ORIGINAL ARTICLE

Isolation and screening of endophytic fungi from Eritrean traditional medicinal plant *Terminalia brownii* leaves for antimicrobial activity

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Plants formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years. But, the advent of drug resistance in human pathogenic bacteria and others has prompted a search for more and better antibiotics. This has led to the identification of a new promising source of antimicrobials known as endophytes. Hence, our study was aimed to investigate the ability of endophytic fungi isolated from *T. brownii* to produce secondary metabolites, which can act as antimicrobial agents. In this preliminary investigation, the leaves were used for isolation of endophytic fungi and fermented, and the cell free ferment broth was subjected to antimicrobial screening against six human pathogens; *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans* by using standard protocol of agar well diffusion method. The results of the endophyte isolation gave three fungal isolates named TBF1, TBF2 and TBF3. According to morphological and microscopical characterizations, the isolates were found to be similar to *Rhizophus oryzae* (TBF1), *Aspergillus niger* (TBF2) and *Aspergillus flavus* (TBF3). Two of the three isolated endophytes *i.e.,* TBF2 and TBF3 showed potential antimicrobial activity against *S. aureus* and no inhibition was found against other tested pathogens. The present study has proven that *T. brownii* may be a rich source of endophytic fungi with antimicrobial potential and our findings may form a basis for further studies on endophytic fungi from medicinal plants for antimicrobial activities.

Key words: Agar well diffusion method, antimicrobial activity, endophytic fungi, medicinal plant, *Terminalia brownii*

INTRODUCTION

Resistance to antimicrobial drugs used in the treatment of many infectious diseases, not only of bacterial, but also of fungal origin is increasing, which complicates and renders treatment of critical infectious illnesses more difficult. This encourages research for new antimicrobial agents with novel modes of action to prevent resistance and provide relief from illnesses.[1] Molecules derived from natural products, particularly those products of plants and microbes have an excellent record of providing novel chemical compounds for the development of new pharmaceutical products.[2] The probability of obtaining a novel compound is higher from a novel source. So, we selected endophytic microbes as potential sources of antimicrobial compounds.

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The term endophyte was coined by the German scientist Heinrich Anton De Bary (1884), and is used to define fungi or bacteria occurring inside plant tissues without causing any apparent symptoms in the host.[3] Of the 300,000 plant species that exist on the earth, each individual plant is host to one or more endophytes, thus providing a rich reservoir of microorganisms.[4-8]

The functional metabolites produced by endophytes include alkaloids, terpenoids, steroids, quinones, isocoumarin derivatives, flavanoids, phenols and phenolic acids, and peptides. Some species produced novel antimicrobial agents (e.g., Cryptocandin from *Cryptosporiopsis quercina*), others produced potent anti-cancer compounds (e.g., Taxol from *Taxomyces andreanae*), and yet others produced compounds that can be utilized industrially, such as enzymes and solvents.[7]

In fact, a recent comprehensive study has indicated that 51% of biologically active substances isolated from endophytic fungi were previously unknown.[9] Among the new bioactive molecules discovered are: Novel wide-spectrum antibiotics, kakadumycins, isolated from the endophytic streptomycete associated with the fern-leafed grevillea (*Grevillea pteridifolia*) from the Northern territory of Australia;[10] ambuic acid, an antifungal agent from

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several isolates of *Pestalotiopsis microspora*;[11] and subglutinols A and B, immunosuppressive compounds produced by *Fusarium subglutinans*, an endophyte of *T. wilfordii*.[12]

While plants have received extensive study as sources of bioactive metabolites, the endophytic microbes that reside in the tissues between living plant cells have received scant attention,[7] and represent a poorly investigated group of microorganism that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial arenas.[6]

Terminalia brownii locally known as 'Weiba' is used traditionally by the people of Eritrea to treat jaundice, hepatitis, liver cirrhosis and yellow fever. While much is known about the phytochemistry of the genus, there is no information available about the endophyte biology. Hence, the objective of our study was to isolate the endophytic fungi from the Eritrean traditional medicinal plant '*Terminalia brownii*' and their capacity to produce secondary metabolites that have anti-microbial effect. Since there were no reports of endophytes from Eritrean native plants and the bioactive compounds produced by them, this work may be the first report on endophytic fungi from *Terminalia brownii* in Eritrea. This finding will form a basis for further studies on endophytic microbes from medicinal plants for antimicrobial activity.

