Fungal endophytes of some medicinal plant growing in northwestern coast of Egypt: Isolation, identification and two-way clustering analysis of its antimicrobial activity

Mohammad Magdy El-Metwally (mmmyco@gmail.com)
Damanhour Fac. of science https://orcid.org/0000-0002-3857-8794
Atia Mohamed Eisa
botany and microbiology, fac of science, damanhour university
Amal Al Mekawey
regional center of mycology and biotechnology, alazhar Univ.,
Samy F. Mahmoud
Taif University College of Science
Yasser El Halmouch
Damanhour University Faculty of Science

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Abstract

One of hidden mine of antibiotics is endophytic fungi especially that inhibited medicinal plants. In this regard, leaves, stems, fruits and bulbs of some commonly medicinal plants growing in Northwestern coast of Egypt were subjected for isolation of endophytic fungi with screening study of its antimicrobial activity. Practically, more than one hundred (101) endophytic fungal species isolated from *Scorpiurus muricatus*, *Mellilotus indicus*, *Lotus polyphyllos*, *Ononis vaginalis, Nicotiana glauca, Lycium europaeum, Asphodelus aestivus, Echium angustifolium,Fagonia cretica*, *Pancratium maritimum*,and *Carduus getulus* were tested *in vitro* for their antimicrobial activities against *E. coli, Pseudomonas argenosa, Staphylococcus aureus, Bacillus subtilus, Candida albicanus, Candida glabrata, Penicillium expansum, Aspergillus flavus*. The values of applied diversity indices revealed significant differences in presence, absence and abundance among endophytic fungal isolates. To the best of our knowledge, the present study is the first to report of *Alternaria pluriseptata* as endophytic species with most dominant and most active in its anitimicrobial activity among the isolated species. Seven distinctive groups were revealed from the two-way cluster analysis showing the intensity of antimicrobial activity against tested pathogens: Twenty-five percent of the isolates (26 strains assembled together in group V) exhibited no antimicrobial activity against all tested pathogens while six percent (6 isolates) assembled in group VII revealed high antimicrobial activity against five pathogens.

Introduction

The Northwestern coast of Egypt is one of the richest phyto-geographic regions with unique varieties of medicinal plant species. This coastal strip, with the weather being between pleasant and hot during summer and mild in winter and with its various ecosystems, offers important habitats for natural resources and their development. Hundred and eighty-eight plant species were recorded as medicinal species in the western Mediterranean habitats (Bidak et al.2013). Folk medicine was and still exploiting medicinal plants for thousands of years for their healing effects and health benefits. Isolation of plant-derived drugs (very effective and have no side effects) is rapidly evolved in recent years. However, the natural resources of medicinal plants are challenging over-use practices at which the bioactive products accumulate at low levels in the native medicinal plants. Endomicrobiome of medicinal plant species may be responsible for the production of a wealth of explored and unexplored bioactive compounds (Venieraki et al 2017). Although, medicinal plants harbored many endophytic fungi that are active in the co-production of active metabolites, a very few species were studied relative to their endophytic biology out of 420,000 plant species exist in nature. Endophytes contribute with considerable benefits to the host plant, where they act as a growth-promoting agent for host plants, besides, to enhance resistance to biotic and abiotic stresses (Hardoim et al.2015). Endophytic fungi grow inside plant tissues in specific ecosystems and produce multiples of secondary metabolites such as antioxidant, anticancer, immunomodulatory, antivirus, antituberculosis, anti-parasite and insecticides that show biological activities (Hussain et al.2014). In Egypt, screening medicinal plant species for endophytes and investigating their antimicrobial activities are a promising trend increasingly, attracted the

microbiologist's attention lately. El-Maghraby et al. (2013) has explored three leguminous plant roots for Endophytic fungi in Egypt. Basheer et al. (2018) isolated the endophytic fungi from *Avicennia marina* and investigated the antibacterial and antifungal properties of their crude extracts against some important human pathogens. Plant growth-promoting activities for bacterial and fungal endophytes isolated from *Teucrium polium* L. were investigated by Saad El-Din Hassan (2017). Saad et al (2019) studied the potentiality of endophytic fungi as bio-control agents against the cotton leaf worm, *Spodoptera littoralis*. Although multiple of endophytic fungi studies were carried out on members of the Egyptian medicinal plants, Egyptian flora is full of extraordinary varieties waiting for exploring new good sources of biologically active compounds specifically from endophytic fungi. The present study aims at screening wider base of high value medicinal plants growing naturally in northwestern coast of Egypt (Matrouh area) and investigates the antimicrobial activities against some selective pathogens.

