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PRELIMINARY SCREENING OF ENDOPHYTIC FUNGI FROM *RHAMNUS PRINIOIDES L.* FOR ANTIMICROBIAL ACTIVITY

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Abstract

The purpose of this study was to isolate and identify the endophytic fungi from the roots of *Rhamnus prinioides* (gesho) plant as well as to examine their antibacterial activity. All the root samples were subjected to a three step surface sterilization procedure. After proper sterilization of the surface, roots of *Rhamnus prinioids* were evenly placed in Petri dishes containing potato dextrose agar (PDA) medium. The Petri dishes were incubated at 30 °C and monitored every day to check the growth of microorganism colonies from the root segments. Total 15 root segments of *Rhamnus prinioids* plants were subjected for isolation of endophytic fungi. Out of which 9 fungal strains were isolated and identified as *Alternaria* Sp. (N=3), *Aspergillus* Sp. (N=5) and *Curvularia* Sp. (N=1). Among 9 isolates of endophytes only 6 isolates were effective against selected bacteria. Among 6 endophytes 50% showed to effectiveness *E. coli* and *P. aeruginosa.* Whereas, 66.66% isolates were effective against *S. aureus.* Two isolates of *Alternaria* Sp. and 3 isolates of *Aspergillus* Sp. were effective against all selected bacteria. Whereas, 1 isolate of *Curvularia* Sp. was effective against only *S. aureus.* This study addressed the scientific knowledge gap and explored whether Ethiopian plant endophytes, may be a potential natural resource to yield useful biologically active compounds.

Keywords: Endophytic fungi, Medicinal plants, Antimicrobial activity.

Introduction

In recent years, entophytes has received increasing attention as a promising supplement or alternative to chemical control. The strategic use of naturally occurring organisms to control pest populations and increase production of major crops represents a viable option to host-plant resistance and pesticide-based pest and pathogen control. Endophytic microorganisms, microorganisms that grow in the intercellular spaces of higher plants, are recognized as one of the most chemically promising groups of microorganisms in terms of diversity and pharmaceutical potential[1]. Beneficial endophytic microorganisms comprise especially fungi and bacteria that colonize internal plant tissues without causing visible damage to their hosts[2]. Furthermore, the endophytic microorganisms are not considered as saprophytes since they are associated with living tissues, and may in some way contribute to the well-being of the plant.

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Endophytes exist in a range of tissue types within a broad range of plants, colonizing the plant systemically with bacterial colonies and biofilms, residing latently in intercellular spaces, inside the vascular tissue or within cells[3].

Endophytes, microorganisms that reside in the tissues of living plants, are relatively unstudied and potential sources of novel natural products for exploitation in agriculture. That is, the plant is thought to provide nutrients to the microbe, while the microbe may produce factors that protect the host plant from attack by animals, insects or microbes[4]. Studies on microorganisms from plant species are recently becoming more frequent, since these fungi and bacteria have been studied for biological control and production of compounds with pharmacological properties.

The purpose of this study was to isolate and identify the endophytic fungi from the roots of *Rhamnus prinioides* plant as well as to examine their antibacterial activity in order to provide additional data for the utilization of the antimicrobial metabolites from the endophytic fungi. The *Rhamnus prinioides* plant has many uses amongst Africans. All parts of the plant being used for nutrition, medicine or religious purposes. In Ethiopia, the plant is known as *gesho*, used in a manner similar to hops.

Fig. 01: *Rhamnus prinioides* Plant (Gesho)

Material and methods

Plant specimen collection

The healthy plants samples selected and uprooted from Samunaber, Gondar, Ethiopia. The root samples were cut by alcohol sterile scissor and wrapped with Parafilm before they were placed in zip-lock plastic-bags and stored less than 72h prior to the isolation of endophytic fungi.

Surface sterilization

All the root samples were subjected to a three step surface sterilization procedure. Initially all the roots 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 roots were washed in sterile distilled water for 2 minutes.

Isolation of Endophytic Fungi

After proper sterilization of the surface, parts of *Rhamnus prinioids* were evenly placed in Petri dishes containing potato dextrose agar (PDA) medium. The Petri dishes were incubated at 30 °C and monitored every day to check the growth of microorganism colonies from the root segments. After isolation, the strains were cultivated and preserved by periodic replications (once a week) on Yeast-Malt agar (YM: 10 g.L-1 glucose, 5 g.L-1 peptone, 3 g.L-1 yeast extract, 3 g.L-1 malt extract, pH 6.7).

Morphological and Microscopical Characterization of Endophytic Fungi

The isolated endophytic fungi were characterized morphologically by shape, colony color, texture, topography and microscopically by sticky tape method.

Fermentation

Isolated endophytic fungal strains were further inoculated in to 20 ml of sterile Sabouraud's 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 disc diffusion technique.

