Iranian Journal of Pharmaceutical Sciences
Summer 2010: 6(3): 217-222
ijps.sums.ac.ir

Original Article

Antimicrobial Activities of Isolated Endophytes from Some Iranian Native Medicinal Plants

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Abstract

Random samples from asymptomatic leaves and branches of five native medicinal plants: *Stachys lavandulifolia, Rumex pulcher, Hypericum scabrum, Starja bachteriarica* and *Achillea kellalensis* were collected from Chaharmahal province of Iran and examined for the presence of endophytic bacteria and fungi with biological activity. From 8 isolated endophytic fungi, all displayed considerable activity against at least one indicator fungi. Fungal isolates from *R. pulcher* leaves and branches showed activity against *Aspergillus niger*, *Penicillium spp*, *Alternaria* spp and *S. aureus*. Five Bacillus *spp* strains were isolated from *R. pulcher* leaves and branches, four (80%) showed activity against *S. aureus*, and two strains were active against all indicator fungi. Bacillus *spp* strain isolated from leaves of *H. scabrum* was active against *S. aureus and all* 3 indicator fungi. None of the isolated endophytes showed antibacterial activity against *E- coli*.

Keywords: Antimicrobial activity; Endophytes; Iran; Medicinal plants.
Received: February 3, 2010; *Accepted:* April 11, 2010.

1. Introduction

A variety of microorganisms, mainly bacteria and fungi, inhabit plants and are, therefore, known as endophytic. Bacon and White [1] give an inclusive and widely accepted definition of endophytes: "microbes that colonize living, internal tissues of plants, without causing any immediate negative effect".

Nowadays scientific communities have become interested in bioprospecting these microorganisms due to their potentially important secondary metabolite production for applications particularly in the pharmaceutical and food industries [2]. Novel antibiotics, immunosuppressant and anticancer compounds are only a few examples of what has been found after isolation, culture, purification and characterization of some endophytes in the recent past [3].

The question is whether these substances are produced by the plant itself or as a consequence of a mutualistic relationships with beneficial organisms in their tissue. Many reports showed that in a microbe-plant relationship, endophytes contribute substances

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that possess various types of bioactivity, such as antibacterial and antifungal [2, 4, 5]. In Iran, extracts from many types of local plants are used in traditional manner for treatments of various ailments. Thus, in this study, we focus on the isolation of endophytes from some native medicinal plants of Chaharmahal province in west center of Iran and screening them for antimicrobial activities.

2. Materials and methods

2.1. Collection of plant samples

Random samples from asymptomatic leaves and branches of five native medicinal plants namely: *Stachys lavandulifolia, Rumex pulcher, Hypericum scabrum, Starja bachteriarica* and *Achillea kellalensis* were collected in Chaharmahal province of Iran in Spring 2009.

Leaves and branches portion were thoroughly washed in running tap water, after which they were surface sterilized by submerging them in 75% ethanol for 2 min. The portions were further sterilized sequentially in 5.3% sodium hypochlorite solution for 5 min, and 75% ethanol for 0.5 min. After drying, each leaf was divided into segments. For isolating endophytic fungi 3-4 segments were placed on potato dextrose agar (PDA) supplemented with 50 mg/l chloramphenicol to suppress bacterial growth. Then branch portions were cut to expose their inner tissue and placed on the same medium. All the plates were incubated at 25 °C for up to 3 weeks. Emerging fungi were transferred to fresh PDA plates, incubated for 1 week and periodically checked for purity [4]. For isolation of endophytic bacteria, the disinfected portions were distributed onto the isolation media, yeast extract agar (yeast extract 5 g/l, glucose 10 g/l, agar 20 g/l) (YEA) and peptone agar (15 g/l peptone and 12 g/l agar) (PA) and incubated at room temperature for 4 days [5]. The isolated fungi were preliminary identified according to their macroscopic characteristics. Preliminary bacterial identification was done using Gram staining, catalase and oxidase activity and biochemical tests on demand.

2.2. Assays for antibacterial activity

One gram-positive (Coagulase positive *Staphylococcus aureus* RITCC 1907) and one gram-negative (*Escherichia coli* RITCC 1662) bacteria were used as indicator microorganisms prepared from Razi institute collection in Karaj-Tehran.