Materials And Methods

Plant Collection and Identification

Healthy (showing no visual disease) of eleven plant species belonging to seven families: *Fabaceae* (*Scorpiurus muricatus* L., *Mellilotus indicus* (L.) All., *Lotus polyphyllos* E. D. Clarke and *Ononis vaginalis* Vahl, Symb.) *Solanaceae* (*Nicotiana glauca* Graham and *Lycium europaeum* L.) *Asphodelaceae* (*Asphodelus aestivus* Brot.) *Boraginaceae* (*Echium angustifolium* Mill.) *Zygophyllaceae* (Fagonia cretica L.) *Amaryllidaceae* (*Pancratium maritimum* L.) and *Asteraceae* (*Carduus getulus* Pomel) were collected from Matrouh area at the west northern coast of Egypt. This phytogeographical region was selected according to the richness of habitats; sand dunes, rocky ridges, calcareous rocks and salt depressions that could play a pivotal role of existence and activity of the endophytes. Plant specimens have been identified to the species rank according to Boulos (2002).

Surface Sterilization and Isolation of Endophytic Fungi

Plant materials were washed under running tap water, followed by distilled water. Secondly, surface sterilization of plant materials were then done by sequentially rinsing with 70% ethanol for 30 sec., followed by 0.5% sodium hypochlorite for 2–3 min, and then rinsing in 70% ethanol for nearly 2 min, and finally with sterile distilled water 2–3 times. Plant materials were then dried in two folds of sterile filter papers. After sterilization, the plant materials were further cut (aseptically) to expose the interior surface to the nutrient media. For each plant, five segments from root stem and leaves were transferred to petri dishes containing potato dextrose agar (PDA) amended with chloramphenicol 500 mg/l (Al-Mahi et al.2003). The dishes were incubated at 28 ± 2°C for 3–6 days.

Purification, Selection and Preservation of Endophytic Fungi

Isolation from examined plants was done by the transfer of the hyphal tips to fresh PDA plates free of antibiotics to obtain pure cultures for identification. The purified endophytic fungal isolates were then transferred separately to PDA slants and maintained at 4°C till further use.

Identification of endophytic Fungi

An Image analysis system, soft imaging system GmbH software (analySIS ® pro ver. 3.0) at The Regional Center for Mycology and Biotechnology Al- Azhar University (RCMB) was used for examining the alteration of the tested fungal morphological features. The cultures were examined microscopically after 5–7 days incubation for dermatophytes, 24 hours for yeast and/or yeast-like fungi and 3–4 days for other fungi, using light microscopy at magnification of X 400 for multicellular fungi, while X1000 for unicellular fungi, using either phase-contrast or bright field optics under video camera. For each isolate 3–5 plates were prepared and a minimum of 20 microscopic fields were examined. Raper and Thom C (1949), Gilman (1957), Raper and Fennel (1965), Ellis and Booth (1971) and Domsch et al. (1980).

Fermentation

Twenty five ml of malt glucose broth was distributed in 100 ml conical flasks and autoclaved at 121°C for 15 min. The isolated endophytic fungal strains were inoculated aseptically into all flasks. The flasks were incubated at 28 ± 2°C for 10 days for growth. The flasks were examined periodically for any contamination. After 10 days, culture media were centrifuged at 6,000 rpm for 35 min. Antimicrobial activity of culture supernatants of endophytes was evaluated by agar well diffusion method using Mueller Hinton agar medium for the bacteria and Sabouraud Dextrose agar for yeast and fungi. By using sterile cork borer, about 5 mm size wells were made and 200 µl of each culture supernatant was added into it. The diameter of inhibition zone (millimeter) were measured after incubation at 37 ± 2°C for 24 h for bacteria and at 28 ± 2°C for 3 days for fungi (Sutjaritvorakul et al.2011).

