssinicus, Rumex nervosus* and *Mysrine africana*) are among medicinal plants that are used as traditional medicines for people (Tilahun Teklehaymanot *et al*., 2007; Teshale Mekonen *et al*., 2010; Abid Aslam, 2018).

Estimated floras of 6,500 to 7,500 species of higher plants are originated in Ethiopia and about 12% are endemic to the country (Biruhalem Taye *et al*., 2011). The large part of the knowledge of ethno-medicinal plants is irreversible loss and declining to deterioration due to the oral passage of herbal heritage from generation to generation rather than in writings (Fisseha Mesfin *et al.*, 2009). Ecological degradation, farming growths, cultivation of marginal lands, and sub-urbanization are also posing a significant threat to the future wellbeing of human and animal populations that have relied on these resources to fight several ailments for generations (Devi *et al*., 2009).

2.2. Ecology and occurrence of endophytes

Almost all vascular plant species examined to date were found to harbor endophytic microorganisms (Firakova *et al*., 2007). Endophytes have been recovered from plants growing under different environmental conditions including tropic, temperate, xerophytic, and aquatic environments. Endophytes are present virtually in all organs of the plant host. The endophytes are transferred from plant to plant via seeds. They are transmitted through horizontal or vertical transmission. However horizontal transmission seems to be the predominant mechanism of dispersion among endophytic species. In general, it is likely that the environmental conditions, in which the host plant grows, influence the number and variety of endophytic populations. Plants growing in unique environmental settings, having special ethano-botanical uses, having extreme age or interesting endemic locations produces novel endophytic microorganisms. Fungal endophytes are a higher chemical diver's secondary metabolite and better antimicrobial effect than the other microbial source. Therefore, endophytic fungal able to inhabit the growth of pathogenic multidrug-resistant microbes have been isolated from a range of medicinal plant species and antimicrobial effect have been detected in a wide variety of endophytic fungi such as *Alternaria, Aspargillus, penicillium, Phoma* and *phomopsis* (Strobel and Daisy, 2003).

2.3. Isolation and identification of endophytes from different sources

The diversity and numbers of fungal endophytes vary considerably and their detection depends on, biotic abiotic and experimental factors including the genotype of the plant, the growth stage of the plant, the physiological status of the plant, the type of plant tissues, the environmental condition of the soil in which it is grown, the sampling season, the surface sterility, selective media and culture conditions (Golinska *et al*., 2015). Traditionally, endophytic fungi inside plant tissues can be recognized by two basic techniques, i.e. direct observation (cultivation-independent) and cultivation dependent methods. In the direct observation method, endophytic fungal structures within living plant tissues are directly examined under a light and electron microscope, which can show all endophytic mycobiota within the plant tissue (Lucero *et al.*, 2011). In contrast, cultivation dependent techniques have been routinely employed in endophyte diversity studies. It is important to isolate endophytic fungi for further detailed studies into their characterization, population dynamics, species diversity, or as inoculate to improve plant growth and health, or screening for

novel biologically active secondary metabolites. Detection and recovery of endophytic fungi have relied heavily on dissection of plant organs into small fragments followed by their surface sterilization and subsequent plating of fragments onto a nutrient-rich agar medium (Stone *et al.*,2004) such as agar, potato dextrose agar and any nitrogen or carbon containing media (Strobel and Daisy, 2003).

Conventionally, the identification of endophytes is based on morphological characteristics for fungi. With the development of molecular biology, ribosomal DNA Internal Transcribed Spacer (ITS) sequence analysis is widely used for the identification of microorganisms (Hata and Sone, 2008). Ribosomal DNA (rDNA) ITS was proved to be a valuable source of evidence to resolve phylogenetic relationships at lower levels, such as among genera or species (Youngbae *et al.*, 1997).

2.4. Classification of endophytes

There are two classes in which endophytic fungi are classified as Class I Clavicipitaceae and Class II Nonclavicipitaceous (NC). The Class 1 endophytes are species that are fastidious in culture and limited associated with warm and cool-season grasses. They are host specific, mainly in the grass family *poaceae* and rarely in *cyperaceae*, and are often vertically transmitted through seeds with maternal plants passing fungi on to offspring via seed infections (Saikkonen, 2002). Class 1 endophytes commonly increase drought tolerance, plant biomass, and produce diverse chemicals that are toxic to animals and decrease herbivory. However, the benefits conferred by these fungi appear to depend on the host species, host genotype, and environmental conditions (Faeth, 2003). Class 2 endophytes are highly diverse (Rodriguez *et al.*, 2009), and all of them are members of (*Ascomycota* or *Basidiomycota*). They colonize roots, stems, leaves, or the whole plant. They can be vertically or horizontally transmitted. They can confer habitat-specific stress tolerance to host plants (Saikkonen, 2004).

Nonclavicipitaceous (NC) endophytes represent three distinct functional classes. Class 2, 3, and 4, based on host colonization and transmission. Class 2 endophytes have been the most extensively studided and has been shown to enhance the fitness benefits of their plant host as a result of habitat-specific stresses such as pH, temperature, and salinity. Class 3 endophytes are distinguished based on their occurrence and horizontal transmission. This includes vascular, nonvascular plants, woody

and herbaceous angiosperms in tropical forest and antarctic communities. Class 3 endophytes are mainly known for their great diversity within host tissues, plant, and populations. Class 4 endophytes have restricted to plant roots. They are generally *Ascomycetous* fungi which are conidial or sterile and that form melanized structures like inter and intracellular hyphae and microsclerotia in the roots. NC endophytes have been recovered from every major lineage of land plants, and all terrestrial ecosystems, including both agro-ecosystems and biomes range (Arnold and Lutzoni, 2007).

2.5. Environmental and host-plant factors affecting endophytic fungi

The type of interaction between an endophyte and a plant is controlled by the genes of both organisms and modulated by the environment (Weber *et al*., 2004).

2.5.1. Influences of ecological environments on endophytic fungi

The population structure or distribution pattern of endophytic fungi is significantly associated with the difference in environments. Some environmental conditions, such as temperature, humidity, illumination, geographic location, levels of soil nutrition, and vegetation significantly affected the distribution pattern of endophytic fungi (Song *et al*., 2007). For example, specific conditions determined the distribution of host plants that in arrival determine the species of endophytic fungi and their spore germination, growth, reproduction, and metabolism during the entire life cycle (Wu *et al.,* 2013). In contrast, only certain types of host species could well be grown under the cold climatic conditions and unsuitable rates of respiration, oxygen concentration, and pH value, As a consequence, only a limited number of specific endophytic fungi could colonize in the host plants, resulting a certain degree of regional specificity on the population structure of endophytic fungi (Jiang *et al*., 2010).

2.5.2. Influence of genetic background of host medicinal plants on endophytic fungi

The distribution of certain endophytic fungal populations is only restricted to particular host plant species (or families) and particular genetic background (genotypes) of a species (D'Amico *et al*., 2008). This finding is predominantly essential because the non-random distribution of endophytic fungi will control the production of different secondary metabolites promoted by endophytic fungi

that can be used by humans as drugs (Unterseher and Schnittler, 2010). Thus, the fungus-host plant relationships should be regarded as flexible interaction, determined by slight differences in fungal gene expression in response to the host reaction, or conversely, by host plant recognition and response to the endophytic fungi. Hence, slight genetic changes in the genomes of both plants and fungi controlled the outcome of the symbiosis (Moricca and Ragazzi, 2008). Thus, the population structure of endophytic fungi was significantly affected by the genetic background of associated host plants. The fitness of the endophytic fungi largely depended on the fitness of the host medicinal plants, that the host plants largely determined the colonization and distribution of endophytic fungi in the host plants (Saikkonen *et al*., 2004). Furthermore, the age of host plants and tissues may also influence the species and composition of the endophytic community (Sieber, 2007).

2.6. Soil microbial communities and related functions

Soil microbial communities play several important ecological and physiological functions (e.g., soil organic matter decomposition, regulation of plant mineral and nutrient availability, atmospheric nitrogen fixation, the formation of mycorrhiza and production of biologically active substances able to stimulate plant growth) enhancing soil physical and chemical conditions and consequently, soil habitat ability for plants (Narula *et al*., 2009).

2.6.1. Rhizosphere fungal diversity and their effects on medicinal plants

The rhizosphere is the soil compartment influenced by plant roots. The rhizosphere is a narrow zone around the root which is interconnected to the root exudates (proteins and sugars), respiration, and biogeochemical reactions (Narula *et al*., 2009). The rhizosphere is having high microbial diversity which showed a critical link between plants and soil (Morgan *et al*., 2005). Fungi abundance 10–20 times found in rhizosphere than in the rhizoplane soil. Rhizosphere fungi have more potential of spreading through the soil than rhizobacteria (Ortíz *et al*., 2009). The root exudates in the zone of rhizosphere may control disease and play important role in nutrient cycling. A broad range of important organic compounds secreted by plant roots in the rhizosphere acts as a nutrient source for microbes enhancing microbial population and activity in the rhizosphere compared to the rhizoplane (Smith and Read, 1997). Also, rhizosphere microbes play a significant

role in improving the medicinal values of plants, they can affect plant physiology by conveying numerous useful effects such as nitrogen fixation, nutrient uptake, and production of diverse secondary metabolites in the medicinal and aromatic plants (Guo *et al*., 2006).

2.6.2. Medicinal plant rhizosphere

The various bioactive compounds secreted by plant roots into the rhizosphere soil carry out several functions (Pandey and Malviya, 2014). The organic materials from the roots of the medicinal plant provide the driving force for the development of active microbial biomass around the root than in the bulk soil (Qureshi *et al*., 2011). For example, Allelochemicals can inhibit the growth of other microorganisms in the rhizosphere, so plant microbes interactions are very complex (Solaiman and Anawar, 2015). The rhizosphere is divided into three zones based on their relative proximity to, and thus influence from, the root. Endorhizosphere includes parts of the endodermis and cortex where microbes and cations can occupy the free space between cells, rhizoplane is the medial zone which is directly adjacent to the root and includes the root epidermis and the mucilage and the outermost zone called the ectorhizosphere extends from the rhizoplane out into the bulk soil (Ramesh *et al*, 2012). Besides, populations of microbes can flourish or reduce in the space in response to the changes in soil conditions such as moisture, temperature, or substrates like carbon. Rhizosphere effect is selective and significant on specific fungal genera such as (*Fusarium, Aspergillus,* and *Penicillium*) which are stimulated and the mycelial forms are more dominant in the field (Thombre *et al*., 2016).

2.7. Biotechnological potential of endophytic and rhizospheric fungi

Fungi are now considered as an untapped resource for producing valuable natural products offering the potential for medical, agricultural, and industrial exploitation (Suryanarayanan *et al.*, 2009). Many of them are capable of synthesizing bioactive compounds that can be used as potential sources of pharmaceutical that leads for novel drug discovery such as anti-microbial, anti-cancer, anti-parasitic, immunosuppressive properties, and anti-oxidant activities (Strobel and Daisy, 2003).

2.7.1. Antimicrobial activity

Natural products are secondary metabolites or chemical compounds produced by the living organisms and that have the bioactivity. Fungi serve as a potential candidate for antimicrobial agents, and many more property (Gouda, 2016). Antimicrobial agents from endophytes are also an alternative source to overcome the increasing drug resistance pathogens. Many fungal bioactive antimicrobial compounds are found in *Pencillium, Aspergillus*, *Alternaria,* and *Phoma* spp. Antimicrobial agents are low molecular weight organic natural substances produced by microorganisms that are active at low concentrations against other microorganisms (Owen and Hundley, 2004). The first antimicrobial agent was discovered by Fleming in 1928 from the microbial source, a fungus from the genus *Penicillium*. Subsequently, Streptomycin and aminoglycoside antibiotic was obtained from the soil bacterium *Streptomyces griseus*. Chloramphenicol, tetracycline, macrolide, and glycopeptide were discovered from soil bacteria (Saga, 2009).

2.7.2. Anticancer activities

Natural products from plant microbes have played important role in cancer drug discovery resulting in a large number of clinically useful agents. In contrast investigation of fungal metabolites have not led to a clinical cancer drugs although significant research efforts revealing a different number of fungi derived natural products with promising anti-cancer activities (Evidente *et al*., 2014). The discovery of taxol (paclitaxel), a potent anti-cancer drug from an endophytic fungus, was isolated from *Taxomyces sandreanae* of yew plant *Taxus brevifolia* Nutt for the treatment of ovarian and breast cancer. Due to attracted worldwide attention on drug discovery and increased their relevance (Stierle *et al*, 1993). Later, several endophytes were reported as taxol producer, viz. *kukenani, Tubercularia sp, Pestalotia sp., Fusarium sp., Alternaria sp., Pithomyces sp., Monochaetia sp*. which was isolated from plants (Evidente *et al*., 2014).

2.7.3. Antiviral compounds

There are only limited numbers of compounds reported as antiviral agents from endophytes. However, the fewer compounds that have already been isolated have been reported to show promising results and thereby provide an alternative means of antiviral drug production. The main limitation of antiviral compound discovery is most probably related to the absence of antiviral screening systems in most of the compound discovery programs. Two novel compounds cytogenic acid A (C32H36O10) and B (C32H36O10) have been isolated from *Cytonaema sp*. These compounds are reported to be the novel human cytomegalovirus protease inhibitors (Guo *et al.*, 2000). Another compound Hinnuliquinone (C32H30N2O4), a potent inhibitor of the HIV-1 protease antiviral compound, from the endophytic fungi inhabiting the leaves of Oak trees (*Quercus coccifera*). Four new compounds have been also isolated from *Pullularia* sp. The compounds isolated were Pullularins A–D (cyclo-hexadepsipeptides). Out of these compounds, Pullularin A exhibited activities against the herpes simplex virus type-1 and also against the malaria parasite *Plasmodium falciparum* (Isaka *et al*., 2009). The other compound Pestalotheol-C isolated from the fungal endophyte *Pestalotiopsistheae* an important antiviral compound, identified from a tree on Jianfeng Mountain, China. The isolated compound showed anti-HIV properties (Erwei *et al*., 2008).

2.7.4. Antiparasitic activities

Parasitic disease is an infectious disease caused by a parasite. These infections are major causes of human chronic diseases in most countries of the tropics. The parasites include protozoa and helminths, infect billions of people, and the resulting diseases because of debilitating injuries such as blindness and disfigurement, or death in millions of people. According to the World Health Organization (WHO) estimates, 25% of the human population is infected with parasitic worms. Out of various species, *Plasmodium falciparum* causing cerebral malaria is considered to be a fatal one (Larsen *et al*., 2005). The major problem with most of the anti-malarial drugs apart from their cost is resistance to drugs over time. Hence bioactive compounds with antimalarial properties seem to be the better choice. Antiparasitic compounds from endophytes would be acting as a source of novel antimalarial drugs. Phomoxanthones A and B produced by *Phomopsis* species show a remarkable antimalarial activity (Robert, 2001). Another *P. archeri* endophytic fungus of *Vanilla*

albindia produces Phomarcherins A-C, aromatic sesquiterpenes with antimalarial action. *Leishmania* and *Trypanosoma* are other pathogenic protozoans. Various fungi as *Cochliobolus* sp. in *Piptadonia adiantoides* produce Cochlioquonone A against *Leishmania* (Hemstein *et al*., 2011). An analog of Cercosporin produced by *Mycosphaerella* sp. inhabiting *Psychotoria horizontal* has been reported to be effective against *Plasmodium falciparum, Leishmania donovani* and *Trypanosoma cruzi* (Campos *et al*., 2008).

2.7.5. Immuno-suppressant activity

Immunosuppressive drugs are used for the treatment of autoimmune disorders and the prevention of allograft rejection in the case of organ transplantation. An intensive search for suitable immune-modulatory compounds is going on to deal with the problems related to the autoimmune system. Fungi possess the capability to synthesize compounds with immunosuppressive action. Cyclosporin A- an immunosuppressant has been isolated from the endophytic fungus *Tolypocladium inflate* (Zhang *et al.,* 2009). Noncytotoxic diterpene pyrones Sub-glutinol A and B were isolated from *Fusarium subglutinans* fungi inhabiting *Tripterygium wilfordii* (Borel and Kis, 1991). Also mycophenolic acid is an immunosuppressant used for the treatment of autoimmune disorders and prevention of rejection reaction in organ transplantation has been reported to be yielded by several endophytic fungi like *Aspergillus, Penicillium, Septoria* and *Byssochlamys* (Bentley, 2000).

