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Anita Tilwari
Senior Scientist, Center of Excellence in Biotechnology, M.P. council of Science and Technology, Bhopal, M.P., India

Prachi Dixit
Center of Excellence in Biotechnology, M.P. council of Science and Technology, Bhopal M.P. and Department of Biotechnology, Kamla Raja Girls, Autonomous PG College, Jiwaji University Gwalior, Madhya Pradesh, India

Correspondence
Anita Tilwari
Senior Scientist, Center of Excellence in Biotechnology, M.P. council of Science and Technology, Bhopal, M.P., India

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Isolation and screening of endophytic fungus from medicinal plant *Saraca asoca* for antibacterial activity

Anita Tilwari and Prachi Dixit

Abstract
Ashoka is the most ancient tree of India, generally known as a "ashok briksh", botanically known as a *Saraca asoca* (Roxb.). *Saraca asoca* has many uses mainly in the ayurvedic medicine. The endophytic fungus have developed a strong bonding with the plant during the co-evolution between them which resulted in production of bioactive Secondary metabolites which are originally the phytochemicals associated with the host plant. The aim of the present study was to isolate and observe antibacterial activity of the endophytic fungi against some pathogenic bacteria. Endophytic fungi (*Syncephalastrum rasemosum*, *Syncephalastrum racemosums328*) were isolated from mature healthy leaves and bark of medicinal plant *Saraca asoca* were collected from the Human Herbal Garden in M.P. Council of Science and Technology, Bhopal, Madhya Pradesh (India). Screening of endophytic fungi for *in-vitro* antibacterial activity against three pathogenic bacteria *i.e. Streptococcus mutans*, *Lactobacillus acidophilus* and *Enterococcus faecalis* were observed by agar disk diffusion method. The diameter of inhibition zone was measured after 48 hrs incubation using a ruler. Antibacterial activity was calculated by measuring zone of inhibition produce by endophyte against pathogenic microbe. In the present study, our results indicate the broad antimicrobial spectrum of the bioactive components of fungal extracts isolated from *Saraca asoca*. Conclusively, the antimicrobial activity of these endophytic fungi is due to the extra cellular and intracellular bioactive components.

Keywords: *saraca asoca,* Antibacterial activity, Endophytic fungus, bioactive compounds, Secondary metabolites.

Introduction

The need for new a fungus is a eukaryote that digests food externally and absorbs nutrients directly through its cell walls. Most fungi reproduce by spores and have a body (thallus) composed of microscopic tubular cells called hyphae. Open most introductory mycology books and you'll see that there are four main groups (phyla) of true fungi Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota (eg., Alexopoulos *et al.* 1996 [1]; Webster and Weber 2007) [27]. Recent studies have provided support for the recognition of additional phyla, such as Glomeromycota, a group of fungi once placed in Zygomycota that form an association with the roots of most plants. Hibbett *et al.* (2007) [6] published a comprehensive classification of the Kingdom Fungi, the result of collaboration among many fungal taxonomists. This classification is used in the *Dictionary of the Fungi* (Kirk *et al.* 2008) [9] and other fungal references and databases. However, the classification system will undergo additional changes as scientists use new methods to study the fungi. For example, Jones *et al.* (2011) [7] described the "cryptomycota," a potentially new phylum of organisms within the Kingdom Fungi.

The association of fungi and plants is ancient and involves many different fungi. Fungi are an important group of plant pathogens most plant diseases are caused by fungi but fewer than 10% of all known fungi can colonize living plants (Knogge 1996) [10]. Most fungi are decomposers, utilizing the remains of plants and other organisms as their food source. An important group of fungi associated with plants is mycorrhizal fungi. Mycorrhiza means 'fungus root', and it refers to a mutually beneficial association between fungi and plant roots. Endophytic fungal are associations differ from mycorrhizas primarily by the absence of a localized interface of specialized hyphae, the absence of synchronized plant-fungus development, and the lack of plant benefits from nutrient transfer the three key defining features of mycorrhizas (Brundrett 2004) [3]. Introduced the form taxon "dark septet endophyte" (DSE) and used it 2 for fungi that form partly or entirely melanised and having

septate thalli within healthy root tissues. (Stoyke and Currah, 1991) [22].

The most common usage of the term "endophyte for organisms whose infections are internal and inconspicuous, and in which the infected host tissues are at least transiently symptomless, is equally applicable to bacterial prokaryotes and fungal eukaryotes (Stone *et al.* 2000; Schulz and Boyle, 2005).