MATERIALS AND METHODS

Chemicals

Saboraud's dextrose agar (SDA) (Himedia Laboratories, Mumbai, India), Saboraud's dextrose broth (Biotec laboratories, UK), Mueller Hinton agar (Oxoid Ltd, Basingstoke, Hampshire, England), ethanol (70%), sodium hypochlorite (2%), crystal violet, saffranin, methylene blue, immersion oil, and distilled water etc.

Plant Collection and Identification

Healthy (showing no visual disease) and mature leaves of Eritrean medicinal plant *Terminalia brownii*, Local name: *Weiba* were collected from Ghindae sub zone, in plastic bags and transported carefully to the laboratory in Asmara. The collected plant material was authenticated by a Taxonomist Dr. Gebrehiwet Medihanie, Department of Biology, Eritrean Institute of Technology (EIT), Mai Nefhi, Eritrea. Fresh plant materials were used for isolation work to reduce the chance of contamination. The collected plant leaves were subjected to surface sterilization within a few hours after sampling.

Surface Sterilization

All the leaf samples were subjected to a three step surface sterilization procedure according to the method described by Petrini.[13]

Initially all the leaves were washed in running tap water for 10 minutes to remove, soil particles and adhered debris, and finally washed with distilled water. This was followed by washing in 95% ethanol for 1 minute, in 2% sodium hypochlorite for 10 seconds and in 95% ethanol for 1 minute. Finally, the leaves were washed in sterile distilled water for 2 minutes. The efficiency of surface sterilization procedure was ascertained for every segment of tissue by imprint method.[14]

Isolation of Endophytic Fungi from *Terminalia brownii* Plant Leaves

After surface sterilization, following two methods were used for isolation of endophytes,

Method 1

The leaves were crushed with sterile distilled water using sterile mortar and pestle. About 1 ml of crushed sample was serially diluted up to 10^{-5} dilutions using 12.5 mM potassium phosphate buffer (pH 7.1). About 0.1-0.2 ml of aliquot from 10^{-2} to 10^{-5} dilutions were taken and spread on Sabouraud dextrose agar media using sterile inoculating loop. Platting was done in duplicates and all the plates were incubated at 30°C. Observation was carried out daily until the growth of endophytic fungi was observed.[15]

Method 2

The outer tissues were removed from the leaves by crushing it with the application of minimal pressure using sterile mortar and pestle and the inner tissues were excised and about 2–3 segments of crushed leaves were placed onto petri plates containing Sabouraud Dextrose agar media and incubated at 30°C. Observation was carried out daily until the growth of endophytic fungi was observed.[16]

Purification, Selection and Preservation of Endophytic Fungi

After incubation, fungal colonies were selected and streaked on sabouraud dextrose agar plates and incubated at 30°C for about 72 hours.

Morphological and Microscopical Characterization of Endophytic Fungi

The isolated endophytic fungi TBF1, TBF2 and TBF3 were characterized morphologically by shape, colony colour, texture, topography and microscopically by sticky tape method.[15]

Screening for Antimicrobial Activity

Test microorganisms

Five reference human pathogenic microorganisms were used for the antimicrobial assay, which includes two Gram positive bacteria, *Staphylococcus aureus* (NCTC 12981/ATCC 25923), *Enterococcus faecalis* (NCTC 12697/ATCC 29212) and two Gram negative bacteria, *Pseudomonas aeruginosa* (NCTC

12903/ACTC 27853), *Escherichia coli* (NCTC 12241/ATCC 25922), and one yeast, *Candida albicans* (NCPF 3179/ATCC 10231). All the test microorganisms were obtained from the National Health Laboratory, Asmara, Eritrea.

Preliminary Screening by Cross Streak Method

Preliminary antimicrobial screening of isolated endophytes were done by following two methods,

Method 1

Endophytic fungi isolates TBF1, TBF2 and TBF3 were streaked centrally on the surface of sabouraud dextrose agar, followed by the overnight culture of clinical isolates (Turbidity adjusted to 0.5 McFarland standard) of bacterial pathogens like *S. aureus*, *E. coli*, *P. aeruginosa*, *Enteroccocus faecalis* and fungal pathogen *C. albicans* were streaked at perpendicular to the original streak and incubated at 30±2°C. The inhibition was observed after 24 hrs and duplicates were maintained for each analysis.[17]

Method 2

This method was a slight modification of Method 1. Endophytic fungi isolates TBF1, TBF2 and TBF3 were streaked centrally on the surface of sabouraud dextrose agar, and then incubated for 24 hrs at 30±2°C. After one day of incubation, the overnight culture of clinical isolates (Turbidity adjusted to 0.5 McFarland standard) of bacterial pathogens like *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Enteroccocus faecalis* and fungal pathogen *C. albicans* were streaked at perpendicular to the original streak and incubated. The inhibition was observed after 24 hrs and duplicates were maintained for each analysis.