Determination of Antimicrobial activity

List of microbial pathogens included *E .coli, Staphylococcus aureus, Bacillus subtilus, Pseudomonas argenosa, C. albicanus, Penicillium expansum, Candida glabrata, Aspergillus flavus* (All microorganisms were maintained at 4°C on nutrient agar slants for bacteria, Malt Glucose Agar slants for fungi.) Agar Disc well diffusion method was used for the antimicrobial susceptibility testing. Antimicrobial potentialities were expressed as the diameter of inhibition zones. Extracts were examined as antimicrobial agent against all microbial isolates. Inoculum suspensions of all bacteria and fungi isolates were spread on the surface media. Four equidistant (1 cm diameter) holes were made in the agar using sterile cork borer in media plates (10 x 10 cm), which had previously been seeded with bacteria and/or fungi tested, were filled by 100 µL of each extract. Discs injected with 100 µL of respective 50 mM sodium phosphate buffer and organic solvent were used as negative control. Standard antibiotic was used as positive control ciprofloxacin (100 µL/disc) (Oxoid) and fluconazole (100 µL/disc) (Oxoid) were used as a positive reference for bacteria and fungi, respectively. Plates were left in a cooled incubator at 4 (± 2) °C for one hour and then incubated at 37 (± 2) °C for 24 hour for bacterial growth and 28ºC for 48 hours for fungal growth. Inhibition zones developed due to active extract ingredients were measured after 24–48 hours of incubation. The experiments were carried out in triplicates and

the inhibition zone was measured with the help of standard scale (Norrel & Messley, 1997)

Data Analysis

Data analysis was accomplished using PC-ORD ver. 5 to perform Two Way Cluster analysis, it is an agglomerative clustering method in which data matrix is classified twice, one time for the rows and the other is for the columns. Clustering dendrogram performed based on Sorensen and Flexible beta of -0.25.

Fungal endophytic Species frequency and Diversity indices

Frequency values of the final list of identified isolates were calculated based on the number of times a particular species presents in each plant organ, **relative frequency** values were calculated by dividing the frequency value for a particular species by the values for all species and multiply by 100%. **The isolation rate (IR, %)** was calculated by dividing the total number of isolates from specific tissues by the overall isolates number.

Species richness (Alpha diversity) was calculated after Whittaker (1972), who described it as the diversity within a particular area or ecosystem and it is expressed by the number of species in each stand.

Shannon Index: $$H' = -\sum_{i=1}^{S} P_i \ln P_i ,$$

Where pi is proportion of S made up of the ith species

$$D = 1 - \left(\frac{\Sigma\, n(n-1)}{N(N-1)} \right)$$

Where n = the total number of organisms of a particular species and N = the total number of organisms of all species

The species evenness (E): calculated after Pielou (1966) by the following formula: **E = H/Ln(S)**

Where H is Shannon value and (S) is the richness for each organ. Evenness defined as a diversity index, a measure of biodiversity which quantity how equal the community is numerically.

Results

Isolation and identification of endophytic fungi

A total of 101 endophytic fungal isolates belonging to 18 genera and 35 fungal species were isolated from vegetative organs of 11 selected medicinal plants growing naturally on the north-western coast of Egypt (Table 1). The isolated genera namely, *Alternaria, Phoma, Mucor, Scytalidium, Pythium, Ulocladium, Spegazzinia, Sarcinomyces Aspergillus, Cladosporium, Penicillium, Clafosporium, Rhizopus, Fusarium, Nigrosora, Trichocladium, Leptosphaeria and Aureobasidium.*

Table 1
List of endophytic fungal species isolated from 11 selected medicinal plants growing naturally on the north-western coast of Egypt