2.7.6. Antioxidant activity

Antioxidants are compounds that inhibit oxidation. Oxidation is a chemical reaction that can produce free radicals, thereby leading to chain reactions that may damage the cells of organisms. Antioxidants protect the cells against free radicals. Due to only a handful of antioxidants recommended for clinical purposes. *Pestalotiopsis microspora* endophytic inside *Terminalia morobensis* yields two important antioxidants known as Pestacin and Isopestacin (Li *et al.*, 2008). Similarly, Graphislactone A was isolated from *Cephalosporin* endophytic fungal species isolated from *Trachelospermum jasminoides* and *in vitro* showed more potent antioxidant activity as compared to Ascorbic acid (Harper *et al.*, 2003). Many other endophytic fungi due to the presence of phenolics and flavonoids in them can act as potent antioxidants. Cajaninstilbene acid is also another antioxidant that has been reported from *Fusarium*, an endophyte of *Cajanus Cajun* (Liu *et al*., 2007), *Chaetomium* sp. in *Nerium oleander* (Song *et al*., 2005), *Xylaria* sp. *Ginkgo biloba* (Huang *et al*., 2007) are among the few to be named here.

2.8. Fungal antibiotics and their Mechanism of action

2.8.1. Inhibiting DNA/RNA replication

The inhibition of nucleic acid synthesis by (topoisomerase II inhibitors) inhibits DNA unwinding enzymes (gyrase) and block replication. DNA synthesis, mRNA transcription, and cell division require the modulation of chromosomal supercoiling through topoisomerase catalyzed strand breakage and rejoining reactions (Kohanski, 2010). DNA gyrase remains an ideal and attractive target for antibacterial and antifungal drugs. The DNA-dependent RNA polymerase mediates the transcription process and is the main regulator of gene expression in prokaryotes. The enzymatic process is essential for cell growth, making it an attractive target for antibiotics (Collin *et al*., 2011).

2.8.2. Inhibitors of protein synthesis

Protein synthesis is an important process necessary for the multiplication and survival of all pathogenic microbial cells. Several types of antibacterial agents target protein synthesis by binding to either the 30S or 50S subunits of the intracellular ribosomes. This activity results in the

disruption of the cellular metabolism of the pathogenic bacteria and inhibition of its growth and multiplication or cause to the death of the organism (McKeegan *et al*., 2002).

2.8.3. Cell wall synthesis disruption

The bacterial cell wall consists of peptidoglycan, which helps maintain the osmotic pressure, conferring the ability to survive in diverse environments. Mechanism of action of antibiotics such as β-lactams is by inhibiting the synthesis of the peptidoglycan layer in the bacterial cell wall especially the Gram-positive bacteria by blocking the action of transpeptidases. Incase of antifungal action of secondary metabolites, the fungal cell wall primarily consists of β-glucans. If the synthesis of these compounds is inhibited, the cell wall integrity will disrupt (Kang, 2010).

2.8.4. Cell membrane disruption

This type of mechanism of action is for antifungal antibiotics are the ergosterols are essential for the cell membrane. If these sterols are bound by antifungal drugs, or the synthesis of them are inhabited by ergosterol biosynthesis inhibitors, the cell membrane's integrity will disrupt. Thereby the membrane becomes leaky (Kim, 2013). In the case of bacteria, the major target site for gram negative bacteria is the lipopolysaccharides present in the cell membrane, it kills bacteria by causing lysis of the outer membrane of the bacterial cell by the disruption of the lipopolysaccharide (Paquet and Carreira, 2006).

2.8.5. Inhibitors of other metabolic processes

Antibiotics act on selected cellular processes essential for the survival of the microbial pathogens. For example, dysfunction of the fungal mitochondria**,** inhibition of the mitochondrial electron transport system, fungi will result in a reduction in mitochondrial membrane potential. The inhibition can occur via inhibition of the proton pumps in the respiratory chain, leading to a reduction in ATP production and subsequent cell death (Kim, 2013).

2.9. Criteria for choosing medicinal plants

The selection of plants for isolation of fungal endophytes for the production of the novel antimicrobial products depends on many criteria including: (i) According to many reports the plants that have ethano-botanical history, used by indigenous peoples and that is related to the

specific use for medicinal purpose (Kharwar *et al*., 2010). (ii) Selection of plant by direct contact with peoples and via local literature. (iii) According to (Kumaresan and Suryanarayanan, 2001) studies, the plants growing in adverse ecological environment possess special capabilities which help them in their survival of microorganisms. (iv)The plants surrounded by pathogen-infected plants with no symptoms of the disease have an internal resistance mechanism and fungal endophytes isolated from such plants are known to produce strong antimicrobial activity (Arnold *et al.,* 2001). (v) Plants that are known to have restricted growth in certain landmass are also more likely to possess endophytes with unique bioactive compounds. Thus, based on these, isolation and characterization of endophytic and rhizospheric fungi associated with five medicinal plants *Rumex abyssinicus, Aloe vera, Solanum incanum, Rumex nervosus* and *Myrsine africana* was investigated.

2.10. Plant description

2.10.1. *Rumex abyssinicus*

Rumex abyssinicus its local Amharic name is (Mekmako) is a perennial herb plant in the family *Polygonaceae*. Which grows up to 3 m tall, with thick and fleshy rhizome (Fig.2). (Eshetu Mulisa *et al.,* 2015). *Rumex abyssinicus* is widespread throughout Ethiopia at altitudes between 1200 and 3300 masl. It also occurs along paths and water, in secondary scrub, grassland, and margins of the rain forest. This plant species will remain locally an essential vegetable. Antimicrobial properties of *R. abyssinicus* roots were used for anti-microbial activities against *Streptococcus pyogenes* (Teshale Mekonnen *et al.,* 2010) have reported that 80 % methanol extract of the rhizomes of *Rumex abyssinicus* had secondary metabolites such as tannins, saponins, flavonoids, steroids, and anthraquinones. Flavonoids and tannins are important for wound healing due to their antioxidant, anti-inflammatory, and antibacterial activities. *R. abyssinicus* is used to treat malaria, gonorrhea, poisoning, hepatitis, constipation, sciatic neuralgia, hypertension, migraine, rheumatism, breast cancer (Abu-Rabia, 2015). Additionally *R. abyssinicus* solve stomach distention, ear ache, liver diseases, hemorrhoids, typhus, rabies, and wound (Teshale Mekonnen *et al*., 2010).

Figure 1: *Rumex abysinicus,* picture was taken during sample collection from Bale Zone of Oromia Regional State.

2.10.2. *Aloe vera*

Aloe vera, or Eret in Amharic, is a species of *Aloe* that is particularly popular for its medicinal properties (Mirutse *et al.*, 2007). The genus *Aloe* belonging to family *Alliaceae* is a perennial and succulent plant. The succulent property enables the species to survive in areas where there is no enough rainfall and can survive in both hot and cold temperatures (Yates *et al.*, 2004). It is a stemless or very short-stemmed plant growing up to 60 to 100 cm hight, which matures in 4-6 years. *Aloe vera* has thick elongated and sharp leaves that grow to about 30 to 50 cm in length and 10 cm in breadth at the base in the adult plant (Boudreaue and Bland, 2006). Colour, pea-green (when young spotted with white) to bright yellow tubular flowers 25 to 35 cm in length arranged in a slender loose spike (Fig. 3). The plant is widely spread and very common in overgrazed range areas or road sides of Ethiopia. In Ethiopia this plant is industrially processed in a wide range of food, healthcare, and cosmetics products due to its nutraceutical qualities (Javed and Atta-ur, 2014). According to the World Health Organization (WHO), *Aloe* is the best source for obtaining a variety of drugs (Darokar *et al*., 2003). It is also well known important source of traditional medicine in Ethiopian communities to treat different ailments including immune-modulatory,

wound and burn healing, hypoglycemic, and anticancer, gastroprotective, antifungal, antibacterial and anti-inflammatory properties. In the northern part of Ethiopia, people use *Aloe* as a traditional medicine to treat urinary retention, cataract, rectal prolepses, Ascaris, infertility, and coughs (Abid, 2018).

Figure 2: *Aloe vera* picture was taken during sample collection from Chancho special zone of Oromia Regional State.

2.10.3. *Solanum incanum*

Solanum incanum or Enbuyi in Amharic is a perennial herb or soft wooded shrub that belongs to *Solanaceae* family. *Solanum incanum* shrub grows up to 1.8 m in height with spines on the stem and calyces and with velvet hairs on the leaves. Flowers pale to deep blue, or purple. The leaves are alternate, egg-shaped in outline with the broad end at the base with slightly wavy margins (particularly on young leaves), with a grey-green upper surface and a green-white lower surface. The fruits are small berries of 2-3 cm in diameter and yellowish-orange or brown when ripe (Fig. 4) (Matu, 2008). *Solanum incanum* is common as a weed, around houses, in overgrazed grassland, and on roadsides. It is also found at forest edges and in bushland and grassland, from sea level up to 2500 masl .

Antimicrobial and anti-fungal effects of *S.incanum* previous reports indicate that the water-soluble crystals derived from unripe fruits of *S.incanum* exhibited marked broad-spectrum antibacterial effect on several gram positive and gram negative bacteria such as *Streptococcus pyogenes,*

Staphylococcus aureus, *Clostridium perfringens*, *Bacillus anthracis* and *Salmonella* species. It also inhibited several fungi like *Microsporum* species, *Trichophyton tonsurans* and *Candida albicans*, etc. Ethanol extract of *S. incanum* (Alamri and Moustafa, 2012) leaf extracts showed antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeuruginosa* (Muchika *et al*., 2011).

Figure 3: *Solanum incanum* picture was taken during sample collection from West Arsi Zone of Oromia Regional State.

2.10.4. *Rumex nervosus*

Rumex nervosus the plant is commonly known as 'Embwacho' in most parts of Ethiopia. It is a shrub of 2 m height or more (Fig. 5). Mostly originated from high altitude areas (above 1000 m) common perennial herbs in Tigray, Gondar, Gojjam, and Wello area of Ethiopia. The leaves and stem of this herb are used for purifying the body by women traditionally, and the leaves are put on fire then they cover the patient body with that hot leaves and blanket so that the vapors and smoke surround all the body. *Rumex* species contains biologically active compounds responsible for anticancer and cytotoxic properties (Wegiera *et al*., 2012). Usually in Ethiopia, the leaves, stems, and roots of *R. nervosus* are used as traditional medicines for different illness such as leaf water extract and aerial parts of *Rumex nervosus* is consumed to reduce non-specific diarrhea, eye disease, hemorrhoids, taeniacapitis, infected wounds, arthritis, abscess, and eczema disorders.

Medicinal importance of the genus *Rumex* was investigated by many investigators toward several bacterial and viral infections (Tilahun Teklehaymanot *et al*., 2007)

Figure 4: *Rumex nervosus* picture was taken during sample collection from Chancho special Zone of Oromia Regional State.

2.10.5. Myrsine africana

Myrsine africana, Kechemo in Amharic, belong to the *Myrsinaceae* family which is a tough slow-growing evergreen shrub that forms a dense upright shrub to 4 to 7 feet tall and 5 feet wide with upright stems bearing tightly overlapping small dark green rounded leaves, with the upper edges slightly cut with fine teeth. Grownup leaves are leathery and have dense, dark-green to red foliage and produce tiny bright purple berries that are edible. Stems have a gray color then new growth, both stems, and leaves have a deep red color (Fig. 6). It is a dioecious plant with insignificant flowers. The plant has harvested from the wild for local use and it is also often grown as an ornamental.

Myrsine africana is selected for the current study that has been used traditionally by many of the country's ethnic groups for treating various illnesses. *Myrsine africana* found in different parts of Ethiopia: Tigray, Gondar, Gojam, Wello, Arsi, Shewa, Welega, Kefa, Sidamo, Bale, and Hararge (Hedberg*et al*., 2003). People mix *Myrsine africana* dry leave powder with honey and take it orally to treat roundworm and tapeworm. The seeds and roots of *M. africana* are widely used for livestock and human as an anthelmintic, especially in the treatment of tapeworms (Gathuma, *et al*., 2004).

Also, the part of the fruit of *Myrsine africana* is used traditionally in many cases such as treatment of roundworm and tapeworm and as the remedy for chest pains and stiff joints.

Figure 5: *Myrsine africana* picture was taken during sample collection from Bale Zone of Oromia Regional State.

2. 11. Justification for choosing pathogens

A large number of microorganisms infect human beings, some selection criteria should be applied based on (WHO, 2011) for this study. (i) Organisms which are proven pathogens. (ii) Organisms that have a high potential for spread in the community and the hospital setting. (iii) Organisms that are known to acquire resistance against currently used and recommended antibiotics. (iv) Organisms that have a standard interpretation of susceptibility test. (v) Organisms that are widespread in the surveillance area and are a frequent cause of disease in the population.

2.12. Pathogenic bacteria

Pathogenic bacteria are bacteria that are capable of causing diseases to the host, pathogenic bacteria including *Escherichia coli, Pseudomonas aeruginosa* and *Entrococcus faecalis* and *Staphylococcus aureus.*

2.12.1. *Escherichia coli*

Escherichia coli is a Gram-negative facultative anaerobic and rod-shaped bacterium commonly found in human and animal intestinal tracts. Most of the *E. coli* strains are harmless, but some serotypes can cause serious food poisoning and becomes intra and extraintestinal pathogen in humans and many other animals (Kaper *et al*., 2004). The most common intestinal pathogens are enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E.coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998).

2.12.2. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram negative, aerobic and rod-shaped bacterium which can survive in a broad range of environmental conditions. *P. aeruginosa* is opportunistic pathogen causing infections in exposed patients with cystic fibrosis (Lucchetti-Miganeh *et al*., 2014). It is an important pathogen in hospitalized patients and causes morbidity and mortality due to its multiple resistance mechanisms (Porras-Gomez *et al*., 2012). This infection affects the urinary tract, surgical site, bloodstream, and wound. *P. aeruginosa* infection can be severe and life-threatening and it is difficult to treat the bacteria since it develops resistance to antibiotics (Adedeji *et al*., 2007).

2.12.3. *Enterococcus faecalis*

The genus *Enterococcu*s consists of Gram-positive, non-spore forming, facultative anaerobic bacteria that can occur both as single cocci and in chains. *Enterococci* belong to a group of organisms known as lactic acid bacteria (LAB) that produce bacteriocins. They are now recognized as the cause of several types of community and hospital-acquired infections, including life-threatening bloodstream infections, endocarditis, meningitis, and urinary tract infections (Upadhyaya *et al*., 2009).

2.12.4. *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterium that is capable of existing in both aerobic and anaerobic environments. Most of *S. aureus* are commensal organism which inhabits the skin, the nose throat and some cause staphylococcal infections, which include impetigo, boils, carbuncles, abscesses and infected wounds (Wertheim *et al*., 2005). The pathological effects of *S. aureus* are associated with the production of enzymes and toxins. *S. aureus* developed methicillin resistance strain in hospitals (Boucher and Corey, 2008). The resistant strains of *S. aureus* produce a plasmid-encoded penicillinase to hydrolyze the β-lactam ring of penicillin deactivating the molecule's antibacterial properties (Chambers and Deleo, 2009). The emergency of methicillin-resistant bacteria led to an increase use of alternative drugs such as in vancomycin (Jones, 2006). Vancomycin affects *S. aureus* bacterial cell wall precursors by binding irreversibly to the terminal D-alanyl-D-alanine of the cell wall component (Costa *et al*., 2013). However, *S. areus* developed vancomycin resistance through the acquisition of resistance genes (*van*A) from vancomycin-resistant *enterococci* (Appelbaum, 2006).

2.13. Pathogenic fungi

Some pathogenic fungi cause human diseases like candidiasis that can cause a serious health problem, especially in immuno-compromised individuals (Odds *et al*., 2006). The clinical use of antibacterial drugs and immunosuppressive agents including chemotherapy taken during organ transplantation, surgery, and cancer are associated with increased risk of human fungal infection (Karkowska-Kuleta *et al*., 2009). *Candida* species is a leading human fungal pathogen to cause invasive fungal infection.

2.13.1. *Candida albicans*

Candida albicans are a commensal fungus that inhabits the oral cavity, the gastrointestinal tract, the vaginal and the urinary tracts. *C. albicans* is the main *Candida* species that cause invasive candidiasis (Brissaud *et al*., 2012). *C. albicans* is an opportunistic fungus that causes infections such as fatigue, weight gain, and joint pain (Pfaller and Diekema, 2007). The biofilm formation in *C. albicans* is one of the factors for the pathogen to be drug-resistant (Al-Fattani and Douglas, 2006).

