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Altheae rosea as a source of endophytic fungi and antimicrobial activity

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ABSTRACT

A study was conducted to isolate and identify of endophytic fungi from *Altheae rosea* (medicinal plant). Nine hundred and thirteenth isolates were recovered from leaves and roots (old and young) of *Altheae rosea* plants, which belonging to 48 species of 25 genera, and deal with the effect of different organ extracts (fruits, leaves, roots and stems) of *Altheae rosea* which extracted by (n-hexane) on *Bacillus subtillus* and *Staphylococcus aureus*. In general the most sensitive bacteria was *Bacillus subtillus* and the most active organs were fruit followed by stems, roots and leaves.

INTRODUCTION

Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value. Recently, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led authors to investigate the antimicrobial activity of medicinal plants[6,22,25,16]. Moreover, the increasing use of plant extracts in the food, cosmetic and pharmaceutical industries suggests that, in order to find active compounds, a systematic study of medicinal plants is very important.

For thousand years, mankind has learnt about the benefits of plant use to alleviate or cure illnesses. The plant kingdom constitutes a source of new chemical compounds which may be important owing to their potential use in medicine and other applications (e.g. food and forage conservation). Plant extracts were regarded by ancient civilizations to be significant for the treatment of various ailments, and about 30% of the worldwide drug sales are based on natural products[14]. It is estimated that there are about 2,500,000 species of higher plants throughout the world, and most of them have not been examined in detail for their pharmacological activities[21]. However, for some decades, there has been increasing interest in plant uses and the detection of their constituents with antibacterial activity. The most important reason for the latter was that infections represent one of the main causes of illness and mortality around the world[47,15]. Endophytic fungi , these include a wide range of fungi characterized by the ability to live for certain period of their life cycle internally and asymptomatically in plants, are recognized as one of the most chemically promising groups of microorganisms in terms of diversity and pharmaceutical potential[17,43,10,35,39]. These endophytic microorganisms are ubiquitous and may increase the plant fitness by improving tolerance to heavy metals and drought, reducing the herbivory or phytopathogen settling[3], so that the relation between plant and endophytic fungi described as mutualism relation. Endophytic commonly present in almost all plants, some species of endophytic fungi have been identified as sources of anticancer, antidiabetic, insecticidal and immunosuppressive compounds[42,43,39].

MATERIALS AND METHODS

Isolation of endophytic fungi from *Altheae rosea*

Samples of leaves and roots of *Altheae rosea* were

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collected from different locations in South Valley University (Qena). They were transported in plastic bags and kept at 4^0C until microbial tests. Plant specimens were thoroughly washed in running tap water for 10 min[30]. Healthy leaves (distal, central and blade) surface were sterilized by immersion in 75% ethanol (1 min) followed by 0.93-1.3 M solution of sodium hypochlorite (3 min) and finally 75% ethanol (0.5 min)[23,11]. While, healthy roots were cut into 1 cm segments, surface sterilized by immersion in 75% ethanol (1 min) and 3.4% of sodium hypochlorite (10 min) and 75% ethanol (0.5min)[32]. Surface sterilized samples were washed with sterile distilled water (3 times) and dried between sterile filter paper. Plant segments were then transferred to glucose-Czapek's agar. Plates were incubated at 28 ± 2^0C for 2-3 weeks with 12 h cycle of dark and light. The growing edges of colonies were transfer to glucose-Czapek's agar slants by hyphal tipping[41], for further investigation.

Antimicrobial activity

Ten samples from each organs (fruits, leaves, roots and stems) of *Altheae rosea* were collected , subjected for washing in running tap water to remove dirt and were allowed to air and afterwards pulverized in grinder. Ten g of the pulverized materials were successively extracted with n- hexane (100 ml) at room temperature for 24 h on shaker [1]. The hexane extract dried with anhydrous Na_2SO_4, concentrated in vacuor and transferred to a glass viel with small amounts of n-hexane and evaporated under air.

Organisms and antibacterial assays

Two pathogenic bacteria (*Bacillus subtillus* and *Staphylococcus aureus*) were used as the test organisms, kindly isolated, identified and employed in the screening on nutrient agar medium at 30^0C.