Name of plants	Plant organs	Fungal code No.	Name of fungal isolates
A- *Scorpiurus muricatus* L.	Fruits	1	*Alternaria pluriseptata*
	Stem	2	*Phoma dennissii*
		3	*Alternaria pluriseptata*
	Leaves	4,5	*Alternaria phragmospora*
		6	*Alternaria alternaria*
		7	*Alternaria pluriseptata*
B- *Faganiu cretica* L.	Leaves	8	*Alternaria pluriseptata*
		9	*Phoma glomerata*
		10	*Scytalidium japonicum*
		11	*Pythium nayoroense*
		16	*Ulocladium atrum*
	Stem	12	*Alternaria pluriseptata*
		13	*Alternaria chlamydospora*
	Fruits	14	*Mucor hiemalis*
		15	*Alternaria pluriseptata*
C- *Mellilotus indicus* (L.)All.	Leaves	17	*Penicillium aurantiogriseum*
		18	*Clafosporium cladosporioides*
		19	*Rhizopus stolonifer*
		20	*Sarcinomyces phaeomriformis*
		21	*Alternaria pluriseptata*
		22	*Aspergillus niger*
	Stem	23	*Penicillium aurantiogriseum*
		24	*Penicillium marneffei*

Name of plants	Plant organs	Fungal code No.	Name of fungal isolates
	Fruits	25	*Alternaria pluriseptata*
		26	*Alternaria phragmospora*
		27	*Spegazzinia pakeri*
		28	*Sarcinomyces phaeomriformis*
		29	*Cladosporium tenuissium*
		30	*Penicillium expansum*
		31	*Penicillium roqueforti*
D- *Carduus getulus* Pomel	Stem	32	*Alternaria pluriseptata*
		33	*Ulocladium alternariae*
		34	*Rhizopus oryzae*
	Leaves	35	*Alternaria pluriseptata*
		36	*Rhizopus microspores*
	Fruits	37	*Pythium elongutum*
		38	*Pythium nayoroerse*
		39	*Alternaria pluriseptata*
		40	*Pencillium expansum*
E- *Pancratium maritimum* L.	Pulp	41	*Mucor hiemalis*
		42	*Aspergillus niger*
	Leaves	43	*Alternaria pluriseptata*
		44	*Alternaria alternaria*
		45	*Pythium nayoroerse*
		46	*Cladosporium nigrellam*
		47	*Cladosporium cladosporioides*
		48	*Penicillium marneffei*

Name of plants	Plant organs	Fungal code No.	Name of fungal isolates
F- *Asphodelus aestivus* Brot.	Leaves	49	*Fusarium verticilloioides*
		50	*Cladosporium nigrellam*
		51	*Rhizopus stolonifer*
		52	*Alternaria pluriseptata*
		53	*Ulocladium alternariae*
		54	*Fusarium oxysporum*
	Stem	55	*Fusarium verticilloioides*
		56	*Fusarium oxysporum*
		57	*Alternaria pluriseptata*
		58	*Cladosporium nigrellam*
		59	*Rhizopus stolonifer*
		60	*Penicillium raistrickii*
G- *Lotus polyphyllos* E. D. Clarke	Stem	61	*Alternaria pluriseptata*
		62,63	*Ulocladium alternariae*
	Leaves	64	*Ulocladium alternariae*
		65	*Alternaria pluriseptata*
		66,67	*Aspergillus niger*
H- *Lycium europaeum* L.	Leaves	68	*Rhizopus stolonifer*
		69	*Trichocladium asperum*
		70	*Pythium nayoroerse*
		71	*Nigrosora sphaeria*
		72	*Mucor hiemalis*
		73	*Rhizopus stolonifer*
		74	*Penicillium marneffei*
		75	*Ulocladium atrum*
		76	*Alternaria chlamydospora*
		77	*Aspergillus niger*