Fermentation

Endophytic fungal strains (TBF2 and TBF3), which showed inhibition over tested bacterial pathogens were further inoculated in to 20 ml of sterile sabouraud dextrose broth, followed by static condition and incubated at 30±2°C for 9 days. After 9 days, culture medium was centrifuged at 10,000 rpm for 30 minutes. After centrifugation, the culture supernatant was collected and subjected to antimicrobial screening by agar diffusion technique.[18]

Antimicrobial Screening by Agar Well Diffusion Technique

Antimicrobial activity of culture supernatants of endophytic fungi (TBF2 and TBF3) was tested by agar well diffusion method using Mueller Hinton agar medium. All the overnight culture (Turbidity adjusted to 0.5 McFarland standard) of test microbes were inoculated into Mueller Hinton agar plates using sterile cotton swab. About 5 mm size well was made using sterile borer and 200 µl of culture supernatant was added into it. All the plates were observed for zone of inhibition after incubation at 37°C for 24 hours for bacterial pathogens and at 30°C for 24 hours for fungal pathogen. Sterile sabouraud dextrose broth was used as negative control. The antimicrobial activities were assessed by the presence or absence of inhibition zones.[19]

RESULTS

Isolation of Endophytic Fungi

From the two methods used for the isolation of endophytic fungi, Method 2 showed better growth of endophytes compared to Method 1. Method 1 showed only one fungal growth named *Terminalia brownii* fungi 1 (TBF1). From Method 2, two endophytic fungal growths were observed and named as TBF2 and TBF3. Isolated fungal endophytes TBF1, TBF2 and TBF3 are shown in Figures 1 and 2.

Morphological and Microscopical Characterization of Endophytic Microbes

Results of macroscopic identification of endophytic fungal isolates TBF1, TBF2 and TBF3 are given in Table 1.

Figure 1: Endophytic fungal isolate TBF1

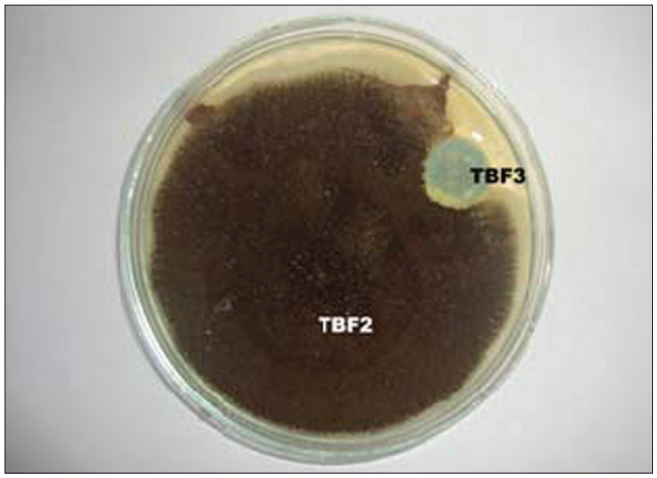

Figure 2: Endophytic fungal isolate TBF2 and TBF3

Endophytic fungal isolate TBF1 showed the following microscopic characteristics, the hyphae are usually aseptate. Unbranched sporongiophores arise opposite rhizoids at the nodes and each sporongiophore support a round spore filled sporangium with a flattened base. Sometimes the sporangia are completely absent or they may be empty. Stolons connect the groups together with each other. Based on macroscopic and microscopic characteristics the endophytic fungal isolate TBF1 may be identified as *Rhizophus spp.*

Microscopically the isolate TBF2 had a mycelium that is septate, unbranched, rough, or smooth conidiophores with a 'foot cell' at their base support a large 'vesicle' at their tip. The vesicles in return support short, flask shaped 'PHIALIDES' in a single or double raw, which produce chains of smooth or rough 'PHIALOCONIDIA'. Based on macroscopic and microscopic characteristics the endophytic fungal isolate TBF2 may be identified as *Aspergillus spp 1.*

Microscopically TBF3 conidiophores are of variable length, rough, pitted and spiny and phialides are uniseriate and biseriate, cover entire vesicle, and point out in all directions. Based on macroscopic and microscopic characteristics the endophytic fungal isolate TBF3 may be identified as *Aspergillus spp 2.*

Screening for Antimicrobial Activity

Preliminary screening

From the two methods used for preliminary screening of antimicrobial activity from endophytes, Method 2 showed antimicrobial potential of endophytic microbes. In the case of *Rhizophus spp* (TBF1), we were not able to conclude of any antimicrobial activity because of its fast growing nature and also it covers the entire surface of tested pathogens. But the endophytic fungal isolates TBF2 and TBF3 showed inhibition against bacterial pathogens and there was no inhibition against the tested fungal pathogen. From the preliminary screening, endophytic fungi isolates that showed inhibitory effect were selected for further studies using suitable fermentation media.

