ANTIMICROBIAL PROPERTIES OF ENDOPHYTIC AND RHIZOSPHERIC FUNGAL SPECIES ASSOCIATED WITH SOME MEDICINAL PLANTS

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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 across the globe. Therefore, the objective of this study was to isolate and identify the antimicrobial properties of endophytic and rhizospheric fungi associated with some medicinal plants. A total of 150 plant parts and 50 soil samples were collected from five medicinal plants. In vitro antimicrobial activities were tested against common resistant pathogens (*Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeroginosa* and *Candida albicans*). A total of 582 fungal isolates were obtained. Accordingly, 78 (19.89%) isolates displayed antimicrobial activities by agar plug diffusion method. Ethyl acetate extracts of isolate 30CRS showed higher significant ($p \leq 0.001$) inhibition zones against *E. coli* (30.33 ± 0.57 mm), *E. faecalis* (25.33 ± 0.28 mm) and *S. aureus* (18.33 ± 0.57 mm) than the positive control, whereas fungal isolate 37BRaL showed significantly higher ($p \leq 0.001$) inhibition zone against *S. aureus* (19.16 ± 0.28 mm) and *C. albicans* (26.83 ± 0.76 mm). The mean minimum inhibitory concentration (MIC) result was 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 *C. albicans*. The minimum bactericidal concentration ranged from 6.25 – 50 mg/ml, while the minimum fungicidal concentration ranged from 12.5 – 50 mg/ml. The potent isolates (30CRS) and (37BRaL) were identified as *Penicillium simplicissimum* and *Talaramycetes flavus* var. *flavus* using Biolog microbial identification system. This research has confirmed the potential of endophytic and rhizospheric fungal species against resistant clinical isolates.

Keywords: Endophytic fungi, Medicinal plant, Multidrug-resistant, Rhizospheric fungi.

INTRODUCTION

Plants have served as a source of medicinal bioactive compounds against numerous forms of diseases for centuries. In recent years, rather than plants themselves, microorganisms associated with plants 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 and have exhibited importance in antagonizing human pathogens (Singh and Dubey, 2015).

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 against human pathogens. Secondary metabolites such as alkaloids, phenols, terpenoids, and steroids play an important role as potential candidates for therapeutic compounds (Pandey and Malviya, 2014).

Diverse microbial populations also inhabit the rhizosphere region of many plants and they principally comprise fungal and bacterial species. In plants, organic materials from the 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). Different compounds secreted by plant roots into the rhizosphere serve as a source of energy and precursors of many metabolites produced by associated microorganisms (Solaiman and Anawar, 2015).

Antimicrobial agents are synthesized from microorganisms, plants, and animal products and are used to treat microbial diseases (Alkhyat and Al-Maqtari, 2014). However, currently, the development of multidrug-resistant human pathogenic microorganisms and the emergence of new diseases are the most challenging problems in the public healthcare system and a major challenge in Ethiopia (Reta et al., 2019). The development of resistant pathogenic bacteria against commonly used antibiotics due to their misuse and overuse in developing countries like Ethiopia is becoming a serious health problem in a hospital setting (Moges et al., 2014). Furthermore, the lack and high cost of new-generation drugs have escalated infection-related morbidity, mortality, losses in productivity, and affected the economy (Mulu et al., 2006). Infections caused by resistant bacteria also adversely affect treatment outcomes, treatment costs, disease spread, and prolonged illness (Moges et al., 2014). Therefore, these problems have prompted the need to search for new drugs with better efficacy from endophytes and rhizospheric microbes against drug-resistant pathogenic

microorganisms as well as for the better treatment of newly emerging diseases (Liang et al., 2012). There are a number of studies on medicinal plants and isolated endophytic fungi with antimicrobial activities which include, *Solanum incanum* (David et al., 2021), *Aloe vera* (Fuad et al., 2021), *Rumex abyssinicus* (Shifa, 2020), *Rumex nervosus* (Asma et al., 2022) and *Myrsine africana* (Hina et al., 2021). However, in Ethiopia, such studies are lacking. Thus, this research was aimed at isolating and identifying medicinal plant-associated fungi from five selected medicinal plants of Ethiopia (*Solanum incanum, Aloe vera, Rumex abyssinicus, Rumex nervosus* and *Myrsine africana* to evaluate their antimicrobial activities.