Name of plants	Plant organs	Fungal code No.	Name of fungal isolates
	Stem	78	*Alternaria alternata*
		79	*Rhizopus stolonifer*
		80	*Alternaria pluriseptata*
		81	*Nigrosora sphaeria*
		82	*Ulocladium atrum*
J- *Echium angustifolium* Mill.	Leaves	83	*Aspergillus terrus*
		84	*Coccidioides immitis*
		85	*Rhizopus stolonifer*
		86	*Alternaria pluriseptata*
		87	*Spegazzinia pakeri*
K- *Nicotiana glauca* Graham	Leaves	88	*Cladosporium nigrellam*
		89	*Rhizopus stolonifer*
		90	*Cladosporium sphaerospermum*
		91	*Alternaria pluriseptata*
		92	*Spegazzinia pakeri*
	Stem	93	*Alternaria chlamydospora*
		94	*Alternaria pluriseptata*
		95	*Ulocladium atrum*
		96	*Leptosphaeria senegalensis*
		97	*Aureobasidium pellalanus*
L- *Ononis vaginalis* Vahl, Symb.	Leaves	98	*Alternaria pluriseptata*
		99	*Ulocladium atrum*
		100	*Aspergillus niger*
		101	*Cladosporium nigrellam*

Endophytes frequency and diversity indices

A list of 101 isolates and their frequencies, relative frequencies were recorded in the investigated plant tissues. The detailed results summarized in Table 2, revealed that 56% isolates were obtained from plant leaf tissues, 27% from the stem, 15% from fruits and 2% from pulps. The dominant endophytic species

recorded with high relative frequencies were *Alternaria pluriseptata* (20%) with 14 active isolates against the tested pathogens, *Rhizopus stolonifer* (8%) four of them were active, *Aspergillus niger* (6%), four of them were active, *Ulocladium atrum, Cladosporium nigrellam, and Ulocladium alternariae* share the same relative frequency (5%) and gave positive results for two, one and two as active strains respectively and *Pythium nayoroense* (4%) with 50% active isolates. The rest of the listed endophytic fungi were recorded with lower relative frequencies.

Table 2
Isolated fungi, frequency (F), relative frequency (RF), isolation rate (IR %), Richness (S), Species diversity, Shannon index (H), Simpson diversity (1-D) and Evenness values (E) tissues.

NO	Endophyte species	(F)	R.F (%)	organs				No of active isolates
				Fruit	Leave	stem	pulp	
1	*Alternaria pluriseptata*	21	20	4	10	7	0	14
2	*Phoma dennissii*	1	1	0	0	1	0	1
3	*Alternaria phragmospora*	3	3	1	2	0	0	3
4	*Alternaria alternaria*	3	3	0	2	1	0	2
5	*Phoma glomerata*	1	1	0	1	0	0	1
6	*Scytalidium japonicum*	1	1	0	1	0	0	1
7	*Pythium nayoroense*	4	4	1	3	0	0	2
8	*Ulocladium atrum*	5	5	0	3	2	0	2
9	*Alternaria chlamydospora*	3	3	0	1	2	0	2
10	*Mucor hiemalis*	3	3	1	1	0	1	1
11	*Penicillium aurantiogriseum*	2	2	0	1	1	0	1
12	*Cladosporium cladosporioides*	2	2	0	2	0	0	1
13	*Rhizopus stolonifer*	8	8	0	6	2	0	4
14	*Sarcinomyces phaeomriformis*	2	2	1	1	0	0	2
15	*Aspergillus niger*	6	6	0	5	0	1	4
16	*Penicillium marneffei*	3	3	0	2	1	0	3
17	*Spegazzinia pakeri*	3	3	1	2	0	0	3
18	*Cladosporium tenuissium*	1	1	1	0	0	0	0
19	*Ulocladium alternariae*	5	5	0	2	3	0	1
20	*Rhizopus oryzae*	1	1	0	0	1	0	1
21	*Rhizopus microspores*	1	1	0	1	0	0	0
22	*Pythium elongutum*	1	1	1	0	0	0	1
23	*Cladosporium nigrellam*	5	5	0	4	1	0	2