2.2.1. A- Endophytic bacterial contents

For isolated endophytic bacteria selected colonies were diluted in peptone water (0.1%) and displayed as drops (Pasteur pipette) in PA and YEA media. Petri dishes were incubated at room temperature and 37 °C for 48 h, simultaneously. The bioassays were conducted using growing colonies in PA and YEA and inactivating them by chloroform (20 min.). Plates were opened (30 min.) to evaporate the substance. At the same time, the reactivation of *S. aureus* - coagulase-positive strain and *E coli* (BHI broth 24 h/37 °C) were made. 200 µl of the each culture properly reactivated were transferred to 10 ml of semi solid BHI medium and shaken. This mixture was deposited onto the surface of plates (YEA and PA) containing chloroform inactivated bacterial colonies. The plates were incubated (37 °C/48 h) for the observation of inhibition halos [5].

2.2.2. B- Endophytic bacterial broth culture:

To test antibacterial activity of endophytic bacterial culture broth, briefly, 100 µl of each indicator bacterial dilution (10^9 CFU ml) was added into 10 ml of YEA at 50 °C, mixed thoroughly and poured into a 9-cm diameter Petri dish. After solidification, two sterilized stainless cylinders (5-mm internal diameter and 10-mm high) were placed open end up on each plate. The culture broth of endophytic bacterial isolates grown in LB broth (18 h incubation at 37 °C), centrifuged at 10000 rpm

Table 1. Antimicrobial activity of the endophytic fungi of selected Iranian medicinal plant species.

Plant	plant part	Isolated fungi	*S. aureus*	*E- coli*	*Asp. niger*	Penicillium	Alternaria
S. lavandulifolia	B	s5	-	-	*# +	-	* # +
H. scabrum	L	b2a	-	-	* +	-	# +
		b2b	-	-	-	#+	#+
R. pulcher	L	b7	-	-	* +	# +	-
	B	s7a	*+	-	-	-	* # +
		s7b	* +	-	-	-	* +
A. kellalensis	L	b8	-	-	-	# +	* +
Sat. bachteriarica	L	b1	-	-	-	-	* +

L stands for leaves, B for branch, * for Filtrated culture broth and # for mycellial extract + Posetive.

for 15 min and filter-sterilized supernatants (100 µl of each) were poured in cylinders on each bacterial plate [6].

2.2.3. C-Endophytic fungi:

Isolated fungal endophytes were grown at 27 oC with shaking in tubes of 5 ml Glucose-yeast extract- trypton broth (YEB) medium (yeast extract 0.05%, glucose 2%, beef extract 0.05%) for one week. The mycellial mass were separated from liquid phase by centrifugation at 10000 rpm for 15 min, weighted and crushed aseptically, dissolved in sterilized distilled water (v/w) and followed by further centrifugation as mentioned. The supernatants were filter sterilized. Two ml of each indicator bacterial suspension (approximately 10^9 CFU/ml) was mixed with 8 ml of YEA at 50 °C, and the mixture was immediately poured in a 9-cm diameter Petri dish. After solidification, sterilized cylinders were placed on each plate. Filter-sterilized supernatant of culture broth and mycellial extract filtrate (100 µl) were added to the cylinders on each bacterial plate, and 48 h after incubation at 37 °C , the diameter of inhibition zone of the bacterial growth was recorded. The tests were repeated once. Tests with inhibition zones of more than 20 mm diameter were considered positive.

2.3. Assays for antifungal activity

Three saprophytic fungi (*Aspergillus niger, Penicillium spp* and *Alternaria spp*) from fungi collection of mycology lab of this college were evaluated as indicator fungi.

To assay antifungal activities of endophytic bacteria, two blocks of each indicator fungi (grown on Sabaroud dextrose agar supplemented with 50 mg/L chloramphenicol) were placed on two points of one YEA media with about 3 cm distance, then 50 µl of filter-sterilized supernatants of endophytic bacterial culture broth (10000 rpm for 15 min) was placed in between the blocks (block assay), [6]. The cultures were kept at 30 °C for 4 days.

For assaying antifungal activity of isolated endophytic fungi, the endophytes were grown in tubes of 5 ml YEB media at 27 °C with shaking, the culture broth filtrate and mycellial mass extracts prepared as mentioned in section C, and antifungal activity assayed as for endophytic bacteria. All tests were repeated once.

3. Results

A total of 7 endophytic bacteria and 8 fungal isolates were obtained from five different types of medicinal plants (Tables 1 and 2). Due to lack of sporulation identification of fungal endophytes were not possible. The results of this study showed that endophytes were more prevalent in the leaves (4/7 or 57.1% for bacteria and 5/8 or 62.5% for fungi) than the branches.

All of 8 fungal isolates displayed considerable activity against at least one indicator fungi. Isolates b2a, b2b, b7 and b8 showed antifungal activities against two indicator fungi.