MATERIALS AND METHODS

Description of the study areas

The samples were collected from Bale, West Arsi, and Chancho Special Zones, Oromia Regional State (Figure 1). The samples were collected from Riverian Fasil Angeso natural forest and the surrounding grazing land in Bale Zone. The GPS coordinates of the sampling site were latitude 6°57'43" to 6°61'14"N and longitude 39°56'60" to 39°57'40"E and with an elevation of 1032 to 1080 masl.

West Arsi Zone is found in the central part of the Oromia National Regional State. GPS coordinates of the sampling site were Latitude 7°05'33" to 7°11'60.00"N and Longitude 38°22'41" to 38°38'03"E and an elevation 1877 to 1937 masl. The sampling sites covered grazing and farming land borderline.

Chancho, Oromia Special Zone, is another sample collection site of the current study. It is located 45 km north of Addis Ababa on the road to Gojam with an elevation of 2555 to 2600 masl. GPS coordinates of the sampling site were 9°15'59" to 9°18'59"N and 38°45'15" to 38°47'15"E.

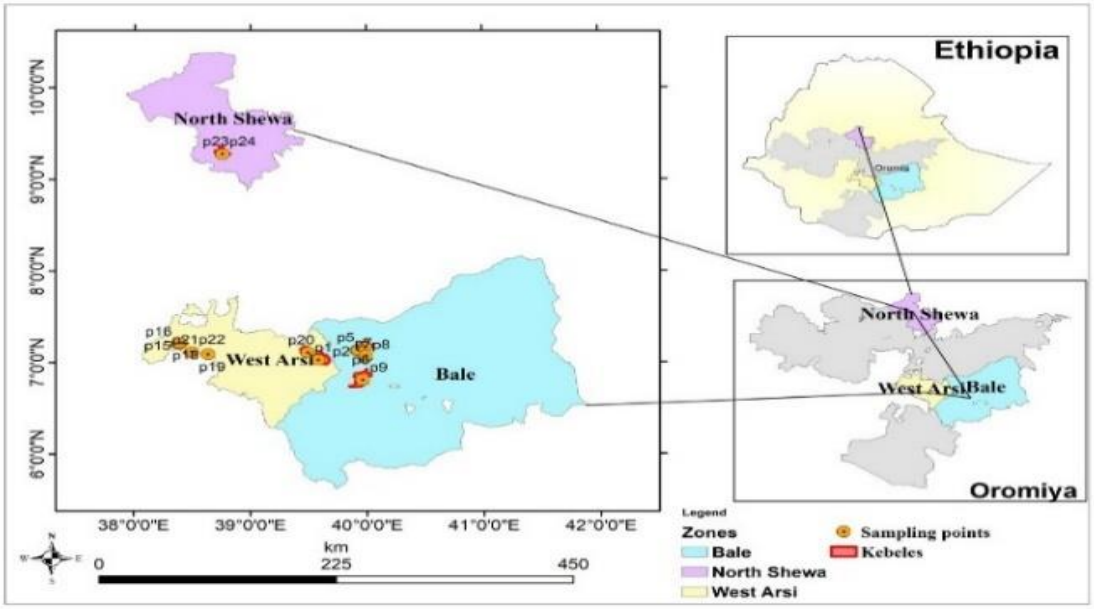


Figure 1. Map of sample collection areas.

Sample collection

Five healthy and young individual plants were selected randomly for each species per location and a total of 150 samples (stem, leaves, root/rhizome) and 50 rhizospheric soils from a depth of 5 cm were collected into sterile plastic bags separately (Table 1). All samples were kept in the refrigerator at 4°C and transported to Microbiology Laboratory of Ethiopian Biodiversity Institute using an icebox.

Isolation of endophytic fungi

About 3 g of plant samples (stem, leaves, root and rhizome) were transferred into a sterile petri dish and washed using running tap water three times to remove dust and soil. The samples were 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 were allowed to surface dry on sterile filter paper and transferred to PDA (HiMedia) for culturing fungi. Also, suspensions from the third wash were plated onto PDA supplemented with 100 mg/L chloramphenicol to check the efficiency of surface sterilization.

Each leaf, stem, and root/ rhizome sample was cut into one-centimeter size using a sterile blade. A total of 900 surfaces sterilized pieces from different tissues of each plant (300 leaves, 240 stems, 300 roots, and 60 segments of rhizome) were taken for the isolation of endophytic fungi. Six pieces from each sample were transferred to PDA containing chloramphenicol (100 mg/L). Inoculated plates were incubated at 27°C for seven days until growth is visible (Deepthi et al., 2018).