2.14. The problems of antimicrobial resistance in the globe and Ethiopia

Across the globe, the emergence of antimicrobial resistance (AMR) is threatening the effective and successful treatment of infectious diseases. Drug resistance proliferates due to the improper use of drugs (overuse or misuse), poor regulation of antibiotics, limited antimicrobial stewardship, poor prescribing habits, and non-compliance with prescription (Bosco *et al*., 2013). Mutation of bacteria genomes by different mechanisms may lead to drug-resistant strains and provides a selective advantage to resistant variants. These, in turn, lead to more common treatment failure, which can result in increased morbidity and mortality, prolonged illness, premature death, and other worsened clinical outcomes (O'Neil, 2016). Globally, it is estimated that 700 000 people die every year from drug resistance in common bacterial infections, HIV, and malaria. This number is believed to be underestimated due to poor reporting and surveillance. Besides, AMR puts a financial burden on resource-limited countries like Ethiopia (Seale *et al*., 2017)

The increased prevalence of resistant bacteria, together with the lack and the high cost of new generation drugs have escalated infection-related morbidity and mortality particularly in developing countries like Ethiopia (Borkotoky *et al*., 2013). In Ethiopia, the misuse of antibiotics has increased by health care providers', unskilled practitioners, and drug consumers, the rapid spread of resistant bacteria and insufficient surveillance contributed to the problem. The problem of increasing antimicrobial resistance is even more threatening when considering the very limited number of new antimicrobial agents that are in development (ECDC/EMEA, 2009) posing a serious challenge to the future of chemotherapy (Feleke Moges *et al*., 2014). In Ethiopia, limitation of resource has not allowed antimicrobial resistance to be prioritized as a major public health concern despite the obvious needs (Demissie Shitaye *et al.,* 2010), Antimicrobial resistance is a multifaceted problem. Other studies conducted across Ethiopia also indicated increasing rates of resistance in *E. coli, Salmonella* spp. and *S. aureus* to commonly prescribed antibiotics such as ampicillin, amoxicillin, penicillin, tetracycline, and trimethoprim or sulfamethoxazole (Teshale Seboxa *et al*., 2015).

3. MATERIALS AND METHODS

3.1. Description of the study areas

The samples were collected from three zones of Oromia region namely, Bale, West Arsi, and Chancho Special Zone (Fig. 6). Bale Zone is located at about 430 km from Ethiopia's capital city, Addis Ababa. It has warm weather with an average annual temperature of 14.4 °C. The minimum and maximum temperatures range from 10-21 °C. The annual mean rainfall is 930 mm. There is significant rainfall throughout the year (Bussmann, 1997). The specific sampling site ranges from latitude 6° 57' 14" N to 7° 10' 18" N and longitude 39° 33' 24" E to 40° 14' 60" E and with an elevation of 2592 to 2743 meters above sea level. The soil of the sampling site has a high moisture content, slightly acidic pH, high organic matter and sand, silt, and clay nature of soil (Maneyahilishal *et al*., 2018). The samples were collected from Riverian Fasil Angeso natural forest and the surrounding grazing land. It is situated at about 5 km from Goba Town near the road of Goba to Delomena. The forest is disturbed by human and livestock encroachment.

West Arsi Zone is found in the central part of the Oromia National Regional State. It is located in the Rift Valley Region. The mean annual temperature of the Zone is found between 20-25 °C in the low land and 10-15 °C in the central high land. On average, the annual mean rainfall of 1020 mm. Shashemane which is the administrative town of the zone is located approximately at a distance of 250 km from Addis A baba. The sampling site GPS coordinates of Latitude 7° 05' 33" N to 7° 11' 60.00" N and Longitude 38°22'41" E to 38°38' 03" E and an elevation 1877 to 1937 masl. The sampling sites were covered grazing and farming land borderline. The soil of the zone is fertile and rich in organic macronutrients. The sample collection area falls in slightly acidic pH and the major soil types have characteristics of water holding during rainy seasons due to high clay content and have good base saturation and fertility.

Chancho which is found in Oromia Special Zone is another sample collection site of the current study. It is located 45 km to the north of Addis Ababa on the road to Gojam with an elevation of 2555 to 2600 meters above sea level. It has GPS coordinates of (9° 15' 59"N to 9° 18' 59"N and 38° 45' 15"E to 38° 47' 15"E). The average annual temperature is 14.5 °C in a year. The summer has a good deal of rainfall, the average rainfall is 1098 mm, while the winters have very little. The

soil of the area is characterized by clay texture, neutral pH, and low organic matter. Samples were collected from grazing land, farmland, and borderlines.

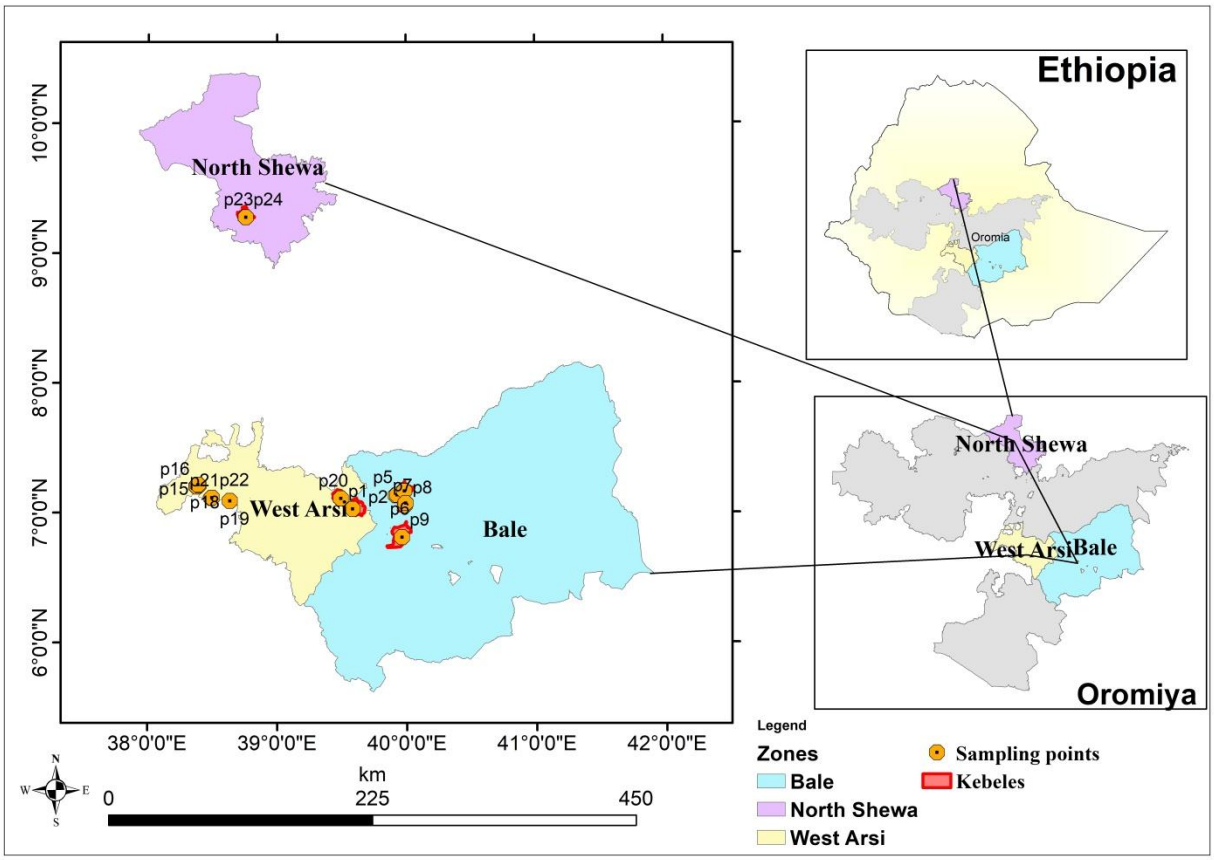


Figure 6: Map of sample collection areas.

3.2. Sample collection

Based on their traditional medicinal value five medicinal plants (*Rumex abyssinicus, Rumex nervosus, Solanum incanum, Aloe vera* and *Mysrine africana*) were selected for this study (Table 1). For each species, two sampling sites were selected based on the abundance of the species. The sample collection sites include forest land (for *Mysrine africana),* grazing land (for *Solanum incanum, Aloe vera,* and *Rumex nervosus*) and medicinal plant gene bank of Ethiopian Biodiversity Institute (*Mysrine africana* and *Rumex abyssinicus*). Five healthy and young individuals were

selected randomly for each species per location and a total of 150 samples (root/ Rhizome, steam and leave) were collected into sterile plastic bags separately. A total of 50 Rhizosphere soil samples were collected from a depth of sapproximately 5 cm around the root of five experimental plants and transferred into separate sterile plastic bags. All the collected samples were kept at 4 °C and transported to Microbiology Laboratory, EBI using an icebox. The plant specimens were identified and authenticated at Forest Directorate, Ethiopian Biodiversity Institute. All the samples were kept at 4 °C until processed.

Table1. Selected medicinal plants for isolation of endophytic and rhizospheric fungi

Family	Scientific Name	Local Name (AfaanOromo/Amharic)	Sample Type	Collection Site
Polygonaceae	*Rumex abyssinicus*	Mekmeko[A]	Leaf, Stem, Rhizome/root and Soil	Bale Zone and West Arsi
Polygonaceae	*Rumex nervosus*	Embuwacho[A]	Leaf, Stem, Root and Soil	West Arsi and Chancho
Myrsinaceae	*Mysrine Africana*	Kechemo[A/AO]	Leaf, Stem, Root and Soil	Bale Zone
Alliaceae	*Aloe vera*	Ret[A]	Leaf, Root and Soil	West Arsi and Chancho
Solanaceae	*Solanum incanum*	Embuayi[A]	Leaf, Stem, Root, and Soil	Bale and West Arsi

Legend: A= Amharic, AO= Afaan Oromo

3.3. Isolation of endophytic fungi

To isolate endophytic fungi about 3 g of plant samples were weighed and transferred into a new sterile Petri dish and washed using running tap water three times to remove dust and soil. The samples surface sterilized with 70% ethanol for 1 min and rinsed three times with sterile distilled water. Plant samples again were washed with 2% sodium hypochlorite solution for 30 seconds followed by rinsing three times with sterile distilled water (Basha *et al.,* 2012). The samples allowed to surface dry on sterile filter paper for culturing fungi, PDA (HiMedia) was prepared in double-distilled water and autoclaved at 121 °C for 15 min. Liquid

from the third wash was plated onto PDA (HiMedia) supplemented with 100 mg/ml chloramphenicol to check the efficiency of surface sterilization.

Each leaf, stem, and root/ rhizome samples were cut to one-centimeter size using a sterile blade. A total of 900 surfaces sterilized segments from different tissues of each plant (300 leaves, 240 stems, 300 roots, and 60 segments of rhizome) were processed for the isolation of endophytic fungi. Six segments from each sample were transferred to PDA (HiMedia) containing chloramphenicol (100 mg/L) using sterilized forceps (Annex 3).Finally, inoculated plates were incubated at 27 °C for 7 days until growth is detectable (Deepthi *et al.*, 2018).

3.4. Isolation of rhizosphere Fungi

Fifty soil samples were serially diluted in sterile distilled water under aseptic conditions (laminar airflow chamber). One gram of rhizospheric soil sample was taken and added into a test tube containing 9 ml of sterile double distilled water. Tenfold serial dilutions (10^{-1} to 10^{-6}) were prepared by pipetting 1 ml from stock suspension into nine ml of sterile distilled water in labeled test tubes with thorough shaking manually to mix the suspension (Nisha *et al.*, 2017). From an appropriate dilution factor, 0.1 ml of the suspension was spread plated onto PDA (HiMedia) supplemented with chloramphenicol (100 mg/L) using a sterile pipette. Finally, inoculated plates were incubated at 27 $^{\circ}$C for 7 days.

3.5. Purification and maintenance of the fungal isolates

Morphologically different fungal colonies from plates were isolated by inoculating each colony onto fresh PDA (HiMedia) plates repeatedly to establish a pure culture. The inoculated plates were incubated at 27 ^{o}C for 7 days. The purified fungal isolates were transferred separately to brain heart infusion preservative medium supplemented with 10% glycerol kept at 4^{o}C and -20^{o}C for screening and identification purposes.

3.6. Source of test organisms

Bacterial and fungal cultures were obtained from the Microbiology Laboratory of Tikur Anbessa Specialized Hospital. The clinical microbes were isolated from patients visiting the Hospital (Annex 6)The test human pathogenic bacteria were *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli,* and *Candida albicans,* a yeast. The bacterial cultures were maintained on nutrient agar medium and Sabouraud agar medium was used to culture the test

yeast. The test bacterial and fungal strains were incubated at 37 ºC for 24 hrs and at 35 ºC for 48 hrs, respectively. The reference human pathogenic microorganisms *Staphylococcus aureus* (ATCC25923), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa (*ATCC 27853), *Escherichia coli* (ATCC 25922) and *Candida albicans* (ATCC 9690) were obtained from Ethiopian Public Health Institute (EPHI), Microbiology Department, Addis Ababa. The selected microorganisms were identified and confirmed at EBI.

3.7. Standard antibiotics

Standard antibiotics 30 µg chloramphenicol were used as a positive control for the antibacterial susceptibility test by disc diffusion, 25 µg fluconazole was employed for antifungal test and 2% DMSO was used as a negative control (Matilde Fernández 2011).

3.8. Preparation of inoculum

0.5 McFarland Standard which was made ready ahead by mixing 0.5 ml of 0.048 M $BaCl_2$ (1.175% W/V $BaCl_2.2H_2O$) with 99.5 ml 0.18 M H_2SO_4 (1% V/V) and followed by thoroughly mixing to ensure that it is evenly suspended.The standard was distributed into a test tube of the same size and volume as those used to prepare the test inoculums. The inocula was prepared from the culture of bacteria and yeast species grown on nutrient agar (HiMedia) at 37 ºC for 24 hrs and Sabouraud agar (HiMedia) at 35 °C for 48 hr respectively, 2-3 colonies were taken aseptically and suspended in 5 ml of 0.085 % saline solution. The suspended inocula were vortexed for 15 seconds and compared visually with standardized 0.5 McFarland (turbidity adjusted spectrophotometrically to (0.08-0.10 at OD $_{625}$ nm) against a white paper background and contrasting black lines (Andrews, 2006).

3.9. Preliminary screening

The preliminary screening of antimicrobial activity was carried out following the fungal agar plug method (Devaraju and Satish, 2011). Nutrient agar (HiMedia) and Sabouraud agar (HiMedia) were poured into Petri plates and inoculated with 100 µl of the pathogenic bacterial and yeast suspensions. All the test organisms' suspensions were standardized on 0.5 McFarland Standard and spread uniformly using a sterile swab. The mycelial discs (6 mm) of each endophytic and rhizospheric fungal isolate (14 days-old) grown at 27 ˚C on PDA (HiMedia) were obtained from

actively growing margins using a sterile cork borer and placed on the surface of the Nutrient Agar (HiMedia) and Sabouraud agar (HiMedia) that were seeded with test organisms. Chloramphenicol (30μg) and fluconazole (25 μg) were used as positive control for bacteria and yeast respectively. PDA medium (6 mm discs) devoid of any fungal colony but inoculated with test yeast was used as a negative control. Moreover, nutrient agar inoculated with test bacteria was also used as control. The plates were sealed using Parafilm and incubated at 37 °C for 24 hrs and 35 °C for 48 hrs for bacteria and fungus, respectively. After incubation, antimicrobial activities were confirmed by the visualization and measurement of inhibition zones. Each experiment was carried out in triplicates.

3.10. Fermentation and extraction of fungal crude metabolites

The fungal isolates that showed good anti-microbial activity in primary screening were subjected to fermentation for metabolite production**.** The potent fungal isolates were cultivated on the surface of PDA (HiMedia) plates and incubated at 27 °C, for 7 days. A log phase growth stage of the culture, five plugs (6 mm diameter) were cut from fungal culture using sterilized cork borer and were transferred into a flask containing 250 ml of PDB (HiMedia) medium (pH 6.8) the fungal cultures were incubated at 27 °C for 14 days. Then, mycelia were filtered and the filtrates were centrifuged at 10,000 rpm for 15 minutes. All the supernatants were collected by new sterilized test tubes and filtered again using Whatman filter paper No. 1 to remove the remaining mycelia. Thereafter, the culture filtrate was extracted with an equal volume of ethyl acetate. The solution was mixed well by vortexing for 10 min and kept for 5 minutes until two clear immiscible layers (medium layer and ethyl acetat layer) were formed. The upper layer of ethyl acetate containing the extracted compounds was separated using separating funnel. The extract was concentrated by removing the solvents under reduced pressure at 40 °C to get concentrated crude extract. Finally, the extracts were dissolved in 2% DMSO (Dimethyl sulfoxide) at equal concentration and stored at 4 °C for further use (Sutjaritvorakul, 2011).