Modified agar diffusion method[5] was used to determine antibacterial activities. The bacterial cell suspension was prepared from 24 h old culture and adjusted to 1×10^6 spore per ml. Sterile nutrient agar was inoculated with bacterial cells (200 Ml of bacterial cell suspension in 20 ml medium) and poured into dishes to give a solid plate.

The Altheae rosea extract (0.1mg) was evaluated by paper disc diffusion techniques (whatman No.3, 1cm) according the method of Janssen & Scheffer[19] and Janssen et al.[20]. the discs were placed on the plates seeded with spore suspension of bacteria and the plates were incubated at 37°C. The inhibition zone of bacteria growth, was measured with Vernier caliper and correlated with the degree of antibacterial activity.

RESULTS

Fungal endophytes of *Altheae rosea*

Nine hundred and thirteenth isolates were isolated from leaves and roots (old or young) of *Altheae rosea* plants which belonging to 48 species of 25 genera, *Aspergillus, Chaetomium, Cirrenalia, Cladosporium, Eurotium, Fusarium, Phoma,Tourla,* sterile mycelium 1,2 and unidentified fungi were the commonest among the fungal endophytes, which isolated from two organs in two stages (TABLE 1). *Fusarium* sp. (7 species), and *Chaetomium* sp (6 species) were the most common endophytic genera accounting for 17.7% and 11.9% of the overall total isolates, respectively. Comparatively fungal endophytes of the other genera and sterile mycelium were recovered less frequently which accounting 8.3% for *Aspergillus* (6 species) , 7.3% for *Cirrenalia* (1 species), 5.04% for *Cladosporium* (2 species), 8.3% for *Eurotium* (1 species), 4.1% for *Phoma* (1 species) , 1.6% for *Tourla* (1 species), 3.9% for sterile mycelium (black), 8.4% for sterile mycelium (white yellow), 2.4% for unidentified fungi of the overall total isolates.

Fungal endophytes of the remaining genera were isolated with high, moderate or low frequencies but not in all organs or all stages. It can be seen from the results the number of isolates from old leaves or roots were major than the number of isolates from young leaves or roots (TABLE 1)

Endophytes of leaf samples

Into the common genera isolated from leaf samples in two stages, *Alternaria, Aphanocladium, Drechslera, Microascus, Monilia, Nigrospora, Penicillum, Ulocladium* and sterile mycelium (yellow) were recoverd in two stages with moderate or low frequencies, comprising 2.1, 2.6, 1.2, 3.6, 1.9, 4.1, 4.3, 2.1 and 0.96% of total leaf fungal counts in two stages, respectively. While, the other species were isolated in one stage of leaf age. *Acremonium, Emericella* and *Myrothecium* were isolated from old leaves only which comprising 6.7, 0.7% and 2.8% of total isolates, re-

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TABLE 1 : Total counts (calculated per 150 young or old leaves segments), percentage counts (%C, calculated per total fungi) of fungal genera and species recovered from young and old leaves of *Altheae rosea* plants on glucose-Czapek's agar at 28±2°C