Table 1. Selected medicinal plants for isolation of endophytic and rhizospheric fungi

Family	Scientific Name	Local Name (Afaan Oromo/Amharic)	Sample Type	Collection Site
Polygonaceae	*Rumex abyssinicus*	Mekmeko[A]	Leaf, stem, rhizome/root and rhizosphere soil	Bale Zone and West Arsi
Polygonaceae	*Rumex nervosus*	Embuwacho[A]	Leaf, stem, root and rhizosphere soil	West Arsi and Chancho
Myrsinaceae	*Mysrine africana*	Kechemo[A/AO]	Leaf, stem, root and rhizosphere soil	Bale Zone
Alliaceae	*Aloe vera*	Ret[A]	Leaf, root and rhizosphere soil	West Arsi and Chancho
Solanaceae	*Solanum incanum*	Embuayi[A]	Leaf, stem, root, and rhizosphere soil	Bale and West Arsi

[A]= Amharic, [AO]= Afaan Oromo

Isolation of rhizosphere fungi

One gram of rhizospheric soil samples were taken and added into a test tube containing 9 ml of sterile distilled water. A tenfold serial dilution (10^{-1} to 10^{-6}) was prepared by pipetting 1 ml from stock suspension into nine ml of sterile distilled water, and thoroughly shaking manually to mix the suspension (Nisha et al., 2017). From an appropriate dilution factor (10^{-4} and 10^{-6}), 0.1 ml of the suspension was spread plated onto PDA supplemented with chloramphenicol (100 mg/L) and incubated at 27˚C for 7 days.

Purification and maintenance of the fungal isolates

The purified fungal isolates were transferred separately to brain heart infusion preservative medium supplemented with 10% glycerol and were maintained at two different temperatures (4ºC and -20ºC) for screening and identification purposes.

Source of test organisms

Clinical isolates (CI) and reference strains (RS) of human pathogenic microorganisms, *Staphylococcus aureus* (ATCC25923), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa (*ATCC 27853), *Escherichia coli* (ATCC 25922) and *Candida albicans* (ATCC 9690) bacterial and fungal cultures were obtained from Tikur Anbessa Specialized Hospital and Ethiopian Public Health Institute (EPHI), Microbiology Department, Addis Ababa, Ethiopia. The cultures were maintained on nutrient agar and Sabouraud dextrose agar (SDA) medium. The test bacterial and fungal strains were incubated at 37^{o}C for 24 hrs. and at 35^{o}C for 48 hrs., respectively.

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 the antifungal test and 2% dimethyl sulfoxide (DMSO) was used as a negative control (Matilde, 2011).

Preliminary screening

The preliminary screening of antimicrobial activity was carried out using the agar plug diffusion method (Devaraju and Satish, 2011). 100 µl of tested pathogenic bacteria and yeast, at concentration of 0.5 McFarland standards, were inoculated into nutrient agar (HiMedia) and Sabouraud dextrose agar (HiMedia) and spread uniformly using a sterile swab. The mycelial discs (6 mm) of each fungal isolate (14 days old) grown at 27°C on PDA were obtained using a sterile cork borer and placed on the surface of the media that were seeded with the test organisms. Chloramphenicol (30 µg) and fluconazole (25 µg) were used as a positive control for bacteria and yeast, respectively. Moreover, nutrient agar inoculated with test bacteria was also used as a control. The plates were incubated at 37°C for 24 hrs. and 35^{o}C for 48 hrs. for bacteria and fungus, respectively.

Fermentation and extraction of fungal crude metabolites

The potent fungal isolates in primary screening were cultivated on the surface of sterilized PDA plates and incubated at 27°C for 7 days. At a log phase growth stage of the culture, five plugs (6 mm diameter) were transferred into sterilized 250 ml of potato dextrose broth medium (HiMedia) and incubated at 27°C for 14 days. Then, the cultures were filtered and the filterate was centrifuged at 10,000 rpm for 15 minutes. All the supernatants were filtered again using Whatman No. 1 filter paper with pore size 11 µm to remove the remaining culture. Thereafter, the 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 acetate layer) were formed. The extract was concentrated by removing the solvents under reduced pressure at 40°C using rotary evaporator. Finally, the extracts were dissolved in 2% DMSO at an equal concentration and stored at 4°C (Sutjaritvorakul, 2011).