Fungal endophytes may inhabit tissues of roots, stems, branches, twigs, bark, leaves, petioles, flowers, fruit, and seeds. Plants housing the endophytic fungus are often more healthy with enhanced nutrient absorption capacity (Krishnamurthy *et al.* 2008) and it is estimated that there are over 1 million fungal endophytes exist in nature (Petrini 1991) [17].

The alarming situation of antibiotic resistance is presumed to be solved by utilizing the metabolites obtained from these funguses (Levy 2002) [13]. That is the main reason for focusing on endophytic fungus of medicinal plants, for developing various drugs, although they are present in very lower concentration, but are assumed to be more fruitful, safer and, better than synthetic drugs (Tan and Zou 2001) [25]. Endophytic fungus can be directly exploited, by in-vitro culturing, for the large scale production of active secondary metabolites, for various pharmaceutical and agro industries.

Materials and Methods

Collection of plant materials

Mature healthy leaves and Bark of medicinal plants (*Saraca asoca*) were collected from the Human Herbal Garden in M.P. Council of Science and Technology, Bhopal, M. P. (India) geographically located at 23'15''49.3N-77'24''22.5.560 E. Samples were immediately brought to laboratory in sterilized sealed packets and were used within 8 hrs. The age of plant was also considered during sample collection and isolation of endophytes.

Selection of plant for isolation of endophytes

It is very important to know the rationale used to make available the best opportunities to isolate endophytic microorganisms as well as production of novel bioactive compounds. There is a need to have creative strategies to quickly search for endophytes displaying bioactivity (Mittermeier *et al.* 1999) [14]. A selection criterion of plant is that it should have ethno botanical history. Plants that are endemic, and having an unusual longevity, also have occupied a certain ancient land mass and Plants growing in temperate region have huge biodiversity with prospects of hosting endophytes with enormous biodiversity.

Isolation of endophytic fungi from medicinal plant

Surface Sterilization

The removal of epiphytic fungi and other contaminations, washing and surface sterilization of samples is done. The samples were washed in running tap water to remove soil particles and adhered debris, and finally washed with distilled water. Subsamples were prepared from each sample for further isolation of endophytes. Samples were immersed in 70% ethanol for 1-3 min and 4% aqueous solution of sodium hypochlorite 1.5 min, l min with 70 % ethanol again and finally rinsed 4-5 times with sterile distilled water. Samples were selected by aseptic cutting using sterile knife and inner tissues were excised. Later the segments were rinsed three times with sterile distilled water and were blotted on sterile blotting paper. All the work was performed in the laminar air flow using sterile glassware and mechanical instruments, such as scissor, forceps, scalpel, blades during all experiments. Samples were cut into small pieces of 1 cm long and 3-4 mm broad in size.

Isolation of Endophytic Fungi

The dissected and sterilized tissues were placed on potato dextrose agar, with50mg/l chloramphenicol. Endophytic fungi usually began to produce hyphal filaments after 5-6 days of incubation at 27 ^{0}C. The hyphal tips appeared on plate were carefully picked up and transferred to potato dextrose agar (PDA) plates as stock culture. Long time preservation of plant material was avoided and precaution taken to avoid further contamination after collection of plant material. The imprint of isolation plates were examined for the sterility. If any growth observed on the imprint plate then the whole batch was discarded.

Preservation of fungal cultures

Endophytic fungal pure culture were preserved on the Potato Dextrose slant at 6 ^{o}C and each tube was labeled with code number of the host plant and isolate code with date of isolation. Replicate were made for each isolates and appropriate media was used according to the need of the organisms.

Fungal extraction using Ethyl acetate

The endophytic fungi were cultivated in an Erlenmeyer flasks containing Potato Dextrose Broth. The flasks were then kept in a shaker incubator in order for the fungal balls to grow. After formation of the fungal balls, it was then separated from the filtrate, by filtering it using a Whatman filter paper No.1 and a funnel. The Fungal balls or the cell mass was then immersed in Ethyl Acetate. After 4 hrs, the fungal balls were then crushed using mortar and pestle and it was filtered again. The Filtered filtrate was kept for air dry. After complete evaporation of ethyl acetate, extract was scrapped off by adding 1ml of Dimethyl sulphoxide (DMSO).

Antibacterial test using Disk diffusion method

Anti-bacterial activity was screened using dual culture method in which both endophytes and test microbes were inoculated in same media plate. Antibacterial potential was tested against test bacteria like *Lactobacillus planatarum* (MTCC-1325), *Streptococcus mutans* (MTCC- 890), *Streptococcus mutans* (MTCC-497), *Lactobacillus acidophilus* (MTCC-10307), *Enterococcus faecalis* (MTCC- 439).