However, none of fungal isolates were

Table 2. Antimicrobial activity of the endophytic bacteria of selected Iranian medicinal plant species†. Endophytes with negative antimicrobial effects are not shown

Plant	plant part	Isolated bacteria	*S. aureus*	*E- coli*	*Asp. niger*	Penicillium	Alternaria
S. lavandulifolia	L	G+ Cocci	+	-	-	-	-
H. scabrum	L	Bacillus spp	+	-	+	+	+
R. pulcher	L	Bacillus spp[a]	#*+	-	-	-	-
		Bacillus spp[b]	*+	-	+	+	+
		Bacillus sppf	#+	-	+	+	-
	B	Bacillus spp[a]	#* +	-	-	-	-
		Bacillus spp[b]	-	-	+	+	+

L stands for leaves, B for branch, * for Filtrated culture broth and # for cell contents †Antifugal effects assayed for filtrated culture broth.

active against all 3 saprophytic fungi. Fungal isolates from *R. pulcher* leaves and branches showed activity against *Aspergillus niger, Penicillium spp, Alternaria spp* and *S. aureus*. Antifungal activity of fungal endophytes of *A. kellalensis* leaves and *S. lavandulifolia* branches were positive against the *Penicillium* spp, *Alternaria* spp, and *Aspergillus niger*. No antibacterial activity was observed by the latter endophytes. Details are summarized in Table 1.

Five Bacillus spp strains were isolated from *R. pulcher* leaves and branches, four (80%) showed activity against *S aureus*, and two strains were active against all indicator fungi. Likewise Bacillus spp strain isolated from leaves of *H. scabrum* was active against *S. aureus* and all 3 indicator fungi, (Table 2).

4. Discussion

In this study, we demonstrated that filtrates from the culture of endophytic bacteria and fungi grown aerobically in LB and YEB media displayed antibacterial, and antifungal activities. These results suggest the presence of either good antimicrobial potency of the filtrates or of a high concentration of some active principles in the filtrates of strains showing positive biological activities. Other endophytic fungal and bacterial filtrates which showed low or lack of antimicrobial activity in the bioassays may have active compounds but probably in smaller amounts and/or the screened filtrates could yield more potent compounds once they had undergone some purification [7]. Also, extracts which showed no antimicrobial activity in these assays may be active against other microbes which were not tested.

There have been some studies on isolating and detecting antimicrobial activities of fungal endophytes from other medicinal plants. For example, Sette *et al*. [4] isolated and identified 25 fungal strains from Coffea Arabica and 14 fungal strains from Coffea robusta. Liu *et al*. [8] identified 16 fungal strains from the variant tissues of Argyrosomus argentatus while Son [9] obtained 121 fungal isolates from 62 different types of medicinal plants. However, in our study only eight distinct fungal endophytes were isolated from five examined medicinal plants. It is possible that there is less diversity of the fungal endophytes in the examined plants or in isolation methods some modification are required, so some other endophytes have not been isolated so far.

In our screening assays of *R. pulcher* endophytic culture filtrates, a wide variety of biological activities were detected (Tables 1and 2). Four (out of 5) isolated endophytic bacteria from this plant showed anti *S. aureus* activity, an important human pathogen. Slepecky and Hemphill [10] reported that most of the antibiotics produced by *Bacillus spp*. are active against gram-positive organisms, although there are exceptions.

Moreover culture filtrates of *Bacillus* spp[b] isolated from leaves and branches of *R. pulcher*, and *Bacillus* spp from leaves of H. *scabrum* inhibited mycellial growth of all 3 indicator fungi, indicating that the antifungal compounds in the filtrates exhibit a wide

spectrum of antifungal activity.

All filtrates from fungal endophytes of *R. pulcher* showed antifungal activity against at least one indicator fungi, and four (out of 5) isolated endophytic bacteria showed anti *S. aureus* activity. This plant is used for treatment of various ailments by local people. So, we suggest a more detailed study on endophytes of this medicinal plant. Eight out of 15 (53.3%) isolated endophytes from these medicinal plants showed anti *S. aureus* activity. Reports indicate endophytic microorganisms from other medicinal plants also show this activity [4, 5, 11].

None of the isolated endophytes showed antibacterial activity against *E-coli*. However, anti *E-coli* activity of endophytes from other medicinal plants is reported [11].

Data exhibited here suggests that examined Iranian medicinal plants are good source to search endophytic microorganisms, emphasizing the potential of natural compounds that can be used in agriculture, clinics and pharmaceutical industry.

In conclusion, endophytic microorganisms are a very promising source for production of bioactive compounds. Further investigations is suggested in order to classify the microorganisms and exploit the potential of the substance produced to inhibit pathogenic microorganisms.

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