3.11. Secondary Screening by Agar well diffusion method

The secondary screening of antimicrobial activities of the fungal extracts was carried out using the agar well diffusion method (Moussa *et al.*, 2011; Onyegbule *et al*., 2014). Concentrations of 50 mg/ml were prepared for all the fungal extracts by dissolving the extracts in 2%DMSO. Twenty (20) mL of molten Mueller Hinton Agar (HiMedia) and Sabouraud Dextrose Agar (HiMedia) were

poured into sterile Petri plates (90 mm) (for bacterial and for yeast, respectively) and allowed to set. All clinical test organisms' suspensions were standardized based on 0.5 McFarland Standard was spread uniformly using a sterile swab. Then, Holes 6 mm diameter and 4 mm deep wells were made in the spread agar plates using a sterile cork borer. A hundred microliters (100 µl) of the diluted extracts were added in each hole under aseptic condition by using a micropipette and kept at room temperature for 1 hr to allow the crude metabolites to diffuse into the agar medium. Chloramphenicol (30 µg) and fluconazole (25 µg) served as a positive control for bacteria and yeast, respectively, while 2% DMSO was used as the negative control. The MHA plates were then incubated at 37 ^{o}C for 24 hrs but the SDA plates were incubated at 35 ^{o}C for 2 days. Finally, the inhibition zones diameters (IZDs) were measured and recorded using caliper in mm. The size of the cork borer (6 mm) was deducted from the values recorded for the IZDs to get the actual diameter. This procedure was conducted in triplicate.

3.12. Determination of the Minimum Inhibitory Concentrations (MICs)

The Minimum inhibitory concentration (MIC) of the solvent extracts was determined by agar dilution methods as described by the European society of clinical microbial and infectious diseases (ESCMID, 2000). Nineteen ml of molten Muller Hinton and Sabouraud agar (HiMedia) media and one ml of crude fungal solvent extracts were prepared at different concentrations (50, 25, 12.5, 6.25, 3.125 mg/ml) and mixed thoroughly and poured on Petri dish. One ml of bacteria and yeast culture in 0.85 % saline solutions adjusted to 0.5 McFarland were swabbed on Muller Hinton and Sabouraud agar and incubated 37 °C for 24 hrs bacteria and 35 °C for 48 hr for yeast, respectively. The negative control contained only the media and the positive control contained one ml tested pathogens plus media for comparison. The plates were incubated for 24 h at 37 °C for bacteria and 35 °C for 48 for yeast. The MIC was determined by observing the growth of the test pathogens.

3.12.1. Minimum Bactericidal Concentrations (MBCs) and Minimum Fungicidal Concentrations (MFCs)

The loop full tests from the last MIC were subcultured by streak onto a fresh Muller Hinton and Sabouraud dextrose Agar (HiMedia) then, incubated at 37 °C for 24 hrs for bacteria and 35 °C for 48 hrs for yeast, respectively. The lowest concentration of the extracts showing no growth on the

Muller Hinton and Sabouraud dextrose Agar plates was recorded as MBC/MFC (Espinel-Ingroff, 2002; Joshua and Takudzwa, 2013).

3.13. Optimization of fungal secondary metabolite production and antimicrobial activities

3.13.1. Effect of the incubation period on secondary metabolite production and antimicrobial activities

Incubation periods ranging from 8-13 days (8, 9. 10, 11, 12, and 13) were used to determine the effect of incubation period on the bioactive production and antimicrobial activity for fungal isolates. Culture flasks containing 250 ml PDB (HiMedia) medium (pH 6.6) with 6 mm mycelia grown at 27 ^{o}C for 7 days, then flasks were incubated at 27 °C in an orbital shaker (150 rpm) for different days. Broth cultures were filtered using 12.5 cm Whatman filter paper No.1.The bioactive metabolite production was measured and culture filtrates were tested for antimicrobial activity by agar well diffusion method (Singh *et al*., 2009).

3.13.2. Effect of carbon source on secondary metabolite production and antimicrobial activities

To optimize the carbon source, 6 mm mycelia disks of seven day old fungal culture grown at 27^{o}C for 7 days were inoculated into 250 ml Potato broth medium supplemented with 1% of five different carbon sources (glucose, dextrose, lactose, fructose, and sucrose) under aseptic conditions. Inoculated flasks were incubated at 27 °C for 14 days. After incubation, broth cultures were filtered using 12.5 cm Whatman filter paper No.1.The bioactive metabolite production was measured and culture filtrates (extract) were tested for antimicrobial activity against the test microbes by agar well diffusion method (Singh *et al.,* 2009).

3.13.3. Effect of nitrogen source on secondary metabolite production and antimicrobial activities

To study the effect of different nitrogen sources, beef extract, yeast extract, peptone, urea, ammonium chloride, and sodium nitrate were used. About 1% of each nitrogen source was added to 250 ml PDB (HiMedia) pH 6.6. Flasks containing the medium were inoculated with 6 mm mycelial disks of seven day old fungal cultures grown at 27 °C under aseptic condition. The flasks

were incubated for 14 days at 27 °C. Culture filtrates were measured and used for testing antimicrobial activity against test microbes by agar well diffusion assay. The antimicrobial production was recorded at the end of the incubation period (Singh *et al.*, 2009).

3.13.4. Effect of Temperature on secondary metabolite production and antimicrobial activities

To determine the effect of temperature for the growth of fungal bioactive production and maximum antimicrobial activity of the crude extract, five different temperature values (20 °C, 25 °C, 30 °C, 35 °C and 37°C) were evaluated. Two hundred fifty milliliters (250 ml) of potato dextrose broth (HiMedia, pH 6.6) was inoculated with 6 mm diameter culture discs that are previously grown at 27 °C for 7 days. Then, broth inoculants were incubated at 27 °C for 14 days and followed by filtration using sterile 12.5 cm diameter whatman.No 1 filter paper and the culture filtrates were used for measuring crude metabolites and testing antimicrobial activity against test microbes by agar well diffusion assay (Ripa *et al*., 2009).

3.13.5. Effect of pH on secondary metabolite production and antimicrobial activities

Five pH values (3, 5, 7, 9, and 11) were taken to determine the optimum pH for the growth of fungal isolates on bioactive production and antimicrobial activities of crude extract. Two hundred fifty ml Potato dextrose broth (HiMedia) was adjusted to the desired pH by adding 0.1 N NaOH or 0.1 N HCl and autoclaved at 121 °C for 15 minutes. Under the aseptic condition, 6 mm mycelial disks of seven-day old fungal cultures grown at 27 °C were inoculated into 250 ml broth and incubated at 27 °C for 14 days. The culture filtrates were collected using 12.5 cm diameter of whatman filter paper No. 1 and crude metabolites were extracted using ethyl acetate. Finally, bioactive metabolite production was measured and antimicrobial activity against the selected pathogenic microbes was tested by agar well diffusion method (Nisha *et al*., 2017)(Fig.7).

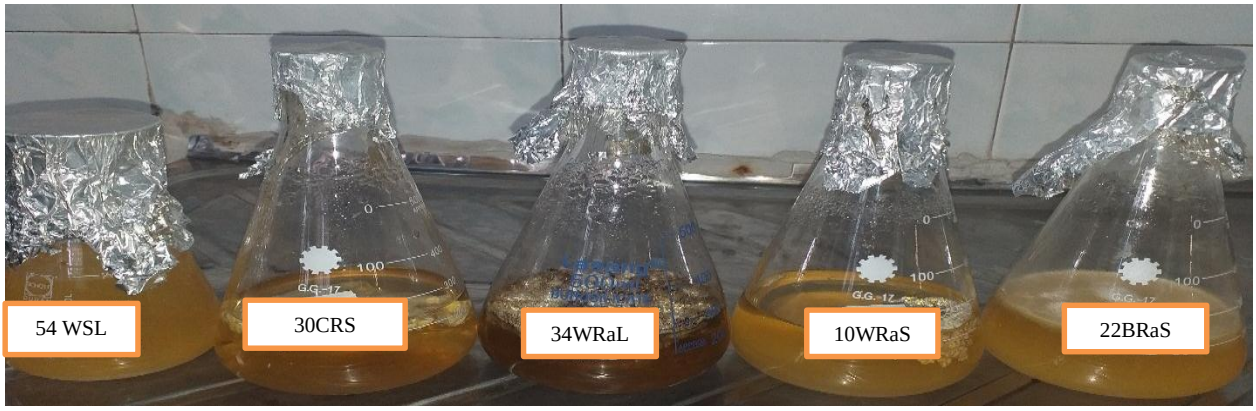


Figure 7: Production and extraction of secondary metabolite

3.14. Qualitative screening of fungal metabolites

The ethyl acetate extracts of potential fungal isolates were checked for the presence of different secondary metabolites having antimicrobial activities such as Alkaloid, Phenols, Flavonoids, Saponins, Steriods, Cardiac glycosides, Terphenoid, and Tannins by following standard procedures.

3.14.1. Alkaloids

Wagner's test was performed to evaluate the presence of alkaloids. One milliliter (1ml) of fungal crude extract was dissolved in 2 N HCl solutions. The mixture was treated with three drops of Wagner's reagent (3 ml of potassium iodide solution mixed with 2 ml of iodine solution). The red-brown precipitate indicates the presence of alkaloids (Handunnetti, 2009).

3.14.2. Flavonoids

Flavonoids test was performed as described by (Cai *et al.*,2004). Three drops of 20% NaOH solution were added to the test tube containing 1 ml of fungal extract resulted in the formation of yellow color. Then two drops of concentrated H_2SO_4 solution were added to the mixture. Finally, as the change of color from yellow to colorless solution depicts the presence of flavonoids, present, and absent data were recorded.

3.14.3. Phenols

To test the presence of phenol compounds in the fungi extracts one milliliter (1ml) of the fungal extract was dissolved in 5 mL of distilled water. To this mixture, 5 µl of neutral 5% ferric chloride solution was added. Dark green color indicates the presence of phenolic compounds (Cai *et al*., 2004) and accordingly present and absent data were recorded.

3.14.4. Tannins

The presence of a tannin compound was tested using a ferric chloride test as described in (Yadav and Agarwala, 2011). One milliliter fungal extract was treated with 0.5 ml of 5% ferric chloride reagent. The occurrence of the blackish-blue color showed the presence of gallic tannins and a green-blackish color indicated the presence of catechol tannins.

3.14.5. Cardiac glycosides

Keller-kiliani test was performed to assess the presence of cardiac glycosides. One milliliter (1ml) fungal extract was treated with 1 ml of $FeCl_3$ reagent (a mixture of 1 ml of 5% $FeCl_3$ solution and 99 ml of glacial acetic acid). To this solution, 1 ml of concentrated H_2SO_4 was added. The appearance of greenish-blue color within a few minutes indicates the presence of cardiac glycosides (Yadav and Agarwala, 2011).

3.14.6. Steroids

Libermann-Burchard reaction method was used to assess the presence of steroids. One milliliter of the fungal extract was added to one ml of chloroform solution. The mixtures were treated with two ml of acetic anhydride. Thereafter, 2 drops of concentrated H_2SO_4 was added. The appearance of a blue-green ring indicates the presence of steroids (Nameirakpam *et al*., 2012).

3.14.7. Saponins

The presence of saponins was determined by the Frothing test (Sujana and Sridhar, 2013). One milliliter of the fungal extract was vigorously shaken with 3 ml of distilled water and allowed to stand for 10 min. Formation of more than 1.5 cm stable froth (foam) indicates the presence of saponin (Foam test and Lieberman Bourchard) method were used.

3.14.8. Terpenoids

One milliliter (1 ml) of the fungal extract was mixed in 2 ml of chloroform. Then 3 ml of concentrated H_2SO_4 was added. The formation of a reddish-brown colored precipitate at the interface indicates the presence of the terpenoids Salkowski method (Yadav and Agarwala, 2011).

3.15. Morphological characterization

Morphological characterization was performed using macroscopic and microscopic characteristics of the isolates following the methods described in (Barnet and Hunter, 2006). Morphological studies were done by plating the fungi on PDA and incubating it for one week at 28 °C. The growth appearances were observed at both the top and bottom sides of the culture plates such as (colonies color, colonies upside color, surface texture, and margins). Microscopic identification of the selected isolates was done based on the slide culture method. Pure PDA (HiMedia) medium was cut (5 mm square), picked up carefully, and transferred to the center of a sterile slide in a sterile Petri plate. The four sides of the agar square were inoculated with 7 days old culture of the fungus to be examined. A cover glass was placed on the inoculated slide and incubated at 27 ˚C for 48 hrs. After incubation, the cover glass was taken carefully and flooded with Lactophenol Cotton Blue (LCB). The glass slide was observed under 10x (40x) light microscope (euromex, Holland) to see the fungal structure (Kumar *et al*., 2015). The microscopic study included conidia and conidiophores and their arrangements(Barnet and Hunter, 2000).

3.16. Identification of fungi isolates by using Biolog™ System

Fungal isolates were identified using the Biolog Microstation™ ID System following procedures described in the manufacturer's user guide (Biolog™, Hayward, CA). Fungal isolates were cultured on PDA (HiMedia) and grown at 27 ºC for one week and transferred to malt extract agar medium (Biolog™) by incubating at 27 ºC for 3 days. Then, pure colonies were picked with a sterile wooden cotton swab (Biolog) and rubbed around the walls of a test tube containing filamentous fungus inoculation fluid (FF-IF, Biolog™) to prepare a fungal suspension. The optical density of the suspension was adjusted to 47 % transmittance using the Biolog™ turbidimeter. A 100 µL of the fungal suspension was transferred into each well of FF microplates (Biolog™) using a multichannel pipettor and incubated at 27°C. The Microplates were read using BioLog Microstation™ microbial

identification systems at every 24 hrs incubation period for seven days at the National Animal Health and Diagnosis center. Each metabolic profile of incubated fungal isolates at each reading time was compared with the Biolog Microstation™ database (Biolog™) and identified at the species level.

4. DATA ANALYSIS METHODS

All experiments were carried out in triplicates and results are expressed as mean ± SD using Minitab®version 17.1 statistical software. One way ANOVA was conducted to test the significance levels of variables using R stat version 3.6.3. Significant differences among treatment means were separated using the least significant difference (LSD) at 5% Fisher's probability level. Inhibition zone, minimum inhibitory concentration, minimum bactericidal/fungicidal concentration, optimization parameters of the tested fungi samples were used for mean separation.

5. RESULTS

5.1. Isolation of endophytic and rhizospheric fungi

A total of 582 (316 endophytic and 266 rhizospheric) fungal isolates were obtained from the entire samples of the current study. In terms of total isolates under the studied plant taxa, 151 (25.9%) from *solanum incanum*, 147(25.3%) from *Rumex abyssinicus*, 127(21.8%) from *Mysrine africana*, 117(20.1%) from *Rumex nervosus* and 40 (6.9%) from *Aloe vera* of fungal isolates were obtained (Table 2).With respect to sample types, 173 (54.7%) fungal isolates were obtained from leaves, 120 (37.9%) from stem, 5(0.001%), from root segmens and 18(5.7%) from rhizomes (Table 2). From a total 266 rhizospheric fungal isolates 69 (25.9%) of fungal isolates were recovered from *Solanum incanum*, 62(23.3%) from *Mysrine africana*, 53(19.9%) from *Rumex nervosus*, 52(19.5%) from *Rumex abyssinicus* and 30 (11.3%) from *Aloe vera* (Table 2).

Table 2. Isolation of endophytic and rhizospheric fungi

Plant Species	**Number of fugal isolates obtained from different sample types**				
	Rhizosper	Root/Rhizome	Stem	Leaf	**Total**
Solanum incanum	69	0	28	54	151
Rumex abyssinicus	52	18	23	54	147
Rumex nervosus	53	0	38	26	117
Mysrine africana	62	0	31	34	127
Aloe vera	30	5	0	5	40
Total	**266**	**23**	**120**	**173**	**582**

5.2. Preliminary screening for antimicrobial activity

Five hundred eighty two fungi isolates (316 endophytic and 266 rhizospheric) were screened for antimicrobial activity by agar plug method (Table 2). Accordingly, a total of 78 (13.4%) isolates displayed antimicrobial activity at least against one clinical and standard pathogenic test organism. Seventeen of these isolates were obtained from soil samples while the remaining sixty-one isolates were from different plant parts. Out of 78 isolates, 32 (41.0%) isolates inhibited *E. faecalis*, 31 (39.7%) isolates *E.coli,* 13 (16.7%) isolates showed potential inhibition against *S.aureus* and only 7(9%) isolates suppressed the growth of *P.aeruginosa*. Likewise, some isolates (10.2%) were effective against *C. albicans.* From 78 positive isolates, 18 fungal isolates revealed better antimicrobial properties based on inhibition zone (Table 3).