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). Concentrations of 50 mg/ml were prepared for all the fungal extracts by dissolving the extracts in 2% DMSO. Molten Mueller Hinton Agar (20 ml) and Sabouraud dextrose agar (20 ml) were prepared for bacterial and yeast, respectively. All the clinical suspensions of the test organisms were standardized based on 0.5 McFarland Standard and spread uniformly. Then, holes, 6 mm in diameter and 4 mm depth were made in the inoculated agar plates using a sterile cork borer. The diluted extracts (100 µl) were added into each hole by using a micropipette and kept at room temperature for one hour to allow the crude metabolites to diffuse into the agar medium. Chloramphenicol (30 µg) and fluconazole (25 µg) were used as a positive control, while 2% DMSO was used as a negative control. Inoculated plates were incubated at 37^{o}C for 24 hrs. but the SDA plates were incubated at 35^{o}C for 2 days.

Determination of the minimum inhibitory concentrations

The minimum inhibitory concentration (MIC) of the solvent extracts was determined by agar dilution methods as described by (ESCMID, 2000). Molten Muller Hinton and Sabouraud agar media were used for this test. Accordingly, 1 ml of crude fungal solvent extracts were prepared at different concentrations (50, 25, 12.5, 6.25, 3.125 mg/ml) and mixed thoroughly with the respective molten agar medium and poured into sterile petri dishes. One milliliter of bacteria and yeast suspensions was adjusted to 0.5 McFarland standards and swabbed on Muller Hinton and Sabouraud agar. The seeded plates were incubated at 37°C for 24 hrs. for bacteria and at 35°C for 48 hrs. for yeast culture, respectively. The MIC was determined by observing the growth of the test pathogens.

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

A loopful of the test cultures from the last MIC were sub cultured by streaking onto a fresh Muller Hinton and Sabouraud dextrose Agar media and incubated at 37°C for 24 hrs. and at 35°C for 48 hrs. for bacteria and yeast respectively. The lowest concentration of the extracts that showed no growth on the media was recorded as MBC/MFC (Takudzwa, 2013).

Qualitative screening of fungal metabolites

Wagner's test was performed to evaluate the presence of alkaloids. One milliliter 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).

The 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 the fungal extract resulting in the formation of yellow color. Then two drops of concentrated H_2SO_4 solution were added to the mixture. Finally, the change of color from yellow to colorless solution depicts the presence of flavonoids.

To test the presence of phenol compounds in the fungi extracts, 1 ml 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).

The presence of tannin compound was tested using a ferric chloride test as described in Yadav and Agarwala (2011). Fungal extract (1 ml) 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.

Keller-kiliani test was performed to assess the presence of cardiac glycosides. A 1 ml 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).

Libermann-Burchard reaction method was used to assess the presence of steroids. Fungal extract (1 ml) was added to 1 ml of chloroform solution. The mixtures were treated with 2 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). The presence of saponins was determined by the Frothing test (Sujana and Sridhar, 2013). A 1 ml 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 saponins.

For testing terpenoids, 1 ml of the fungal extract was mixed in 2 ml of chloroform and 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 terpenoids (Yadav and Agarwala, 2011).

Morphological characterization

Microscopic characterization was done based on the slide culture method. Pure PDA medium was cut (5 mm square) and picked up carefully to transfer 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 grown at 28°C of the fungus to be examined.

A cover glass was placed on the inoculated slide and incubated at 27°C for 48 hrs. and, the cover glass was taken carefully and flooded with Lactophenol Cotton Blue (LCB). The glass slide was observed under 40x light microscope (Kumar et al., 2015). The microscopic study included conidia and conidiophores and their arrangements (Barnet and Hunter, 2000).

Identification of fungi isolates by using Biolog™ System

Fungal isolates were identified using the Biolog Microstation™ ID System at the National Animal Health and Diagnosis Center, Sebeta, Ethiopia, following procedures described in the manufacturer's user guide (Biolog™, Hayward, CA). The testing was performed in a pre-filled microplate format to measure metabolic reactions. The characteristic metabolic pattern generated by an unknown organism was recorded and compared to hundreds of identification profiles in a corresponding Biolog Database. Fungal isolates were cultured on PDA and grown at 27ºC for one week and transferred to malt extract agar medium (Biolog™) by incubating at 27ºC for 3 days. Pure colonies were transferred into 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 system at every 24 hrs. incubation period for seven days.