Fungal genera and species	Young leaves		Old leaves		Overall	Young roots		Old roots		Overall
	TC	C%	TC	C%		TC	C%	TC	C%	
Acremonium Link	0	0	19	6.7	4.5	6	3.3	11	3.5	3.4
Alternaria alternata (Fr.) Keissler	4	2.9	5	1.8	2.5	0	0	0	0	0
A. phanocladium album(Preuss) W.Gams	3	2.2	8	2.8	2.625	0	0	0	0	0
Aspergillus	12	8.9	21	7.4	7.9	16	8.7	27	8.7	8.7
A. flavus link	0	0	0	0	0	5	2.7	9	2.9	2.8
A. niger Van Tieghem	4	2.9	4	1.4	1.9	3	1.6	7	2.3	2.02
A ochraceus Wilhelm	5	3.7	6	2.1	2.6	0	0	2	0.6	0.41
A terreus thom	1	0.7	5	1.7	1.4	5	2.7	9	2.9	2.8
A.versicolor (Vuill.)Tiraboschi	2	1.5	3	1.1	1.2	3	1.6	0	0	0.61
A..ustus (Bain.) Thom &Church	0	0	3	1.1	0.7	0	0	0	0	0
Chaetomium	3	2.2	43	15.1	10.9	28	15.3	35	11.3	12.8
Ch. atrobrunneum Ames	2	1.5	5	1.8	1.7	5	2.7	11	3.5	3.2
Ch. barilochense Calviello	0	0	0	0	0	17	9.3	10	3.2	5.5
Ch. dreyfussii V.Arx	0	0	7	2.4	1.7	0	0	0	0	0
Ch. hexagonosporium Carter& Malloch	1	0.7	5	1.8	1.4	0	0	0	0	0
Ch. globosum Kunze	0	0	25	8.8	5.9	6	3.3	14	4.5	4.05
Ch. variostiolatum Carter	0	0	1	0.3	0.24	0	0	0	0	0
Cirrenalia sp.	4	2.9	12	4.2	3.8	16	8.7	35	11.3	10.3
Cladosporium	10	7.4	19	6.7	6.9	13	7.1	4	1.3	3.4
Cla. cladosporioides (Fresen) de Vries	10	7.4	7	2.5	4.1	9	4.9	4	1.3	2.6
Cla. sphaerosperum Penzig	0	0	12	4.2	2.9	4	2.2	0	0	0.81
Domingella asterinarum *Petrak&Ciferri*	2	1.5	0	0	0.5	0	0	0	0	0
*Drechslera halode*s (Drechsler)Subeam.& jain	3	2.2	2	0.7	1.2	0	0	0	0	0
Emericella nidulans(Eidam) Vuillemin	0	0	2	0.7	0.48	0	0	5	1.6	1.01
Eurotium repens De Bary	18	13.3	26	9.2	10.5	7	3.8	25	8.04	6.5
Fusarium	38	28.15	32	11.3	16.7	33	18.03	59	18.9	18.6
F. anthophilium (A.Braun) Wollenw	2	1.5	4	1.4	1.4	0	0	0	0	0
F. dimerum (Corda) Sacc	3	2.25	0	0	0.72	0	0	0	0	0
F. merismoides *Corda*	0	0	0	0	0	0	0	2	0.64	0.41
F. moniliforme Sheldon	32	23.7	19	6.69	12.2	25	13.7	31	9.9	11.3
F. oxysporum Schlecht	0	0	1	0.35	0.24	0	0	3	0.97	0.61
F. sambucinum Fuckel	1	0.7	8	2.8	2.1	8	4.4	22	7.1	6.1
F. scripi Lambotte & Fautr	0	0	0	0	0	0	0	1	0.32	0.20
Microascus trigonosporus Emmons&Dodge	1	0.7	14	4.9	3.6	0	0	0	0	0
Monilia fructigena *Pres. Ex Fr*	2	1.5	6	2.1	1.9	0	0	0	0	0
Myrothecium roridium Tode	0	0	8	2.8	1.9	4	2.2	11	3.5	3.04
Nigrospora oryzae (Berk.&Br.)	5	3.7	12	4.2	4.1	0	0	0	0	0
Penicillum	9	6.7	9	3.2	4.3	1	0.55	0	0	0.20
P. chrysogenum Thom	1	0.7	2	0.7	0.72	0	0	0	0	0
P. *corylophilum* Dierckx	2	1.5	0	0	0.48	0	0	0	0	0
P. jenseni Zaleski	4	2.9	0	0	0.96	0	0	0	0	0
P. *verrucosum* Peyronel	2	1.5	7	2.5	2.1	1	0.55	0	0	0.20
Paecilomyces variotii Bain	1	0.7	0	0	0.24	0	0	0	0	0
Phoma sp.	4	2.9	17	5.9	5.01	4	2.2	12	3.9	3.2
Rhizopus stolonifer	0	0	0	0	0	0	0	4	1.3	0.81
Saccharomyces sp	0	0	0	0	0	4	2.2	0	0	0.81
Tourla herbarum(Pres.) Link	3	2.2	3	1.1	1.4	7	3.8	2	0.64	1.8
Ulocladium botrytis Preuss	2	1.5	7	2.5	2.1	0	0	3	0.97	0.61
Verticillum lateritium Berkeley	0	0	0	0	0	2	1.1	6	1.9	1.62
Sterile mycelium (black)	2	1.5	5	1.8	1.7	3	1.6	26	8.4	5.87
Sterile mycelium(white yellow)	4	2.9	11	3.9	0.96	33	18.03	29	9.3	1.01
Sterile mycelium (yellow)	2	1.5	2	0.7	3.6	0	0	5	1.6	12.55
Unidentified fungi	3	2.2	1	0.35	0.96	6	3.3	12	3.9	3.64
Gross total count	135		284			183		311		
No. of Genus	20		20			15		16		
No of Species	32		31			23		27		