Table 3. Antimicrobial activities of fungal isolates by plug agar method

Isolate code	Antimicrobial potential fungal isolates against test organisms									
	E. coli		*E. faecalis*		*S. aureus*		*P.aeroginosa*		*C. albicans*	
	CI	RI	CI	RI	CI	RI	CI	RI	CI	RI
34WRaL	+++	+++	++	++	-	-	-	-	-	-
22BRaS	++	+++	++	++	++	++	++	++	-	-
54WSL	-	-	-	-	-	-	++	++	-	-
34BSSoil	-	++	-	-	-	-	+++	++	-	-
30CRS	+	+++	++	+++	++	++	++	++	++	+++
37BRaL	++	+++	+	++	+	++	-	-	+	+
1BRaS	++	+++	-	+	-		-	-	-	-
10WRaS	+	+	+	++	++	-	-	-	-	-
15BRaS	+	++	+	+++	-	-	-	-	-	-
41WRaS	++	++	+	+	-	-	-	-	-	-
68BMS	++	++	+++	+++	-	-	+	+	+++	+++
67BMSoil	-	+	+	+	-	-	+	+	+	+
74WSS	-	-	++	+++	-	-	+++	+++	-	-
75WRaR	-	-	-	+	+	++	-	-	++	+++
78BRaR	-	+	++	++	-	-	-	-	++	+
72BML	-	+	-	+	-	-	-	-	++	++
63BML	++	+++	-	++	-	-	-	-	-	-
28BML	+	++	-	+	-	-	-	-	-	-

Chloraphenicol(C_{30})	++	+++	++	+++	+	++	+	+	-	-
Fluconazole (FLC_{10})	-	-	-	-	-	-	-	-	+	++
2%DMSO	-	-	-	-	-	-	-	-	-	-

CI=Clinical Isolates, RI=Reference Isolates

Note: Inhibition zone diameter index: +++,> 20 mm; ++, >10 mm; +, <10 mm; – no inhibition

34WRaL-west arsi Rumex abyssinicus Leaf, 22BRaS -Bale Rumex abyssinicus Stem, 54 WSL-West Arsi Solanum Incanum Leaf, 34BSSoil- Bale solanum Incanum Soil, 30CRS- Chancho Rumex nervosus Stem, 37BRaL- Bale Rumex abyssinicus Leaf, 1BRaS- Bale Rumex abyssinicus Stem, 10WRaS-West Arsi Rumex abyssinicus Stem, 15BRaS-Bale Rumex abyssinicus Stem, 41WRaS-West Arsi Rumex abyssinicus Stem, 68BMS- Bale Mysrine africana Stem, 67BMSoil - Bale Mysrine africana Soil, 74WSS-West Arsi Solanum incanum Stem, 75WRaR-WestArsi Rumex abyssinicus Rhizome, 78BRaR- Bale Rumex abyssinicus Rhizome, 72BML Bale Mysrine africana Leaf, 63BML- Bale Mysrine africana Leaf, 28BML- Bale Mysrine africana Leaf.

5.3. Antimicrobial activities of the crude fungal extracts

Among 78 fungal isolates that showed antimicrobial activities in primary screening, crude extracts obtained from 58 (74.3%) displayed antimicrobial activity against at least one clinical resistant test pathogen with inhibition zones ranged from 1.00 $\pm$ 0.00 to 40.33$\pm$ 0.57 mm (Table 4). Forty five of these fungal extracts were obtained from fungal endophytes while the remaing threen fungal extracts were from rhizospheric fungal isolates. Out of the 58 positive extracts more than half of them 32(55.2%) displayed activity against *E. faecalis*, 24 (41.3%) against *E.coli*, 10 (17.2%) against *S.aureus* and 8 (13.8 %) inhibited growth of *C. albicans*. However, only 5 isolates (8.6%) inhibited *P. aeruginosa*. Eighteen isolates from primary screening was also positive for secondary screening out of 58 isolates (Table 4). Interms of plant species, most of the active isolates were from *Rumex abyssinicus* (30.4%) followed by *Solanum incanum* (22.5%), *Mysrine africana* (17.9%), *Rumex nervosus* (4.3%) and *Aloe vera* (1.9%). With regards to medicinal plant parts, 62% of the effective isolates were obtained from leaves and 38% from stem but a few (5%) were from roots.

The initial fermentation process production varied from one fungus isolate to the next with the largest residue production of 94 mg and the least yielded 50 mg from 250 ml fermentation broth. The highest antibacterial activity was exhibited by isolate 30CRS and 37BRaL for the tested with inhibition of most of the test organisms. Fungal isolate 30CRS was highly significant ($p< 0.001$) difference which showed large inhibition zone against test organisms aganist *E.coli* (30.33 $\pm$ 0.57mm), *E. faecalis* (25.33 $\pm$ 0.28 mm) and *S.aureus* (18.33 $\pm$ 0.57), respectively compared with positive control, chloramphenicol (Table 4). Isolate 37BRaL which showed strong inhibition zone against *S.aureus* (19.16$\pm$ 0.28 mm) and *C.albicans* (26.83 $\pm$ 0.76 mm) respectively, and highly significant difference ($p<0.001$) compared with positive control Chloramphenicol and Fluconazole (Table4). However, clinical *P.aeruginosa* showed strong resistance for isolate 37BRaL fungal extract tested and weak inhibition zone (2.33 $\pm$ 0.28mm) diameter was measured.

Table 4. Antimicrobial activity of crude ethylacetate extracts of fungi using agar well diffusion method including the well (6mm diameter)

Isolates code	Inhibition zone of different human pathogen microorganisms (Mean + standard deviation)				
	E. coli	*E. faecalis*	*S. aureus*	*P. aeruginosa*	*C. albicans*
1BRaS	20.33 ± 0.57^{e}	3.00 ± 0.50^{i}	4.16 ± 0.28^{efgh}	3.16 ± 0.28^{fg}	2.50 ± 0.00^{hi}
10WraS	15.16 ± 0.28^{g}	20.16 ± 0.28^{c}	23.33 ± 0.57^{b}	5.00 ± 0.00^{e}	2.00 ± 0.00^{i}
15BRaS	18.16 ± 0.28^{f}	18.33 ± 0.57^{d}	4.33 ± 0.28^{efg}	3.00 ± 0.00^{fg}	4.66 ± 0.57^{g}
22BRaS	23.70 ± 0.60^{c}	17.16 ± 0.28^{d}	19.25 ± 0.43^{c}	3.16 ± 0.28^{fg}	4.50 ± 0.86^{g}
28BML	22.33 ± 0.57^{d}	0.00 ± 0.00^{j}	0.00 ± 0.00^{k}	0.00 ± 0.00^{i}	0.00 ± 0.00^{j}
30CRS	30.33 ± 0.57^{a}	25.33 ± 0.28^{b}	18.33 ± 0.57^{c}	18.16 ± 0.28^{d}	20.33 ± 0.57^{d}
34WRaL	30.66 ± 0.57^{a}	25.33 ± 0.57^{b}	3.50 ± 0.50^{fghi}	3.33 ± 0.57^{f}	3.33 ± 0.57^{ghi}
34BSSoil	3.33 ± 0.28^{h}	5.00 ± 0.00^{g}	2.16 ± 0.28^{j}	32.33 ± 0.57^{a}	2.66 ± 0.28^{gh}
37BRaL	23.33 ± 0.57^{cd}	17.16 ± 0.28^{d}	19.16 ± 0.28^{c}	2.33 ± 0.28^{g}	26.83 ± 0.76^{b}
41WraS	20.33 ± 0.57^{e}	15.33 ± 0.57^{e}	5.000 ± 0.00^{e}	1.00 ± 0.00^{h}	2.00 ± 0.000^{i}
54WSL	1.83 ± 0.28^{i}	4.16 ± 0.28^{ghi}	2.833 ± 0.76^{ij}	29.36 ± 0.32^{b}	3.83 ± 0.76^{gh}
63BML	24.33 ± 0.57^{bc}	0.00 ± 0.00^{j}	0.00 ± 0.00^{k}	0.00 ± 0.00^{i}	0.00 ± 0.000^{j}
67BMSoil	0.00 ± 0.00^{j}	14.50 ± 0.70^{e}	5.00 ± 0.00^{d}	18.50 ± 0.70^{d}	12.50 ± 0.70^{f}
68BMS	14.33 ± 0.57^{g}	12.33 ± 0.57^{f}	3.16 ± 0.28^{ghij}	19.33 ± 0.57^{c}	35.33 ± 0.57^{a}
72BML	0.00 ± 0.00^{j}	0.00 ± 0.00^{j}	0.00 ± 0.00^{k}	0.00 ± 0.00^{i}	20.33 ± 0.57^{d}

74WSS	0.00 ± 0.00^{j}	20.33 ± 0.57^{c}	40.33± 0.57^{a}	0.00± 0.00^{i}	0.00 ± 0.000^{j}
75WRaR	0.00 ± 0.00^{j}	30.33 ± 0.57^{a}	3.00 ± 0.00^{hij}	0.00± 0.00^{i}	29.33± 0.57^{b}

Table 4. Contd…

Isolates code	Inhibition zone of different human pathogen microorganisms (Mean + standard deviation)				
	E. coli	*E. faecalis*	*S. aureus*	*P. aeruginosa*	*C. albicans*
78BRaR	0.00 ± 0.000^{j}	0.00 ± 0.00^{c}	0.00 ± 0.00^{k}	0.00 ± 0.00^{i}	15.33± 0.57^{e}
Chloramphenicol	25.33 ± 0.57^{b}	16.33+0.28^{e}	15.33± 0.57^{d}	8.33± 0.57^{e}	0.00 ± 0.00^{j}
Fluconazole	0.00 ± 0.00^{j}	0.00 ± 0.00^{j}	0.00 ± 0.00^{k}	0.00 ± 0.00^{i}	24.00 ± 0.00^{c}

Note: Results displayed are representative of the mean of triplicate determinations ± sum of standard deviation. Means followed by different letters (a, b, c, d, e, f, g, h, i, k) within the row are significantly different at $p< 0.05$.

Selection of the best performed isolates for identification

From eighteen (18) potential fungal isolates five fungal isolates were selected which inhibit all pathogenic test organism with higher inbition zone at least more than two test organism during seconday screening

Table 5. Means of inhibition zone of the fungal isolates against test organisms using agar well diffusion method

Test organisms	Mean inhibition zone in mm						MSerror	P-value	LSD
	30 CRS	37 BRaL	68BMS	22BRaS	34WRaL	Control			
E. coli	30.33[a]	23.33[c]	14.33[d]	23.70[c]	30.67[a]	25.33[b]	0.339	<0.05	1.60
E.faecalis	25.33[a]	17.16[b]	12.33[c]	17.17[b]	25.33[a]	16.33[b]	0.542	<0.001	1.30
S.aureus	18.33[b]	19.17[a]	3.166[d]	19.25[a]	3.500[d]	15.33[c]	0.212	<0.001	0.81
P.aeruginosa	18.16[ab]	2.33[c]	19.33[a]	3.16[c]	3.33[c]	8.33[bc]	34.875	>0.2	10.50
C.albicans	20.33[d]	26.33[b]	35.33[a]	4.50[e]	3.33[f]	24.00[c]	0.35	<0.001	1.05

Note: Means followed by different letters (a, b, c, d, e and f) within the row are significantly different at $p < 0.05$. Resistant *E.coli*, *E.faecalis*, *S.aureus*, *P.aeruginosa* and *C.albicans*. LSD: Least Significant Difference at alpha=0.05

5.4. Minimum Inhibitory Concentrations (MICs), Minimum bactericidal concentration and Minimum fungicidal concentration (MB/FCs)

For the selected fungal isolates, the MIC values ranged from 3.125 - 50 mg/ml for gram positive bacteria, 6.25 - 50 mg/ml for gram negative bacteria and 12.5 – 50 mg/ml for yeast test organism (Table 6). The mean MBC of fungi isolates from different sources against the tested microbes is indicated (Table 6). There was no visible colony observed against *E. coli* and *E. faecalis* at 6.25 mg/ml and *S. saureus, P. aeruginosa* at 50mg/ml and MFC of *C. albicans* at 50 mg/ml concentration by 30CRS. Isolate 37BRaL also killed *E. coli* and *E. faecalis* at 12.5 mg/ml, *S. aureus* at 50 mg/ml and MFC for *C. albicans* at 25 mg/ml concentration, respectively. *P.aeruginosa* was resistant for isolate 37BRaL.

Table 6. Minimum inhibitory concentration (MIC) and Minimum bactericidal/fungicidal Concentration of EtOAc extract at different concentration.

Isolates code	MIC and MB/FC against the test organisms (mg/ml)									
	E. coli		*E. faecalis*		*S. aureus*		*P. aeruginosa*		*C. alcicans*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
30CRS	6.25	6.25	3.125	6.25	50	50	50	50	50	50
34 WRaL	25	25	50	50	-		-	-	50	50
37BRaL	12.5	12.5	12.5	12.5	50	50	-	-	25	50
68BMS	6.25	6.25	25	25	-	-	50	50	12.5	12.5
22BRaS	50	50	12.5	25	25	25	-	-	-	-

MIC: Minimum Inhibitory Concentration (MIC), MBC: Minimum Bactericidal Concentration
MFC: Minimum Fungicidal Concentration

5.5. Optimization of fungal secondary metabolite production and antimicrobial activities

5.5.1 Effect of incubation period on fungal secondary metabolite production and antimicrobial activities

Antimicrobial activity test of the crude extracts obtained from different incubation period showed effects on bioactive production and antimicrobial activities. The increase of incubation period from 8 to 13 days enhanced the production of secondary metabolite and antimicrobial activities of the two fungal isolates optimized (Table7). The highest bioactive metabolite (67mg/250 ml) with mean inhibition diameter (28.32mm against *E. coli*) was obtained from 30CRS fungal isolates followed by 54mg/250ml with 26.33 mm against *C. albicans* for 37 BRaL fungal isolate.

Table 7. Bioactive metabolite and inhibition diameter respectively at different days of incubation period

Fungal isolates and test organisms		**Effect of Incubation period on fungal bioactive metabolite production and antimicrobial activity**											
		8 days incubation		9 days incubation		10 days incubation		11days incubation		12 days incubation		13 days incubation	
		Crude extract (mg/250ml)	ID (mm)	Crude extract (mg/250 ml)	ID (mm)	Crude extract (mg/250ml)	ID (mm)	Crude extract (mg/250 ml)	ID (mm)	Crude extract (mg/250ml)	ID (mm)	Crude extract (mg/250ml)	ID (mm)
30CRS	*E. coli*	52.9	11.23	54.4	17.33	58.2	20.00	59.3	20.38		28.00	67.0	28.32
	E.faecalis		-		-		19.33		22.14		22.32		25.00
	S.aureus		-		-		-		11.28		12.00		15.00
	P.aeruginosa		-		-		-		6.00		10.00		18.00
	C.albicans		-		16.16		18.32		19.11	64.1	20.21		20.22
37BRaL	*E. coli*	38.0	12.00	41.5	12.23	44.44	18.12	48.9	20.13	50.2	20.20	54.0	22.16
	E.faecalis		-		-		13.24		15.00		17.50		17.53
	S.aureus		-		-		10 .00		14.27		17.37		18.17
	P.aeruginosa		-		-		-		-		-		-
	C.albicans		-		17.17		22.17		22.40		24.13		26.33

ID: Inhibition Diameter

Note: Results displayed are representative of the mean of triplicate at different Incubation time,-no inhibition zone.

5.5.2. Effect of pH on fungal secondary metabolite production and antimicrobial activities

Crude extracst obtained from fungal isolates grown at pH3-pH9 range exhibits antimicrobial activities for both 30CRS and 37BRaL isolates.Funga isolate 30CRS with the maximum mean inhibition value of 28.32 mm againest *E.coli* and 22.20 against *C.alcicans* was obtained at pH5 with bioactive metabolite of 73 mg/250 ml followed 20.32 mm against *S.aureus* at pH7 with 64 mg/250ml, respectively. Fungal isolate 37 BRaL was also showed strong activities at pH 5 and pH 7 (Table 8). A bioactive metabolite of 57 mg/250 ml was obtained with good activity, (25.23 mm) against *E.coli* and (22.20 mm and 22.24 mm) aganist *E. faecalis,* respectively (Table 8). Both bioactive metabolite and antimicrobial activities of optimized two isolates were considerably low at pH11.