Test bacterial Culture

Preserved bacterial cultures were revived in nutrient broth and incubated at 37 ^{0}C for 24 hrs. 5mm disks were prepared using Whatman filter paper (40 No.) and were autoclaved to avoid contamination.

Disk Diffusion Assay

For disk diffusion method the bacterial suspension of 24 hrs old culture of pathogenic bacteria was spread on sterile nutrient agar plate with sterile cotton tipped swab to form an even lawn. Sterile paper disks (6 mm in diameter) impregnated with extract was placed on the surface of each nutrient agar plate using a sterile pair of forceps. The plates were incubated aerobically at 37 ^{o}C for 24 hrs. The diameter of inhibition zone was measured after 48 hrs incubation using a ruler.

Identification of Endophytes Fungus

Cultures on PDA media were assessed according to their morphology. Colony appearance, mycelium color and structure, shape of conidiomata, conidia and conidiophore (size, color, ornamentation, etc.) and characters of conidiogenous cells were observed for morphological classification of isolated fungi using a light microscope with 5X, 10X and 40X objective lenses for magnification.

Results

Plants are important source of potentially useful structures. But due to their depletion, concerns have shifted upon their endophytic fungi and bacteria to harness those novel structures. In the present study, our results indicate the broad antimicrobial spectrum of the bioactive components of these fungal extracts. Conclusively, the antimicrobial activity of these endophytic fungi is due to the extracellular and intracellular bioactive components.

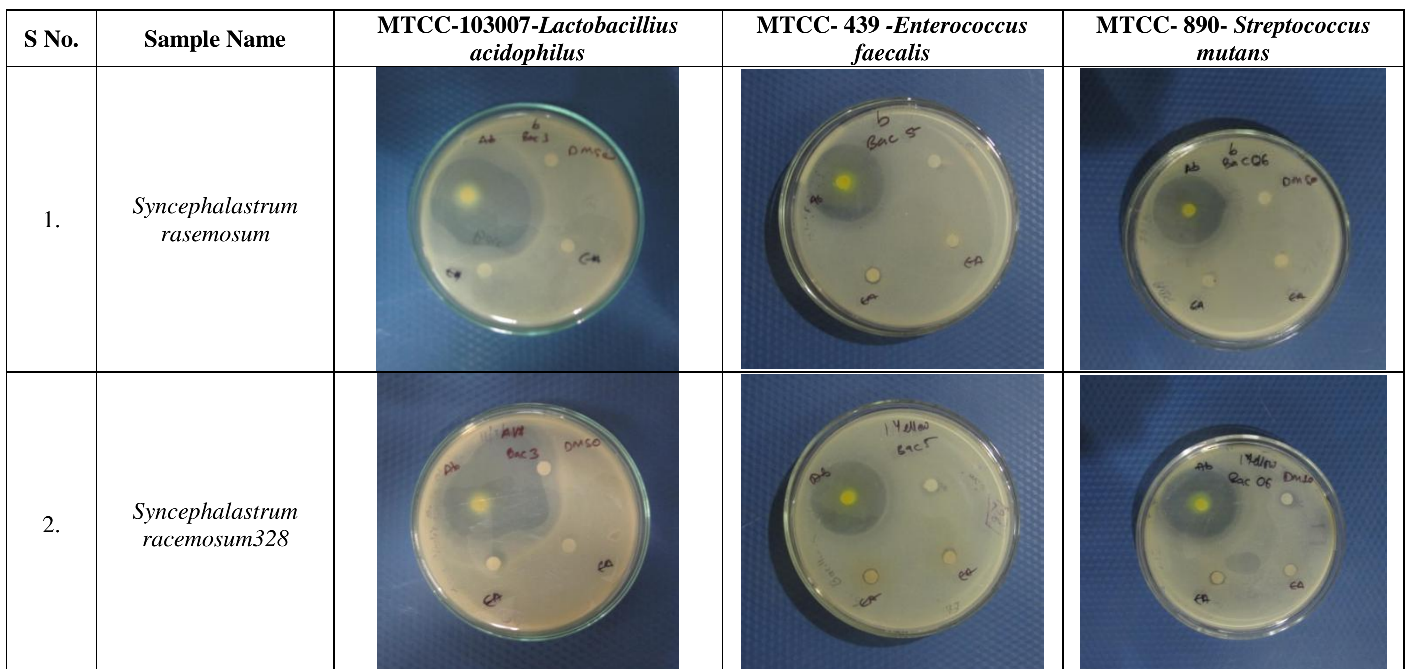
Antibacterial test by disk diffusion method