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spectively. Both *Domingella* and *Paecilomyces* were recovered from young leaves with low frequencies accounting for 1.5 and 0.7 % of total leaf isolates, respectively.

Endophytes of root samples

Acremonium, Myrothecium and *Verticillium* plus *Aspergillus, Chaetomium, Cirrenalia, Cladosporium, Eurotium, Fusarium, Phoma, Tourla,* sterile mycelium (black, white & yellow) and unidentified fungi, were the most common endophytic fungi isolated from *Altheae rosea* root samples in two stages comprising 3.4, 3.04 and 1.6% of total root isolates, respectively. In contrast, *Emericella, Ulocladium, Rhizopus* and sterile mycelium (yellow) were isolated from old roots only. *Rhizopus* and *Ulocladium* accounting 1.3 and 0.96% of total root isolates, respectively , while each of the other genera comprising 1.6% of total isolates. *Penicillum* and *Saccharomyces* were recovered from young roots only comprising 0.5 and 2.2% of total fungi, respectively.

Antimicrobial activity of *Altheae rosea* organ extracts

The study deals with the effect of different organs (fruits, leaves, roots and stems) extracts (n-hexane) of *Altheae rosea* (each, 10 samples) against two bacteria species(*Bacillus subtillus* and *Staphylococcus aureus*).The inoculated plates were incubated at 30^0C for 1-2 days. It can be seen that fruit extracts of *Altheae rosea* had the highest activity against the two species. All extracts (10 extracts) had clear effect, on the growth of *Bacillus subtillus*, which the activity ranged between 11-20 mm. But *Staphylococcus aureus* was less (4/10 extracts, 5-7 mm). Stem extracts occupied the second place in activity, where 9/10 extracts were activity against *Bacillus subtillus* (9-18 mm), while no effect could be detected of stem extracts on *Staphylococcus aureus* growth. The root extracts ranked the third place of the activity, (7/10 extracts and 7-11 mm), on the growth of *Bacillus subtillus* , while (1/10 extract) appeared low activity. No clear effect on the growth of *Staphylococcus aureus*. The leaf extracts do had not any effects on the growth of two bacteria species.

TABLE 2 : Effect of different *Altheae rosea* organ extracts (n-hexane) on some bacteria growth (mm)

Samples	Fruits		Leaves		Roots		Stems	
	B.subtillus	*S.aureus*	*B.subtillus*	*S.aureus*	*B.subtillus*	*S.aureus*	*B.subtillus*	*S.aureus*
1	11	7	-	-	-	-	-	-
2	19	7	-	-	-	-	17	-
3	19	-	-	-	10	-	12	-
4	17	-	-	-	8	-	16	-
5	18	-	-	-	9	-	15	-
6	19	-	-	-	10	-	17	-
7	10	-	-	-	7	-	14	-
8	20	-	-	-	9	-	16	-
9	20	6	-	-	8	-	9	-
10	20	5	-	-	11	-	18	-

DISCUSSION

In this study with deal with endophytic fungi and medicinal plants, because endophytic fungi are associated with living tissues, and may in some way contributed to the well being of the plant. 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[27,36,26,24,24]. Whereas, medicinal plants have been used as sources of medicine in virtually all cultures[4]. And endophytic fungi considered a rich source of bioactive metabolites which used as antibacterial, anticancer, antifungal or antitumor[35,39,50,34,44]. The results were presented in this study showed that the number of fungal species were isolated from old leaves and roots(284, 311 isolates, respectively) are more than the number of fungal species that isolated from young leaves and roots (135, 183, respectively). Many investigations showed that foliar and stems endophytes are that overall infection frequencies increase with the age of host organs and tissues[12,38,29,30,31]. Hoff et al.[18] explained that endophytic fungi usually occur in above ground plant tissue but are also found in root. Unlike mycorrhizal fungi, fungal endophytes of roots lack extraradical (outside the root) hyphal networks and mantles(sheaths around the roots).