Table 8. Effect of different pH on bioactive metabolite production and antimicrobial activity of fungal extracts

Fungal isolates and test organisms		**Effect of pH on fungal bioactive metabolite production and antimicrobial activity**									
		pH3		pH5		Ph7		pH9		pH11	
		Crude extract (mg/250ml)	ID(mm)	Crude extract (mg/250ml)	ID(mm)	Crude extract(mg/250ml)	ID(mm)	Crude extract (mg/250ml)	ID(mm)	Crude extract (mg/250ml)	ID(mm)
30CRS	*E. coli*	52	10.13	73	28.32	64	12.46	49	-		-
	E.faecalis		-		12.26		20.46		14.36		-
	S.aureus		10.16		15.16		20.32		12.52		-
	P.aeruginosa		-		15.20		-		-		-
	C.albicans		16.16		22.20		20.00		15.4	31	-
37BRaL	*E. coli*	42	-	57	25.23	57	25.23	34	-	28	-
	E.faecalis		-		22.20		22.24		-		-
	S.aureus		-		11.16		-		-		-
	P.aeruginosa		-		-		-		-		-
	C.albicans		-		24.00		26.23		-		-

ID: Inhibition Diameter

Note: Results displayed are representative of the mean of triplicate at different pH,-no inhibition zone

5.5.3. Effect of Temprature on fungal secondary metabolite production and antimicrobial activities

A result obtained from incubation temperature experiment of the current study demonstrated increase of incubation temperature from 20 to 30 ºC enhances bioactive production and antimicrobial activities of both tested fungal isolates. Contrarily, bioactive metabolite production and antimicrobial activities of the isolates decrease beyond 30 ºC incubation temperature (Table 9). The 30CRS fungal isolate showed maximum bioactive metabolite production (69 mg/250ml) with highest mean inhibition diameter 20.20 mm against *S.aureus* and 23.23 mm against *C.albicans* was recoreded at 30 ºC optimum incubation temperature whereas 37BRaL fungal isolate obtained 57 mg/250ml bioactive production with maximum inhibition diameter of 19.30 mm aganist *E.faecalis*, 22.20 mm against *S.aureus* and 29.5 against *C.albicans* at 30 °C (Table 9).

Table 9. Effect of different temperature on bioactive metabolite production and antimicrobial activities of fungal extracts

Fungal isolates and test organisms		**Effect of Temprature (°C)on fungal bioactive metabolite production and antimicrobial activity**									
		20 °C,		25 °C,		30 °C		35 °C		37 °C	
		Crude extract (mg/250ml)	ID(mm)	Crude extract (mg/250ml)	ID(mm)	Crude extract (mg/250ml)	ID(mm)	Crude extract (mg/250 ml)	ID(mm)	Crude extract (mg/250ml)	ID(mm)
30CRS	*E. coli*	46	7.16	58	19.3	69	28.20	65	22.30		-
	E.feacalis		12.00		23.13		24.20		21.3		-
	S.aures		10.00		15.00		20.20		-		-
	P.aeruginosa		-		10.2		18.00		15.3		-
	C.albicans		-		16.3		23.23		21.26	49	-
37BRaL	*E. coli*	41	-	49	20.2	57	23.20	50	9.13	42	-
	E.faecalis		-		15.5		19.30		16.26		-
	S.aureus		16.16		18.18		22.20		16.16		11.1
	P.aeruginosa		-		-		5.23		-		-
	C.albicans		-		17.36		29.50		25.76		-

ID: Inhibition Diameter

Note: Results displayed are representative of the mean of triplicate at different temprature range,-no inhibiton zone

5.5.4. Effect of carbon source on fungal secondary metabolite production and antimicrobial activities

Among the various carbon sources tested, glucose was the best carbon source for metabolite production for 37BRaL fungal isolate weight of 50 mg/250 ml. Crude extract obtained from the isolate also exhibited highest antimicrobial activity with 27.23 mm and 20.12 mm inhibition diameter against *E.coli* and *E.faecalis* respectivly. On the other hand dextrose was found to be best carbon source for bioactive production 56mg/250ml and antimicrobial activities with value of 18.00 mm with mean inhibion diameter against *P.aeruginosa* and 54mg/250ml was obtained fructose supplemented medium with antimicrobial activities 20.12 mm against *S. aureus* for 30BRS fungal isolate. Contrarily, the growth of both tested isolates was completely suppressed and there was no antimicrobial activity observed from the extracts that supplemented with sucrose (Table 10).

Table 10. Effect of different carbon sources on bioactive metabolite production and antimicrobial activities of fungal exreacts

Fungal isolates and test organisms		**Effect of Carbone source on fungal bioactive metabolite production and antimicrobial activity**									
		Glucose		Dextrose		Lactose		Fructose		Sucrose	
		Crude extract (mg/250ml)	ID(mm)	Crude extract (mg/250ml)	ID(mm)	Crude extract (mg/250ml)	ID(mm)	Crude extract (mg/250ml)	ID(mm)	Crude extract (mg/250ml)	ID(mm)
30CRS	*E. coli*	49	14.64	56	28.38	45	12.00	54	20.22		-
	E.faecalis		23.00		17.00		-		19.00		-
	S.aureus		14.00		16.00		13.26		20.12		-
	P.aeruginosa		16.22		18.22		-		-		-
	C.albicans		13.00		19.00		11.12		18.21	32	-
37BRaL	*E. coli*	50	27.23	45	21.12	42	17.42	36	15.32	28	-
	E.faecalis		20.12		15.32		13.00		12.12		-
	S.aureus		16.00		14.00		13.64		10.22		-
	P.aeruginosa		4.00		5.00		2.00		2.00		-
	C.albicans		25.62		26.20		11.00		8.00		-

ID: Inhibition Diameter

Note: Results displayed are representative of the mean of triplicate determinations i.e. 1 % carbon source supplementation,- no zone of inhibition

5.5.5. Effect of nitrogen source on fungal secondary metabolite production and antimicrobial activities

Among the nitrogen sources tested, maximum bioactive metabolites production (62 mg/250ml) and antimicrobial activity (24.00 mm) zone of inhibition against *C. albicans* was recorded for 30BRS fungal isolate grown in beef extract supplemented medium (Fig. 11). This value was greater than normal PDA basal medium. On the other hand, the optimal bioactive production (53mg/250ml) and antimicrobial activities (23.00mm) mean inhibition diameter against *E. faecalis* and (23.00 mm) against *S .aureus* was obtained with peptone supplemented medium for 37BRaL fungal isolate. This result higher than normal medium. Urea supplemented medium completely suppressed both bioactive production and antimicrobial actives for both isolates (Table 11).

Table11. Effect of different nitrogen sources on bioactive metabolite production and antimicrobial activities of fungal extracts

Fungal isolates and test organisms		**Effect of nitrogen source on fungal bioactive metabolite production and antimicrobial activity**											
		Ammonium nitrate		Sodium nitrate		Urea		Peptone		Beef extract		Yeast extract	
		Crude extract (mg/250ml)	ID(mm)	Crude extract (mg/250ml)	ID(mm)	Crude extract (mg/250ml)	ID(mm)	Crude extract (mg/250 ml)	ID(mm)	Crude extract (mg/250ml)	ID(mm)	Crude extract (mg/250ml)	ID(mm)
30CRS	*E. coli*	46	24.00	40	10.23	32	-	56	19.00		8.00	38	-
	E.faecalis		10.00		12.00		-		14.00		9.00		-
	S.aureus		13.00		8.21		-		16.00		17.24		-
	P.aeruginosa		9.00		13.00		-		17.00		-		-
	C.albicans		13.00		11.00		-		20.00	62	24.00		16.00
37BRaL	*E. coli*	48	17.10	41	9.00	24	-	53	13.32	48	17.00	43	-
	E.faecalis		12.00		16.28		-		23.00		16.00		12.00
	S.aureus		12.31		17.00		-		23.00		15.00		16.00
	P.aeruginosa		-		-		-		-		-		3.00
	C.albicans		19.00		15.00		-		10.00		22.00		10.00

ID: Inhibition Diameter Note: Results displayed are representative of the mean of triplicate determinations i.e. 1 % nitrogen source supplementation,-nozoneofinhibition

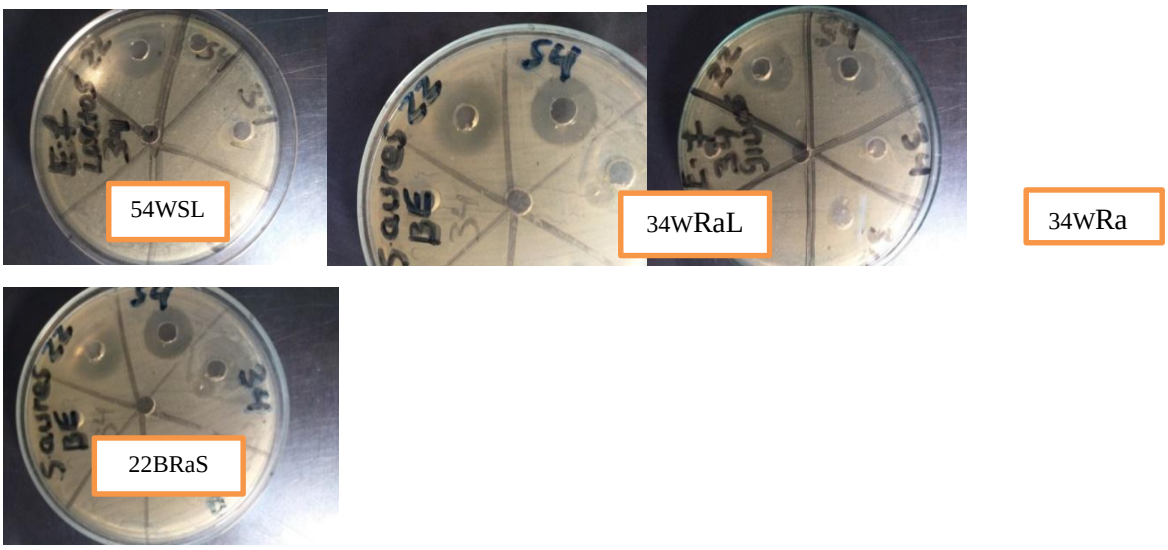


Figure 8: Positive fungal isolate for carbon and nitrogen source

5.6. Detection of secondary metabolite

In the present study out of 18 tested fungal isolates ethyl acetate extracts of 12 of them were found to be positive for at least one secondary metabolite group. Fungal isolate 37BRaL extracts contained flavonoid, phenol, cardiac glycosides and saponin (Annex 11) where as 30CRS fungal isolate extracts had only alkaloid and saponin (Table 12) (Fig. 9).

Table 12. Phytochemical analysis of endophytic and rhizospheric soil fungi

Fungal Extract	Alkaloid	Flavinoid	Phenol	Tanin	Cardiac glycosides	Steroid	Saponin	Terphenoid
34WRaL	-	-	-	-	-	-	+++	++
22 BRaS	-	+	-	-	-	+++	-	+++
54 WSL	+	-	-	-	-	+++	-	++
34 BSSoil	-	-	-	-	-	++	-	+++
30CRS	++	-	-	-	-	-	+++	-
37BRaL	-	+++	+++	-	+++		++	-
1 BraS	-	-	-	+	-	+++	+	+++
10WRaS	-	-	-	-	-	+++	-	++
15BRaS	-	++	-	+	-	-	-	++
41WRaS	-	-	-	-	-	+++	-	-
68BMS	-	-	-	-	-	+++	++	-
67BMSoil	-	-	-	-	-	-	-	-
74WSS	-	-	-	-	-	-	-	-
75WRaR	-	-	-	-	-	-	-	-
78BRaR	-	-	-	-	-	-	-	-
72BML	-	-	-	-	-	+	-	-
63BML	-	-	-	-	-	-	-	-
28BML	-	-	-	-	-	-	-	-

Note: +++, Potent activity, ++, Moderate activity, +, Less activity,-, No activity

Alkaloid Flavonoids Saponine Tanine

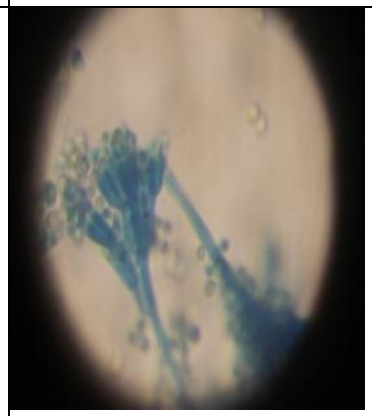
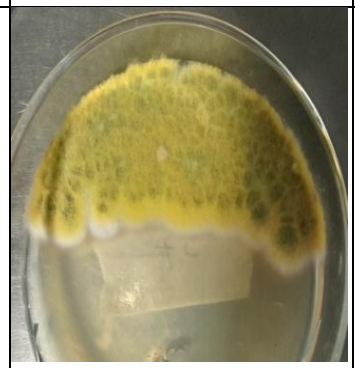
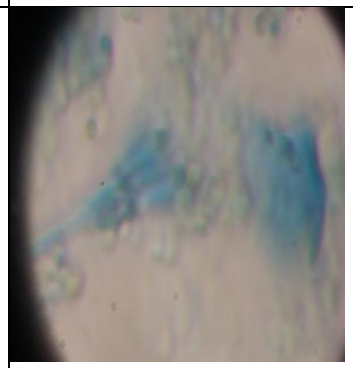
Figure 9. Posetive secondary metabolite analysis of endophytic and rhizospheric fungi

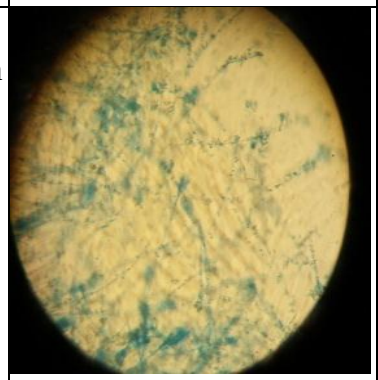
5.7. Identification of the fungal isolates

Morphotyping and biochemical tests of the five top fungal isolate with antimicrobial activities assigned them into *Penicillium* sp., *Talaramycetes* sp., *Aspergillu*s sp and *Trichoderma* sp. (Table 13). BioLog ID system was identified two fungal isolates while the remaing three isolates were not identified. Fungal isolate 30CRS was identified as *Pencillium simplicissimum* while fungal isolate 37BRaL was identified as *Talaromyces flavus var flavus* with similarty of 99.8% and 56.46%, respectively. The Biolog analysis of *P. simplicissimum* and *T. flavus var flavus* also analyzes fungal growth via turbidimetric analysis. The turbidities of *P. simplicissimum* were significantly high in wells containing terbidity N-Acetyl-D Glucosamine, Adonitol, L-Arabinose, Arbutin, D-Cellobiose, i-Erythritol, D-Fructose, α-D-Glucose, α-D-Lactose, Lactulose, Maltitol, Maltose, Maltotriose, D-Mannitol, D-Mannose, D-Melezitose, D-Melibiose, α-Methyl-D Galactoside, α-Methyl-DGlucoside, β-Methyl-DGlucoside, Palatinose, D-Raffinose, L-Rhamnose, D-Ribose, L-Sorbose, Stachyose, Sucrose, D-Trehalose, Turanose, Xylitol, D-Xylose those shows that fungal species has ability to utilize and oxidize different carbone sources (Annex 14). These groups of carbon sources are mainly catagorised as carbohydrates, polymer such as (Dextrin), Miscellaneous (Glycerol) and Amines/amides (D-Glucosamine).

The other identified species *T.flavus var flavus*, also utilized and oxidized different groups of carbohydrates such as Adonitol, D-Cellobiose, i-Erythritol, D-Fructose, Gentiobiose, α-D-Glucose, Maltotriose, D-Melezitose, L-Rhamnose, Sedoheptulosan, Sucrose, Turanose, Xylitol etc (Table 14).