Antimicrobial activity

The previously isolated pathogenic bacteria *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* obtained were inoculated in Petri dishes containing solid BHI (Brain Heart Infusion) medium and incubated at 30 °C. After 24 h, one full loop of each pathogenic culture was transferred to 50 mL Erlenmeyer flasks containing 10 mL of liquid BHI medium, and then was placed on the shaker at 30 °C and 150 rpm. After 24 hours,

the absorbance was adjusted with pure liquid BHI between 0.080 and 0.100 (625 nm). Then, 100 µL of each microorganism was spread in petri dishes containing solid BHI. Thereafter, three filter paper discs (6 mm diameter) were placed on each BHI Petri dish previously inoculated with pathogenic microorganisms. The extracts of endophytic cultures (10µL) were dispensed to each disc. After incubation at 30 ºC for 24 h, the presence of inhibition zones around the discs was analyzed.

Results

Isolation of endophytic fungi and their characterization

Total 15 root segments of *Rhamnus prinioids* plants were subjected for isolation of endophytic fungi. Out of which 9 fungal strains were isolated and identified as *Alternaria* Sp. (N=3), *Aspergillus* Sp. (N=5) and *Curvularia* Sp. (N=1) (Table No. 01). *Alternaria* Sp. isolated from root segments produced olivaceous black color colonies. Reverse side of colonies were dark brown with colorless hyphae and in small group of conidiophores which were smooth in appearance. The conidia were formed in long chains and ovoid in shape with oblique septa. *Aspergillus* Sp. grown slowly, 2-3 cm in diameter in 9 days produced pale yellow color droplets of exudates on colonies and reverse of the colony were tan in color. The conidial heads were globose to radiate. The conidiophores were smooth and vesicles were thick walled and globose. Whereas, *Curvularia* Sp. was grown rapidly, colonies were woolly, blackish brown in color. The conidiophores were geniculate, septate and brown in color. The central cell of the conidium was typically and elongated as compare to the end cells.

Table No. 01: Endophytic fungi isolated from root segments of *Rhamnus prinioids* plant

Endophytic species	No. of isolates	Isolated from
Alternaria Sp.	3	Root
Aspergillus Sp.	5	Root
Curvularia Sp.	1	Root

Antimicrobial activity

Extracts of endophytes were assessed for their antimicrobial activities against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus.* Percentage of susceptibility testing of bacteria from endophytic fungal extract using disc diffusion was calculated. Among 9 isolates of endophytes only 6 isolates were effective against selected bacteria. Among 6 endophytes 50% showed to effectiveness *E. coli* and *P. aeruginosa.* Whereas, 66.66% isolates were effective against *S. aureus* (Table No. 02).

Table No. 02: Percentage of susceptibility testing of bacteria from endophytic fungal extract using disc diffusion method

Endophytic fungi	Bacteria tested		
	E. coli	***P. aeruginosa***	***S. aureus***
Crude extract N=06	3 (50%)	3 (50%)	4 (66.66%)

The antimicrobial susceptibility pattern of endophytic fungal extract showed in table no. 03. Two isolates of *Alternaria* Sp. and 3 isolates of *Aspergillus* Sp. were effective against all selected bacteria. Whereas, 1 isolate of *Curvularia* Sp. was effective against only *S. aureus.*

Table No. 03: Antimicrobial pattern of endophytic fungal extract against bacteria

Bacteria	Effect of endophytic fungal extract (Zone of inhibition in mm)		
	Alternaria Sp.	Aspergillus Sp.	Curvularia Sp.
E. coli	14.5	12	-
P. aeruginosa	12	10.5	-
S. aureus	17	15.5	12

Discussion

Microorganisms isolated from unexplored area are the obvious choice for development of potential novel bioactive metabolites[5]. 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.[6] Recently, biological controls or the uses of microorganisms or their secretions to prevent diseases offer an attractive alternative or supplement to disease management without the negative impact of chemical control[2].

In this preliminary investigation, the roots were used for isolation of endophytic fungi and extracts were collected and subjected to screening against three human pathogenic bacteria standard protocol of disc diffusion method. The endophyte isolates indicated that the plant *Rhamnus prinioids* is enriched with various fungal populations. Among isolates the most dominants were *Aspergillus* Sp. followed by *Alternaria* Sp. and *Curvularia* Sp. These endophytic fungi were identified based on colony morphology, conidia and conidiophore characteristics.

Most of the isolates, 66.66% showed antimicrobial activity against *S. aureus* followed by 50% of the isolates showed antimicrobial activity against both *E.coli* and *P. aeruginosa*. Whereas, antimicrobial pattern of endophytic fungal extract revealed that maximum effect was observed by *Alternaria* Sp. All the tested bacteria were susceptible for both *Alternaria* Sp. and *Aspergillus* Sp. and *Curvularia* Sp. was effective only on *S. aureus.*

However, many studies have showed that several endophyic fungi exhibit antibacterial activity. Several metabolites of the marine isolate, *Aspergillus niger*, *Pichia guilliermondii* isolated from *Paris polyphylla var. yunnanensis*[7] and endophytic fungi isolated from *Acanthus ilicifolius* and *Acrostichum aureum*[8] showed broad spectrum antimicrobial activity.

The altersetin isolated from two endophytic Alternaria species, showed potent inhibition against several pathogenic Gram-positive bacteria[9]. In another study, the crude extracts from endophytes isolated from *Smallanthus sonchifolius* inhibited the growth of *S. aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* [10].

This study addressed the scientific knowledge gap and explored whether Ethiopian plant endophytes, 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. Further investigations on purification and structure elucidation of the compounds are in progress.

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