Nine hundred and thirteenth isolates were recovered from leaves and roots (old and young) of *Altheae rosea* plants belonging 48 species of 25 genera, *Aspergillus, Chaetomium, Cirrenalia, Cladosporium, Eurotium, Fusarium, Phoma, Tourla,* sterile mycelium (black , white & yellow) and unidentified fungi were the common genera among the fungal endophytes which isolated from two organs in two stages. The above men-

tioned species were previously isolated by several researcher from different plants. [2,46]. Ananda & Sridhar[2] isolated *Aspergillus, Cladosporium, Fusarium oxysporum, Phoma* and four sterile mycelium from root segments of four mangrove plant species on the west coast of India, and isolated all the above species plus *Cirrenalia* sp.from root segments of *Rhizophora mucronata* samples, while Wijeratne et al.[46] recovered *Chaetomium chiversii* as endophytic fungi from Sonoran desert plant, and all of these species were recovered as endophytic fungi from *Amaranthus hybridus, Theobroma cacao* L., *Quercus cerris* plants by Ragazzi et al.[28], Rubini et al., 2005[32], Bloodgett et al., 2007[7], respectively.

Into the common genera isolated from leaves in two stages, *Alternaria, Aphanocladium,Drechslera, Microascus, Monilia, Nigrospora, Penicillum, Ulocladium,* and sterile mycelium(yellow) were recovered from leaves in two stages with moderate or low frequencies, comprising 2.2, 2.6, 1.2, 3.6, 1.1, 4.1, 4.3, 2.2 and 0.96% of total fungal count in two stages. Fisher et al.[13] isolated *Alternaria, Nigrospora oryzae* and sterile mycelium with moderate or low counts from leaves of *Gynoxis deifolia*. Caruso et al.[8] reported that *Alternaria* was isolated from woody tissues and herbaceous tissues of different trees belonging to the genus *Taxus*, and can be considered a resident genus of *Taxus* tissues, in addition to *Drechslera, Penicillium* and sterile mycelium were also isolated, *Ulocladium* sp were also isolated from healthy and declining trees of *Quercus cerris*[28]. *Acremonium, Emericella, Myrothecium, Domingella* and *Paecilomyces* were recovered from leaves of *Altheae rosea* in one stage, most of these species were isolated by several researchers from different plants[41,13,45].

Of root samples

Acremonium, Myrothecium and Verticillium were isolated from roots in the two stages, which comprising 3.4, 3.04 and 1.6% of total isolates. *Acremonium* and *Verticillium* were isolated as endophytic fungi by many authors[28,32,28], isolated *Acremonium* from *Quercus cerris* trees from twigs and buds of healthy trees and declining trees comprising 5.7, 5.3 and 7.1, 6.3% of total fungi, respectively, while Rubini et al.[32] isolated *Acremonium* and *Verticillium* from branches of *Theobroma cacao*. The remaining genera which were isolated from *Altheae rosea* roots were reported a, endophytic fungi by several investigations[8,9,28,45].

Antimicrobial activity

The study deal with the effect of different organs extracts of *Altheae rosea* on *Bacillus subtillus and Staphylococcus aureus* growth, Sarker et al.[33] reported that most of the antibiotics available today come from natural origin, espically from medicinal plants which produce compounds to protect themselve from microbial attack, Youssef[48] showed that the aqueous extracts of 55 kinds of medicinal plants and species were generally more effective on bacteria (Gram +ve and Gram -ve) Shan et al.[37] explain that from the 46 spice and medicinal herb hydrophobic extracts tested, twelve exhibited high antibacterial activities against the five foodborne bacteria, and showed that Gram postive is more sensitive than Gram negative bacteria because its cell wall structure and outer membrane[49]. Al-Fatimi et al.[1] reported that the results of the antibacterial screening of total of 90 extracts of 30 species against five bacteria species, showed 45 extracts were found to be active against *Staphylococcus aureus* and 51 extracts against *Bacillus subtillus*. In general the most active organs was fruit followed by stem, root and leaf.

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