Table 13. Characterization and identification of efficient fungal isolates

Isolatecode	**Morphological characterstic**				**Species identification**	
	Fungi in culture plate	Macroscopic characteristics	Fungi under microscopic	Microscopic characterstic	Morphological identification	Identity using Biolog
30 C*RS*		green colour colonies later turned in to cream colour, flamentus mediume colony size,circular form/shape and flate elevation,		conidiopohores with metulae and phialides and the phialidesbranched. Conidia were budded from the phialides and were oval shape medium size ,blue color arranged in chains. The conidia were ellipsoidal in shape and the walls were smooth.	*Penicillium*	*Penicillium simplicissimum* (oudemans)Thom BGA
37 BRaL				Conidiospore branching Monovalent,phialides branched,condia	*Talaramyces*	*Talaromyces flavus var flavus*(Klocker) Stolk &Samson

34WRaL		gray color colonies, medium size, raised elevation and filamentous form		Conidia were globose and Phialides were flask shaped	*Trhichoderma*	Unidentified

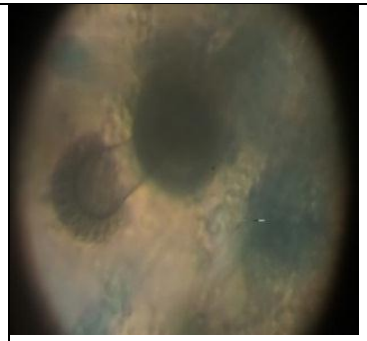
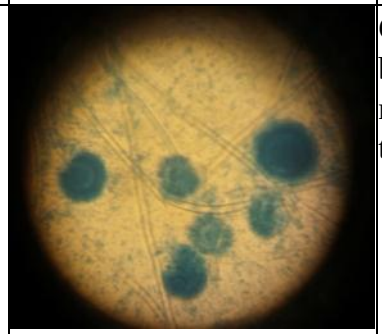
22BRaS		graycolor colonies,medium size, raised elevation and filamentous form		Smooth and colorless conidiophores and spores. Biseriate phialides	*Aspergilus*	Unidentified
68BMS		Black colony color, large colony size, filamentous form,circular shape		Conidiacircular, branchedphialides,smoothconidiophoresandspores.Biseriatephialides	*Aspergillus*	Unidentified

Table14. Metabolic profile of *P.simplicissimum* and *T.flavus var flavus* using Biolog FF Microplate

Different carbone source utilized by endophytic fungi	**Identified fungal species**		**Different carbon source utilized by endophytic fungi**	**Identified fungal species**	
	P.simplicissimum	*T.flavus var flavus*		*P.simpliciss imum*	*T.flavus var flavus*
Water	-	-	D-Ribose	+	+
Tween 80	-	-	Salicin	+	+
N-Acetyl-DGalactosamine	-	-	Sedoheptulosan	-	+
N-Acetyl-DGlucosamine	+	+	D-Sorbitol	+	+
N-Acetyl-DMannosamine	-	-	L-Sorbose	+	+
Adonitol	-	-	Stachyose	+	+
Amygdalin	+	-	Sucrose	+	+
D-Arabinose	+	-	D-Tagatose	-	-
L-Arabinose	-	+	D-Trehalose	+	+
D-Arabitol	-	+	Turanose	+	+
Arbutin	+	+	Xylitol	+	+
D-Cellobiose	-	+	D-Xylose	+	+
α-Cyclodextrin	-	-	γ-Amino-butyric Acid	-	+
β-Cyclodextrin	-	-	Bromosuccinic Acid	+	+
Dextrin	+	+	Fumaric Acid	+	+
i-Erythritol	-	+	β-Hydroxy-butyric Acid	+	+
D-Fructose	-	+	γ-Hydroxy-butyric Acid	-	-
L-Fucose	-	-	p-Hydroxyphenylacetic Acid	+	+

D-Galactose	-	+	α-Keto-glutaric Acid	+	+
D-Galacturonic Acid	-	-	D-Lactic Acid Methyl Ester	-	-
Gentiobiose	-	+	L-Lactic Acid	+	
D-Gluconic Acid	+	+	D-Malic Acid	+	+
D-Glucosamine	-	+	L-Malic Acid	+	+
α-D-Glucose	-	+	Quinic Acid	+	+
Glucose-1- Phosphate	+	+	D-Saccharic Acid	+	+
Glucuronamide	-	-	Sebacic Acid	+	+

Note: + = Utilize carbone, - = No utilize carbon

Table 14. Metabolic profile of *P.simplicissimum* and *T.flavus var flavus* using Biolog FF Microplate

Different carbone source utilized by endophytic fungi	**Identified fungal species**		**Different carbon source utilized by endophytic fungi**	**Identified fungal species**	
	P.simplicissimum	*T.flavus var flavus*		*P.simplicissimum*	*T.flavus var flavus*
D-Glucuronic Acid	+	+	Succinamic Acid	+	-
Glycerol	-	+	Succinic Acid	+	+
Glycogen	+	+	Succinic Acid Mono-Methyl Ester	+	+
m-Inositol	-	-	N-Acetly-LGlutamic Acid	-	
2-Keto-D-Gluconic Acid	+	+	Alaninamide	+	-
α-D-Lactose	-	+	L-Alanine	+	+
Lactulose	-	+	L-Alanyl-Glycine	+	+
Maltitol	-	-	L-Asparagine	+	+
Maltose	-	+	L-Aspartic Acid	+	-
Maltotriose	-	+	L-Glutamic Acid	+	+
D-Mannitol	-	+	Glycyl-L-Glutamic Acid	+	+
D-Mannose	-	+	L-Ornithine	-	+
D-Melezitose	-	+	L-Phenylalanine	+	+
D-Melibiose	-	+	L-Proline	+	+
α-Methyl-DGalactoside	+	-	L-Pyroglutamic Acid	+	+
β-Methyl-DGalactoside	+	+	L-Serine	+	+
α-Methyl-DGlucoside	+	-	L-Threonine	+	-
β-Methyl-DGlucoside	-	+	2-Amino Ethanol	+	-

Palatinose	+	+	Putrescine	+	+
D-Psicose	-	-	Adenosin	+	-
D-Raffinose	-	+	Uridine	-	-
L-Rhamnose	-	+	Adenosine-5'-Monophosphate	+	-

Note: + = Utilize carbone, - = No utilize carbon

6. DISCUSSION

The current study demonstrated that a single plant part colonized by more than one culturable endophytic fungi. Similarly, previous studies have demonstrated that one species of plant can be inhabited by various groups of fungi (Ilyas, 2009). Corresponding to the current study, 30 CRS isolate later identified as *Pencillium simplicissimum* fungal species was reported from some other medicinal plants such as the root of *Alnus glutinosa* (Fisher *et al.*, 1991), twing of *Eucalyptusnitens* (Fisher *et al*., 1993) stem of *Melia azadarach* (Geris dos Santos, 2003), and roots of *Panaxginseng* (Hao, 2013). Similarly, isolate 37 BRaL identified as *Talaromyces flvus var flavus* fungal species was previously retrieved from leaves of *Sonneratia apetala* (Li *et al.*, 2011). Our result is differ from previous, the community and distribution of endophytes are based on plant species, host developmental stage and environmental stage.

In this study, a total of 266 rhizospheric fungal isolates were isolated from five different plant species. According to (Nisha, 2017) mainly antibiotic-producing fungi are isolated from soil such as *Aspergillus* sp., *Fusarium* sp., and *Penicillium* sp from *Azadirachta Indica* Plant. It also depends on the nature of the environment and the texture of the soil. The large population of the microbes is found in the rhizospheric region of the plants comparing to the bulk soil due to the secretion of organic nutrients from the roots of the plants that favor the growth and development of the microbes in the rhizosphere (Nisha, 2017). Also, rhizosphere microbes play a very important role in improving the medicinal values of plants. Rhizospheric microbes affect plant physiology by imparting several useful effects such as nitrogen fixation, nutrient uptake, and production of secondary metabolites in the medicinal and aromatic plants (Guo *et al*., 2006; Raha and Shagufta, 2019).

A large number of fungal isolates exhibited antimicrobial properties against tested clinical and standard strains of human pathogenic microorganisms. During secondary screening, five fungal extracts having antimicrobial properties were selected against more than two clinical test organism with a higher inhibition zone. This indicates *P.simplicissimum* crude extract of the current study has broad-spectrum antimicrobial activities and has a different mechanism of action toward gram positive, gram-negative, and yeast (Table 4). Previous research conducted in Malysia by Tong woei yen (2014) reported that endophytic fungi *penicillium minioluteum* has showed 17.3$\pm$ 1.2 mm zone of inhibition against *S. aureus,* 5.7±1.2 mm zone of inhibition against *E. coli,* and 17.3

± 1.5 mm zone of inhibition against *P.aeruginosa* and did not inhibit *C.albicans* at 50 mg/mL concentration. This difference might be two fungal species categories under the same genus but different species and have different biosynthesis gene clusters for the production of secondary metabolites.

Bibin *et al.* (2016) reported that the extract from *T. flavus* SP5 was found to be more active against various human pathogens at 10 g/100ml of the biomass of ethanol extract, *E. coli* ATCC 52922 (18.3 ± 0.3mm), *E. feacalis* ATCC 29212(14.2 ± 0.7mm), *P.aeruginosa* ATCC 27853 (17.8 ± 0.1mm) and *C. albicans* ATCC 90028 (15.7 ± 0.7mm). These results revealed that the extract from isolated fungus *T. flavus* SP5 acted as a potent antibacterial and antifungal activity. The current result is higher inhibition zone than that of the previous finding on *E.coli*, *E.faecalis* and *C.albicans* but previous finding higher against *P.aeruginosa.* This difference may be due to the application of different concentrations during antimicrobial assay and previous studies used ethanol solvent for extraction. Besides, test organisms that were used in antimicrobial assay were from a different source.

The MIC of 30CRS fungal isolate (*P.simplicissimun*) extracts 3.125 mg/ml against clinical *E. faecalis* followed 6.25mg/ml against clinical *E.coli* was observed than the other clinical human pathogenic tested organisms and 12.5 mg/ml concentration against clinical *E. coli* and *E. feacalis* by 37BRaL later identified as *T. flavus var flavus* (Table 6). Both identified fungal species are poorly studied for their antimicrobial activities with minimum inhibitory concentration. Even though, the mean MIC of the current study exhibited from *P.simplicissimum* is higher than the study conducted by Amina *et al.*(2018) crude ethyl acetate extract of *P. griseofulvum,* who investigated with less MIC of 50 µg/ ml for (*E. coli* ATCC 25922) and 100 µg/ ml for (*S. aureus* ATCC 25923). Akanksha (2015) who reported that *Penicillium frequentans* against less MIC *C.albicans* at 10 mg/ml. On the other hand, *T.flavus var flavus* when compared with the previous research finding made by Fang *et al.*(2012) using *Talaromyces verruculosus* with MIC at 15.6 µg/ ml against *C. albicans.* At concentration 2.5 µg/ ml and 5.0 µg/ ml, against *S.aureus* and *E.coli* respectively. However, the variation observed might be due to differences in secondary metabolite production among compared fungal species. Furthermore, the isolated resistant pathogens of this study might differ with the above-tested pathogens and its wide range of MIC values indicates that

the different susptablility levels of test organisms for a fungal extract which showed a difference in the result.

The mean MBC/MFC of fungal isolates collected from different plant sources against the resistant tested microbes is indicated (Table 6).There was no visible colony observed against *E. coli* and *E. feacalis* at 6.25 mg/ml of fungi extract concentration by isolate 30CRS (*P. simplicissimum*), might kill by less concentration compared with others and isolate 37BRaL (*T. flavus var flavus*) also has MBC in *E. coli* and *E. feacalis* at 12.5 mg/ml was obtained. *P. aeroginosa* was resistant to the tested 37BRaL isolates at given concentrations. *P. aeruginosa* make resistance in different mechanisms intrinsic, acquired and adaptive resistance. The intrinsic resistance of *P. aeruginosa* includes low outer membrane permeability, expression of efflux pumps that expel antibiotics out of the cell and the production of antibiotic-inactivating enzymes. The acquired resistance of *P. aeruginosa* can be achieved by either horizontal transfer of resistance genes or mutational changes The adaptive resistance of *P. aeruginosa* involves formation of biofilm ,where the biofilm serves as a diffusion barrier to limit antibiotic compounds access to the bacterial cells (Zheng *et al*., 2019).

The incubation pariod results of this study indicated that the incubation period affected both metabolite production and antimicrobial activities of both *P. simplicissimum* and *T. flavus var flavus* fungal species. Thirteen days of incubation was found to be an optimum growth period for attaining maximum secondary metabolite production and higher antimicrobial metabolite activity. These disagree with *P. chrysogenum* was the highest antimicrobial activity was achieved from 8 days with inhibition zone diameter of 33 mm for *S. aureus* and this continued till the fourteenth day (Mohammed *et al*., 2015). Therefore, the biosynthesis of the secondary metabolite is directly related to cultural conditions (Demain, 1999). Secondary metabolite production takes place is usually when growth is limited by the exhaustion of one key nutrient such as carbon or nitrogen when time is increased or unoptimal environmental conditions (Vaishnav and Demain, 2010).

Some studies indicated that pH is a significant factor when optimizing conditions for increasing metabolites during the fermentation phase (Thongwai and Kunopakarn, 2007). Fungi digest and release metabolic products into their environments. pH effects microbial metabolisms in different ways. It affects the environmental conditions that are important to microbial growth and survival,

pH describes the chemical activity of protons, a key player in redox reactions and mineral dissolution. These reactions determine the salinity and composition of aqueous solutions and control the bioavailability of nutrients and trace elements. In this study during pH optimization, two selected isolates (later identified as *P. simplicisimum* and *T. flavus var flavus*) were grown well at pH 3-9 and the maximum antimicrobial activity was obtained at pH 5 for species *P. simplicissimum* (28.32 mm) against *E.coli* followed (20.32 mm) against *S.aures* at pH 7,respectively (Table7). Phan *et al*., (2016) *Penicillium chrysogenum* also exhibited antimicrobial activity in the medium at pH7, 28 mm against *S. aureus*, 14 mm against *P.aeruginosa* and 12mm against *E.coli*. The current result is higher than the previous finding inhibiting *E.coli*. On the other hand, *T. flavus var flavus* showed strong inhibition at pH 5 and pH 7, (22.2mm) against *E.fecalis* and (25.23mm) against *E.coli.* These results are slightly similar to those reported by Zannatual *et al*. (2017) the optimum pH for growth of *T.verruculosus* was found to be 6.5 for secondary metabolite production.

In the present study, *P. simplicissimum* was grown well at 30˚C and showed remarkable activity against *S.aureus* and *C.albicans* (Table 8). In another study, *P. marneffei* was grown well at different temperatures with optimum growth range at a temperature of 28 ^{o}C (Cunwei, 2007) in China. Also, the fungus *T.flavus var flavus* grow best when the temperature was maintained at 30°C as noted elsewhere (Goyari *et al*., 2014). According to the report of Bhattacharyya and Jha, (2011), *T. verruca* grew luxuriously at a temperature of 30°C. Temperature is an important parameter that influences the growth and development of the microorganisms to produce the required metabolites (Sujana, 2013). The growth and development of the microorganisms could be decreased at much higher as well as much lower temperatures.

Carbon source optimization of the current study reveals that culture media supplemented with different carbon sources were influenced the cell growth and biosynthesis of a metabolite of both tested fungal species. According to Nisha, (2017) the maximum activity was shown by strain *Aspergillus niger* in the broth having glucose with a maximum zone of growth inhibition (20 mm) against *E. coli*. Simple sugar such as glucose and fructose enhance growth as well as secondary metabolite production by microorganisms compared to complex carbon sources like starch. Because filamentous fungi can utilize a great variety of carbon sources of secreting a wide range of different enzymes. Therefore, fungi appear to have a higher affinity with several known

transporters capable of transporting the simple sugar into the cell for subsequent phosphorylation and conversion into mainly biomass and CO_2 In the case of sucrose, the process involved extracellular sucrose breakdown (invertase) to hexose genes are inactivated (Mchunu *et al*., 2013).

The tested fungal isolates produced antimicrobial compounds with most of the nitrogen sources. The inhibition zone showed at 24.00 mm against *C. albicans* with beef extract supplements as nitrogen sources on *P.simplicissimum.* According to Pranay and Shaffali, (2012) the maximum production of antimicrobial metabolite in *Penicillium* species was observed in the presence of 2% sodium nitrate and displayed 28±0.81mm against *S.aureus*, 28±0.54mm against *E.coli,* 26±0.54mm inhibit *P.aeruginosa* and 28±0.57mm inhibit *C.albicans. T. flavus flavus var flavus* also exhibited the maximum antimicrobial properties with inhibition zone 23.00 mm against *E. faecalis* and 23.00 mm against *S. aureus* were using peptone supplemented medium (Table10). Previous studies by Nisha (2017) on culture filtrate in medium supplemented with peptone showed the zone of growth inhibition of 25.00 mm diameter against *E.coli* by *Aspergillus niger.* (Peighamy-Ashnaei *et al*., 2007) reported that nitrogen sources have an important role in maximizing the growth rate of the fungal strains, biosynthesis of product formation, and expression of genes required for the use of various secondary nitrogen sources of the regulatory mechanism (Yun *et al*., 2008). Filamentous fungi able to use nitrogen sources, GATA transcription factors are the central regulators of nitrogen assimilation as they globally activate the expression of permease and catabolic enzyme. Genes encoding required degrading most complex nitrogenous compounds (Magasanik and Kaiser, 2002). *Ure* gene-encoded urease, the only enzyme of the pathway that has been the utilization of urea. In our case Urease enzyme might be absent in the organisms that cannot be utilized urea as a nitrogen source.