Data analysis

All the experiments were carried out in triplicates and the results were expressed as mean ± SD using R stat version 3.6.3 statistical software. One-way analysis of variance was conducted to test the significance levels. Significant differences among treatment means were separated using the least significant difference (LSD) at 5% Fisher’s probability level.

RESULTS

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 (Table 2). From the total fungal isolates under the studied plant taxa, 151 (25.9%) were from *Solanum incanum*, 147 (25.3%) were from *Rumex abyssinicus*, 127 (21.8%) were from *Mysrine africana*, 117 (20.1%) were from *Rumex nervosus* and 40 (6.9%) were from *Aloe vera*. From a total of 266 rhizospheric fungal isolates, 69 (25.9%) fungal isolates were recovered from *Solanum incanum*, 62 (23.3%) were from *Mysrine africana*, 53 (19.9%) were from *Rumex nervosus*, 52 (19.5%) were from *Rumex abyssinicus* and 30 (11.3%) were from *Aloe vera*.

Table 2. Fugal isolates obtained from different sample types.

Plant Species	**Number of fungal isolates obtained from different sample types**				
	Rhizosphere	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		5	40
Total	**266**	**23**	**120**	**173**	**582**

Preliminary and secondary screening for antimicrobial activity

Out of 582 fungal isolates, a total of 18 fungal isolates revealed better antimicrobial properties based on measured inhibition zone diameters at least against one clinical and standard pathogenic test organism (Table 3; Figure 2).

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	**RS**	**CI**	**RS**	**CI**	**RS**	**CI**	**RS**	**CI**	**RS**
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, RS=Reference strains; + = 10mm-15 mm;++ = 15 mm -25 mm; +++ = >25mm

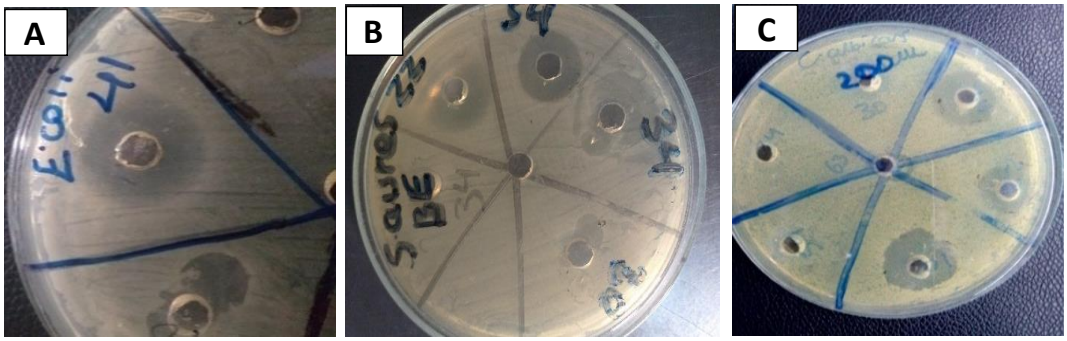


Figure 2. The inhibition zone of selected isolates against tested pathogens (A) *Escherichia coli,* (B) *Staphylococcus aureus*, (C) *Candida albicans* on Mueller Hinton agar medium by agar well diffusion method.

Table 4. Antimicrobial activity of crude ethyl acetate extracts of fungi using agar well diffusion method.

Isolates code	Inhibition zone of different human pathogenic microorganisms (Mean ± SD)				
	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}
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 (SD). Means followed by different letters (a, b, c, d, e, f, g, h, i, k) within the row are significantly different at $p < 0.05$.
Chloramphenicol: R = ≤ 12mm, I=13-17mm, S = ≥ 18mm

Selection of the best-performed isolates for identification

From 18 potential fungal isolates that showed higher inhibition zones against at least one clinical and standard pathogenic test organism, five were selected since they inhibited all the pathogenic test organisms with higher inhibition zone above 15.33 ± 0.57 mm (Table 5).

Table 5. Mean inhibition zones (mm) of the fungal isolates against test organisms using agar well diffusion method.

Test organisms	Mean inhibition zone in mm						MS	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

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.

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

For the selected fungal isolates (n=5), 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 organisms. The fungal extracts showed different values of MBC against the tested microbes (Table 6).