The leaves and bark of the tree have been reported to have Antimenorrhagic Activity, Anticancer Activity, Antibacterial Activity and Antioxytocic Activity, etc. Thus, Ethyl acetate extract of these fungi was prepared to check Antibacterial activity against 3 strains of bacteria (MTCC – 103007–*Lactobacillius acidophilus*, MTCC – 439-*Enterococcus faecalis*, MTCC-890- *Streptococcus mutans)*.

The zone of inhibition was given by *Syncephalastrum rasemosum* against *Lactobacillius acidophilus* (0mm), *Enterococcus faecalis* (1.2mm), *Streptococcus mutans* (0mm) and *Syncephalastrum racemosums328* against *Lactobacillius acidophilus* (0mm), *Enterococcus faecalis* (2mm), *Streptococcus mutans* (2mm).

Table 1: antimicrobial test of 3 bacteria with zone of inhibitions

S No.	Extracts of Endophytic Fungus	Zone of inhibition (in mm)		
		L. acidophillus	*E. faecalis*	*S. mutans*
1.	*Syncephalastrum rasemosum*	-	1.2	-
2.	*Syncephalastrum racemosum328*	-	2	2



S No.	Sample Name	MTCC-103007-*Lactobacillius acidophilus*	MTCC- 439 -*Enterococcus faecalis*	MTCC- 890- *Streptococcus mutans*
1.	*Syncephalastrum rasemosum*			
2.	*Syncephalastrum racemosum328*			

Fig 1: Antimicrobial activity of extracts of endophytic fungi from *saraca asoca.*

Discussion

0ne of the most important properties of endophytic microorganisms, especially fungi is linked to their metabolic potential to produce a large variety of bioactive molecule that can protect the plant against pathogens (Tan and Zou, 2001; Storbel, 2003) [25, 23]. For example, natural compounds synthesized by endophytic fungi have been reported as inhibitors of a wide variety of animal and plant pathogens (Wiyakrutta *et al*. 2004; Gunatilaka, 2006; Zhao *et al*. 2011b) [29, 5, 30]

The isolation and identification of endophytic mycobiota is necessary, since the medicinal properties of a plant can be a consequence of the capacity of its endophytic microorganisms to produce biologically active secondary metabolites (Kaul *et al*. 2012; Kusari *et al*. 1993) [8].

In this study, our results indicate the broad antimicrobial spectrum of the bioactive components of these fungal extracts. Conclusively, the antimicrobial activity of these endophytic fungi is due to the extracellular and intracellular bioactive components.

From the total fungal isolates obtained from plant *Saraca asoca*, similar colonization frequencies were obtained for *Syncephalastrum racemosum* (98%) and *Syncephalastrum racemosum328* (97%). From the endophytes isolated from *Saraca asoca*, 2 were selected and grouped into morpho groups, according to their morphological characteristics, such as colony coloration, pigment formation, development, and growth of mycelial colonies on potato dextrose agar.

By using data from the sequencing analysis of the ITS1-5.8S-ITS2 region of DNA and through BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/) of the Gen Bank database, it was possible to identify 2 fungal isolates from the total of 14 endophytes studied.

Endophytes	Host Plant	Closely related Fungal Sequences	Identity (%)
1	*Saraca asoca*	*Syncephalastrum rasemosum*	98
2	*Saraca asoca*	*Syncephalastrum racemosum328*	97

Conclusion

This study indicates that endophytic fungi isolated from different tissues of *Saraca asoca* have pharmaceutical bioactive compounds with antibacterial potential. This may be due to the fact that endophytic microorganisms produce bioactive secondary metabolites. Crude extracts from endophytes of *saraca asoca* showed in this study a greater antimicrobial activity against some human pathogenic bacteria. So studies on safety and efficacy should be performed for these fungi for use as pharmaceutical drugs. Our results indicate the broad antimicrobial spectrum of the bioactive components of fungal extracts isolated from *Saraca asoca*.

Abbreviations used

DSE: Dark Septet Endophyte; PDA: Potato Dextrose Agar; DMSO: Dimethyl Sulphoxide; ITS: Internal Transcribed Spacer; BLAST: Basic Local Alignment Search Tool.

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