According to Lai (2010) endophytes and rhizospheric fungi have shown the presence of different secondary metabolites profiles viz alkaloids, steroids, phenolic and flavonoids and are known to possess strong antimicrobial activities. They often have unusual structures and their formation is regulated by nutrients, growth rate, enzyme inactivation, and enzyme induction (Suni, 2009) .The result of *T. flavus var flavus* following the previous research report conducted by Ming and Zhai (2016) has a remarkable potential for its secondary metabolites with unique biological activities and is the commonest species of the genus *Talaromyces* (Bohumil, 2010). All of the natural products extracted from the identified fungal species are contained flavinoid, cardiac glycosides,

phenol, and saponin, which have an important role in antimicrobial activity (Chandrappa *et al*., 2013). On the other hand, *P. simplicissimum* was positive for alkaloid and saponin production (Table12). Akanksha *et al*. (2015) reported that a study on *Penicillium frequentans* showed alkaloid, saponin, flavinoid, phenol, taninns, terphenoid and steroids and were major secondary constituents of the crude extracts. In addition Fill *et al*. (2009) reported that *P. brasilianum* produces alkaloid-nature metabolites, Tan and Zou, (2001) also reported that alkaloid was produced from grass endophytic *Penicillium* species. The presence of such active biomolecules in fungi is a good indicator for producing industrial level antimicrobial metabolites.

The characterization and identification using morphological and biochemical fungal identification systems were carried out using a light microscope and Biolog ID system. The two isolates (30CRS and 37BLaS) which were identified as genus *Pencillium* and *Talaromyces* respectively using microscopic methods were also identified as *P.simpicissimum* and *T.flavus var flavus* respectively by Biolog fungal identification system. This indicated morphological characterization support Bolog identification systems which give similar results at the genus level. Such morphological and Biolog identification similarity of the genus *pencillium* were corresponding to the similar previous study result (Suhaila *et al*., 2018). Suhaila and her colleagues were also found similar identification result of microscopic and the Biolog ID system with the molecular identification using ITS for *P.oxalicum*. The Biolog identification method had also been used to characterize the physiology and metabolism of certain endophytic fungi (Papaspyridi *et al*., 2011). Biolog FF Microplate has the ability of the microorganism to assimilate or oxidize compounds from a pre-inoculated panel of different carbon sources. The Biolog FF Microplates analysis was demonstrated that *P.simplicissimum* was utilized different carbon compounds including carbohydrates, amines/amides, polymer, and miscellaneous. *T.flavus var flavus* also utilize carbohydrates (Annex 14). Therefore, the strength of Biolog Microplates is available containing different substrates for which have a single table to allow direct and rapid comparison of the substrates assimilation (Juliet, 2002). Also, the substrate assimilation fingerprint obtained from the Biolog FF Microplate analysis is useful in selecting components for media optimization of maximum biomass production in vitro condition. The remaining unidentified isolates might be novel species (not found in the Biolog database) which did not utilize different carbon sources that tagged into Biolog microplates.

7. CONCLUSIONS AND RECOMMENDATIONS

This research has confirmed the essential role of endophytic and rhizospheric fungal species that could exhibit antimicrobial activities against resistant clinical and reference human pathogenic microbes. Fungi associated with some medicinal plants as endophytes and/or rhizospheric organisms could be an excellent source of diverse biologically active compounds with pharmaceutical importance. The ethyl acetate extracts of *P.simplicissimum* isolated from the stem of *R. nervesus* and *T.flavus var flavus* isolated from the leaf of *R.abbysinicus* exhibited that the stronger antibacterial potential against resistant test organisms such as *S. aureus* and *E. fecalis*, *E. coli* and *P. aeruginosa* and *C. albicans* using plug agar and well diffusion method. *E. coli, E. faecalis* and *S. aureus* was more susceptible to the ethyl extracted of *P. simplicissimum* wereas *S. aureus* and *C.albicans* more susceptible to the ethyl extracted from *T. flavus var flavus* species based on the mean inhibition diameter comparing to the other test organisms. The overall mean of minimum inhibitory concentration and minimum bactericidal or fungicidal concentration was 3.125 mg/ml and 6.25 mg/ml concentration to inhibit *E. feacalis* by *P. simplicissimum*. From the result, the mean least 12.5 mg/ml concentration to inhibit and kill was recorded against *E. coli*, and *E. feacalis* is compared to the tested pathogenic organism by *T. flavus var flavus* ethyl acetate extract. Secondary metabolite profiling tests of the current 18 selected medicinal plant associated endophytes and rhizospheric fungi revealed the presence of major secondary metabolites that include alkaloids, flavonoids, phenol, glycosides, terpnoids, steroid, tanine and saponins.

The results from the antimicrobial properties of endophytic and rhizospheric fungi associated with some medicinal plants of Ethiopia generated the following plausible recommendations:

1. This study found that medicinal plant associated endophytic fungi is very important as a source of the antimicrobial compound. Therefore, intensive isolation, screening, and evaluation of fungi from other medicinal plants are highly important.

2. It can be recommended that there is the need to conduct detailed research works on the characterization of the endophytic fungal species which are associated with different *R. nervesous* and *R. abyssinicus* genotypes/ populations on potential antimicrobial secondary metabolite using NMRI and chromatography techniques. Moreover, further

characterization and ingredient identifications of the crude extracts *P. simplicissimum* and *T. flavus var flavus* is recommended.

3. Thre are different antibiotics that have a different mode of action. Therefor; it is recommended that studies of the mode of action for a fungal metabolite of current isolates that have antimicrobial activities are important.

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9. APPENDIX

Table15: Plant sample collection site with GPS data

Zone	Woredas	Latitude	Longitude	Altitudes
Bale	Angaso	6° 57' 14" N	39° 33' 24" E	2592
		6° 57' 16" N	39° 57' 40" E	2600
		6° 57' 20" N	39° 56' 40" E	2600
		6° 58' 43" N	39° 57' 47" E	2605
		7° 02' 18" N	40° 04' 60" E	2680
		7° 06' 45" N	40° 07' 60" E	2694
		7° 07' 11" N	40° 09' 60" E	2694
		7° 08' 26" N	40° 11' 60" E	2700
		7° 10' 18" N	40° 14' 60" E	2743
West Arsi	Urji korbrcha	7° 05' 33" N	38° 22' 41" E	1877
		7° 05' 43" N	38° 22' 41" E	1877
		7° 07' 10"N	38° 22' 47" E	1885
	Hassawe	7° 10' 48" N	38° 23' 38" E	1894
		7° 10' 05" N	38° 23' 20" E	1890
		7° 11' 49" N	38° 37' 22" E	1925
		7° 11' 60" N	38° 38' 03" E	1937
Chancho Special	koreroba	9° 15' 59" N	38° 45' 15" E	2555
		9° 15' 59" N	38° 45' 54" E	2565
		9° 15' 60" N	38° 45' 55" E	2565
		9° 16' 43" N	38° 45' 20" E	2560
		9° 16' 45" N	38° 45' 26" E	2558
		9° 16' 46" N	38° 46' 29" E	2580
		9° 17' 46" N	38° 46' 24" E	2578

9° 17' 48" N	38° 46' 37" E	2580
9° 18' 42" N	38° 47' 04" E	2594
9° 18' 59" N	38° 47' 15" E	2600

Annex 1: Plant and rhizospher soil samples collection areas

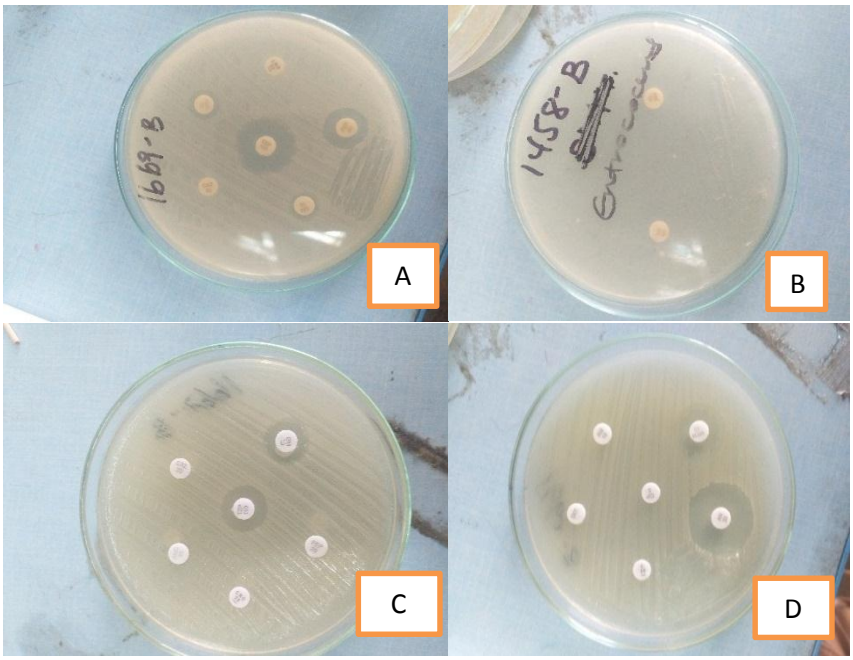
Annex 2: Surface sterilization

A: indicates segmentation of *Rumex nervosus* steam sample

B : indicates segmentation of *Mysrine africana* leaf sample

C: indicates transfer of root segmentaton of *Rumex abysinicus* on Potato dextrose agar medium

Annex 3: Fragmentation of plant parts and transfer of plant fragments in solid media



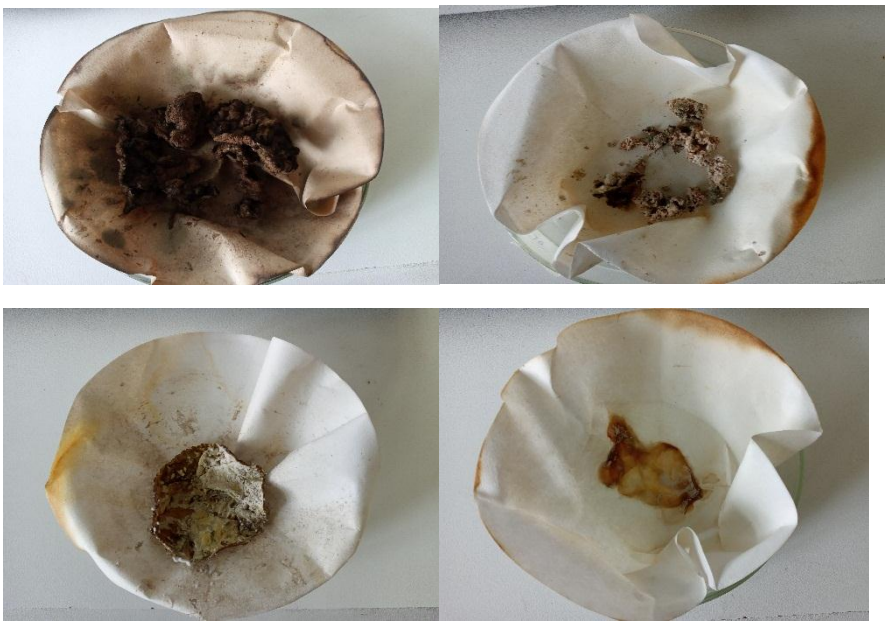
Annex 4: Resistant bactrial isolates taken from patient visiting Tikur Anbessa Specialized Hospital

A: indicates *S.aureures* strain isolated from pecient resistant for Cefoxitin, Erythromycin ,Clindamycin ,Nitrofurantoin and Gentamicin

B: indicates *E.faecalis* strain isolated from pecient resistant for Ampicilin and Vancomycin

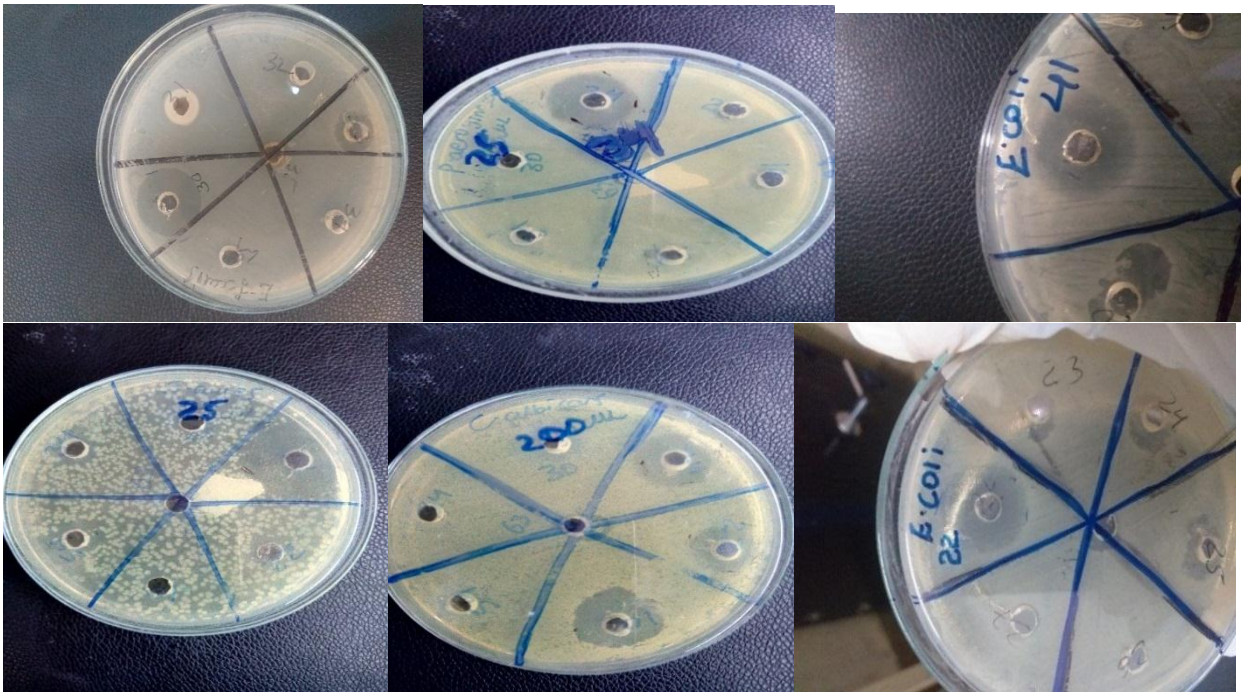
C: indicates *E.coli* strain isolated from pecient resistant Ceftazidim, , Amikacin Ciprofloxacin, Norfloxacin and Tetracycline

D: indicates *E.coli* strain isolated from pecient resistant Ampicilin, Gentamicin, Tobramycin, Amikacin, Cefazolin



Annex 5: Production of fungal biomass

Annex indicates fungi biomass that are grown in potato dextrose broth media there after, filtrated by using Whatman filter paper No1 finally dried at 40°C for 7 days by using heat driyer



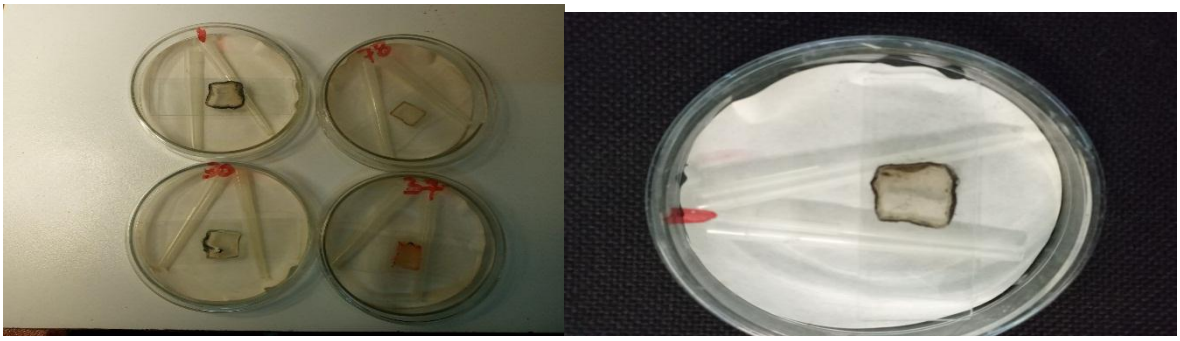
Annex 6: Positive result during secondary screening test by well diffusion method

Annex indicates result obtained from ethyl acetate crude extract of different fungi isolates against resistant clinical *E.coli, P.aeruginosa, C.albicans E.feacalis* and *P.aruginosa* at 50mg/ml

Annex 7: Inhibition zone of crude extract at different carbone and nitrogen source

Annex indicates result obtained from fungi grown in carbon and nitrogen source such as fructose and beef extract at 50 mg/ml against *E.feacalis* and *S.aures*Annex 9: Detection of secondarymetabolite

Annex result obtained from different fungi crude ethylacetate extract by using qualitative approach such as Alkaloid, Flavonoids, Saponine and Tanine



Annex 8: Slide culture techniques for microscopic characterization

Preparation of fungi isolates in order to microscopic characterization to determine conidia and conidospore by using stainig dye lactophenol blue by slide culture techniques