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

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
34WRaL	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

Qualitative screening of fungal metabolites

Out of the 18 tested fungal isolates' ethyl acetate extracts, 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 where as 30CRS fungal isolate extracts had only alkaloid and saponin (Table 7).

Table 7. Phytochemical analysis of endophytic and rhizospheric soil fungi.

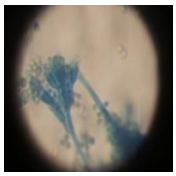
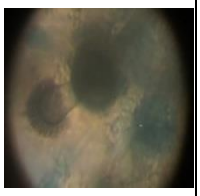
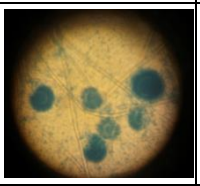
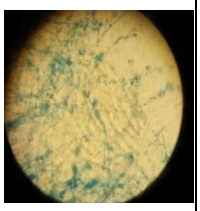
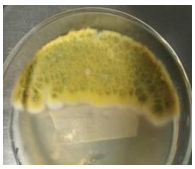
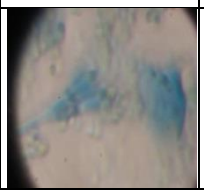
Fungal Extract	Alkaloid	Flavinoid	Phenol	Tanin	Cardiac glycosides	Steroid	Saponin	Terpenoid
34WRaL	-	-	-	-	-	-	+++	++
22BRaS	-	+	-	-	-	+++	-	+++
54WSL	+	-	-	-	-	+++	-	++
34BSSoil	-	-	-	-	-	++	-	+++
30CRS	++	-	-	-	-	-	+++	-
37BRaL	-	+++	+++	-	+++		++	-
1BraS	-	-	-	+	-	+++	+	+++
10WRaS	-	-	-	-	-	+++	-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

+++ = Potent activity; ++ = Moderate activity; + = Less activity; -, No activity

Identification of the fungal isolates

Morphological and biochemical tests of the five top fungal isolates with antimicrobial activities assigned the isolates to *Penicillium sp., Talaramycetes sp., Aspergillus sp*. and *Trichoderma sp*. (Table 8). Fungal isolate 30CRS was identified as *Penicillium simplicissimum*, while fungal isolate 37BRaL was identified as *Talaromyces flavus* var. *flavus* with similarity of 99.8% and 56.46%, respectively.

Table 8. Characterization and identification of efficient fungal isolates.

Isolate code	Morphological characteristic				Species identification		
	Fungi in a culture plate	**Macroscopic characteristics**	**Fungi under microscopic**	**Microscopic characteristic**	**Morphological identification**	**Identity using Biolog**	**Similarity**
30CRS		Green colour, colonies later turned into cream colour, filaments medium colony size, circular form/shape and flat elevation.		Conidiospore with metulae and phialides and the phialides branched. Conidia were budded from the phialides and oval shape medium size, blue color arranged in chains. The conidia ellipsoidal in shape and the walls smooth	*Penicillium*	*Penicillium simplicissimum* (oudemans) Thom BGA	99.8%
22BRaS		Gray color colonies, medium size, raised elevation and filamentous form		Smooth and colorless conidiophores and spores. Biseriate phialides	*Aspergillus*	Unidentified	
68BMS		Black colony color, large colony size, filamentous form, circular shape.		Conidia circular, branched phialides, smooth conidiophores and spores. Bi-seriate phialides	*Aspergillus*	Unidentified	
34WRaL		Gray color colonies, medium size, raised elevation and filamentous form.		Conidia were globose and Phialides were flask shaped	*Trhichoderma*	Unidentified	
37BRaL		Gray color colonies, medium size, raised elevation and filamentous form.		Conidiospore branching Monovalent, phialides branched, condia	*Talaramyces*	*Talaromyces flavus* var. *flavus*	56.46%

Penicillium simplicissimum and *Talaromyces flavus* var. *flavus*, those shows that fungal species has ability to utilize and oxidize different carbon sources (Table 9).

Table 9. Metabolic profile of *P. simplicissimum* and *T. flavus* var. *flavus* using Biolog FF Microplate.