Table 1: Macroscopic identification of endophytic fungal isolates

Colony no	Colour	Size	Texture	Topography
TBF1	White	Large	Cottony or wooly	Flat
TBF2	Dark brown	Large	Velvety	Rugose
TBF3	Green	Small	Velvety	Umbonate and rugose

TBF – *Terminalia brownii* fungi

Antimicrobial screening by agar well diffusion technique

Endophyte culture filtrates were assessed for their antimicrobial activities against *S. aureus, Enteroccocus faecalis, E. coli, P. aeruginosa* and *C. albicans*. Antimicrobial activities of endophytic fungi against human pathogens are given in Table 2.

Among the two endophytic fungi tested, i.e., TBF2 and TBF3, both the isolates were found to be active against *S. aureus*. But there was no inhibition against the other tested bacterial and fungal pathogen. This suggests that fungal isolates showed better activity against *S. aureus*.

DISCUSSION

Microorganisms isolated from hitherto unexplored areas and/or from extreme environments is the obvious choice for development of potential novel bioactive metabolites.[20,21] It has become apparent that an enormous and relatively untapped source of biological diversity is represented by microbial endophytes which are a promising source of novel natural products for use in medicine, agriculture and industry.[22]

In this preliminary investigation, the leaves were used for isolation of endophytic fungi and cell free fermentation broth was collected and subjected to screening against four human pathogenic bacteria and one human pathogenic fungus by standard protocol of well diffusion method. The endophyte isolates indicated that the plant *Terminallia brownii* is enriched with various fungal populations. Fungal growth was initiated within 3 days of inoculation. The day at which first visual growth was observed from plating date was considered as an incubation period for growth.

The reason for better results of Method 2 in preliminary screening by cross streak method could be due to the fact that growth as well as production of secondary metabolites does not take place in the same phase. Generally microbes produce secondary metabolites during the stationary phase of growth. Hence, we believed that Method 2 provided sufficient time duration for the fungal isolate to reach its stationary phase for

Table 2: Antimicrobial activity of fermentation aliquots of endophytic fungi

Endophytes isolated from *Terminalia brownii*	Zone of inhibition (mm)				
	Bacterial pathogens				Fungal pathogen
	Gram +ve		Gram -ve		
	S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*	*C. albicans*
TBF2 (*Aspergillus sp1*)	14.5	-	-	-	-
TBF3 (*Aspergillus sp2*)	12.5	-	-	-	-

TBF – *Terminalia brownii* fungi

the production of antimicrobial substances compared to Method 1.

The results of the antimicrobial activity test showed that from the total of three endophytic fungal isolates, i.e., TBF1, TBF2 and TBF3 of the *Terminallia brownii* leaves; only TBF2 and TBF3 produced antimicrobial substances. Of the five tested microbes, *S. aureus* was the most sensitive to the antimicrobial substances produced by the endophytic fungi. This was demonstrated by the formation of a clear zone surrounding the well, indicating that there was an inhibition of growth of the test microbe *S. aureus*. So TBF2 and TBF3 were considered as good source of antimicrobial compound. This result further indicated that the antimicrobial metabolites produced by active fungal endophytes TBF2 and TBF3 may have specific application where the control of *S. aureus* is desired.

CONCLUSION

This study addressed the scientific knowledge gap and explored whether Eritrean plant endophytes, in particular those isolated from '*Terminallia brownii*' species, may be a potential natural resource to yield useful biologically active compounds. Furthermore, our investigation concludes that the isolated bioactive fungi could be a rich source of novel metabolites with antimicrobial activity, which may represent a potential for pharmaceutical and/or agricultural applications. To the best of our knowledge this is a first study on the antimicrobial activity of fungal isolates from *Terminallia brownii* of Eritrea. Further investigations on purification and structure elucidation of the compounds are in progress.

So far, a great number of antimicrobial compounds have been found in a handful of the one million different endophyte species,[23] it is believed that searching for natural products synthesized by endophyte could be a promising way to solve the problem of resistance and meet the emergent demand of discovering highly effective, low toxicity and low environmental impacted antibiotics to fight against resistant bacterial species.[24]

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