Different carbon 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*
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	+	+
α-D-Glucose	-	+	Quinic Acid	+	+
Glucose-1- Phosphate	+	+	D-Saccharic Acid	+	+
Glucuronamide	-	-	Sebacic Acid	+	+

Note: + = Utilization of carbon source; - = No utilization of carbon source

DISCUSSION

The current study demonstrated that a single plant part is colonized by more than one cultivable 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, 30CRS isolate, later identified as *Penicillium simplicissimum* fungal species, was reported from some other medicinal plants such as the root

of *Alnus glutinosa* (Fisher et al., 1991), twig of *Eucalyptus nitens* (Fisher et al., 1993), stem of *Melia azadarach* (Geris dos, 2003), and roots of *Panax ginseng* (Hao, 2013). Similarly, isolate 37BRaL identified as *Talaromyces flvus* var. *flavus* that was previously retrieved from leaves of *Sonneratia apetala* (Li et al., 2011).

According to Nisha (2017), several antibiotic-producing fungi (*Aspergillus* sp. and *Penicillium* sp.) were isolated from the rhizosphere soil. Presence of these fungi depends on the nature of the environment and the texture of the soil. Generally, rhizosphere-associated microbes play a very important role in improving the medicinal values of plants (Guo et al., 2006; Raha and Shagufta, 2019). Soil microbial communities play several important ecological and physiological functions (Narula et al., 2009).

Five fungal extracts having antimicrobial properties were selected during secondary screening against more than two clinical test organisms. Accordingly, *P. simplicissimum* crude extract of the current study had broad-spectrum antimicrobial activities by inhibit the growth of gram-positive, gram-negative, and yeast at 50 mg/ml. Previous research conducted in Malysia by Yenn et al. (2014) revealed that endophytic fungi *Penicillium minioluteum* showed 17.3 ± 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* but did not inhibit *C. albicans* at 50 mg/ml concentration.

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

The mean MIC of the current study exhibited by *P. simplicissimum* was higher than the finding by Amina et al. (2018) for crude ethyl acetate extract of *P. griseofulvum*, which reported MIC of 50 µg/ml for *E. coli* ATCC 25922 and 100 µg/ml for *S. aureus* ATCC 25923. Akanksha (2015) has reported that *Penicillium frequentans* inhibited *C. albicans* with less MIC (10 mg/ml).

The mean MIC of *T. flavus* var. *flavus* at 25 mg/ml against *C. albicans*, 50 mg/ml against *S. aureus* and 12.5 mg/ml against *E.coli* were higher when compared with the previous research finding by Fang et al. (2012) using *Talaromyces verruculosus* with MIC at 15.6 µg/ ml against *C. albicans*, 2.5 µg/ml against *S. aureus* and 5.0 µg/ml against *E. coli*. The variations might be due to differences in secondary metabolite production among different fungal species and the different susceptibility levels of test organisms for a fungal extract.

Penicillium simplicissimum was positive for alkaloid and saponin secondary metabolite. These results were similar to the findings by Akanksha et al. (2015) who reported that a study on *Penicillium frequentans* showed alkaloid, saponin, flavonoid, phenol, tannins, terpenoid and steroids as major secondary constituents of the crude extracts. Also Tan and Zou, (2001) reported that alkaloid was produced from grass endophytic *Penicillium* species. The result of *T. flavus* var. *flavus* was supported by a previous research conducted by Ming and Zhai (2016). *Talaromyces flavus* var. *flavus* has a remarkable potential for its secondary metabolites with unique biological activities (Bohumil, 2010). According to Lai (2010), endophytes and rhizospheric fungi have shown the presence of different secondary metabolite profiles 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).

Morphological characterization was supported by the Biolog identification systems that gave similar results at the genus level. Suhaila et al. (2018) also found similar identification results of microscopic and the Biolog ID system with the molecular identification using ITS for *P. oxalicum*. *T. flavus* var. *flavus* also utilize carbohydrates. 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 species not available in the Biolog database which did not utilize different carbon sources that were tagged into Biolog microplates.

CONCLUSION AND RECOMMENDATION

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. nervosus* and *T. flavus* var. *flavus* isolated from the leaf of *R. abyssinicus* exhibited the stronger antibacterial potential against test organisms. Further detailed research works on the characterization of the endophytic fungal species which are associated with different *R. nervosus* and *R. abyssinicus* genotypes/populations on potential antimicrobial secondary metabolite using NMRI and chromatography techniques is recommended. Moreover, further studies of the mode of action for a fungal metabolite of current isolates that have antimicrobial activities are important.

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