NO	Endophyte species	(F)	R.F (%)	organs				No of active isolates
				Fruit	Leave	stem	pulp	
24	*Fusarium verticilloioides*	2	2	1	1	0	0	2
25	*Fusarium oxysporum*	2	2	0	1	1	0	0
26	*Penicillium raistrickii*	1	1	0	0	1	0	1
27	*Trichocladium asperum*	1	1	0	1	0	0	0
28	*Nigrosora sphaeria*	2	2	0	1	1	0	1
29	*Aspergillus terrus*	1	1	0	1	0	0	1
30	*Coccidioides immitis*	1	1	0	1	0	0	1
31	*Cladosporium sphaerospermum*	1	1	0	1	0	0	0
32	*Leptosphaeria senegalensis*	1	1	0	0	1	0	1
33	*Aureobasidium pellalanus*	1	1	0	0	1	0	1
34	*Penicillium expansum*	2	2	2	0	0	0	0
35	*Penicillium roqueforti*	1	1	1	0	0	0	0
	Total no of isolates	**101**	**100**	**15**	**57**	**27**	**2**	**75**
	Isolation rate (%)			15	56	27	2	
	Species richness (S)			11	26	16	2	
Species diversity	**Simpson's index of diversity (1 – D)**			0.93	0.95	0.92	1	
	Shannon index (H)			2.14	2.55	2.59	0.7	
	Evenness (E)			0.89	0.78	0.93	1	

On the other hand, the richness and the diversity of the isolated endophytic fungi were calculated; plant leaf tissues settled the highest richness value with 26 species followed by stem (16 species) and 11 species for fruits. Simpson diversity indices values were, 0.95 for leaf tissues, 0.93 for fruits and 0.92 for stem, consistently; Shannon index values (H = 2.55 in leaf tissues), (H = 2.59 in stem tissues) and (H = 2.14 in fruits) while recorded in pulps as the lowest value (H = 0.7). Evenness indice4s for the leaf (E = 0.78), for the stem (E = 0.93) and for fruits (E = 0.89)

Antimicrobial activity of isolated endophytic fungi

Based on the similarity measure between the different endophytes activity, the antimicrobial activity data against eight selected significant pathogens were subjected to two-way clustering analysis. The resulted dendrogram revealed seven contiguous groups of fungi: The superimposed groups are discriminated at high resolution at the cut level of 0.65. Hierarchically, the resulted groups are arranged on the figure (1) and the pattern of each assemblage is clarifying as following:

Group I

this group separated firstly at the base of the tree with 14 isolates and showed high activity range against *E. coli* (mean 23 mm clear zone), Table 3. This group divided into three subgroups. The first subgroup; *Alternaria pluriseptata* (35), *Alternaria phragmospora*(5), *Rhizopus stolonifer* (51 and 68), *Penicillium marneffei* (48), *Aspergillus niger* (77), *Cladosporium nigrellam* (88), and *Ulocladium alternariae* (53) isolated from leaf tissues and revealed high activity only against *E. coli*. *Cladosporium cladosporioides* (18), *Penicillium aurantiogriseum* (23), *Alternaria phragmospora* (26) isolated from *Mellilotus indicus* and inhibited *Aspergillus flavus*, *E.coli*, and *Pseudomonas argenosa* were included in the second subgroup. The third subgroup represented by *Scytalidium japonicum* (10), *Cladosporium cladosporioides* (47), and *Alternaria pluriseptata* (98), isolated from leaf tissues and showed varied activity against *E. coli*, *Pseudomonas argenosa*, *Bacills subtilus*, *Aspergillus flavus*, and *Penicillium expansum*.

Table 3
Two-way cluster groups (I - VII), No. of isolated endophytes in each group, inhibition zone (main/mm) and the most pathogens affected by groups

Groups	No of isolates	inhibition zone main (mm)	Most affected pathogens
I	14	23	*E. Coli*
II	10	20	*Staphylococcus aureus*
III	10	13	*Candida albicans*
IV	15	17	*Pseudomonas argenosa*
V	28	0	Not active
VI	18	21	*Bacillus subtilus*
VII	6	20	*Bacillus subtilus, Staphylococcus aureus, Penicillium expansu, Aspergillus flavus, Pseudomonas argenosa and E.coli*

Group II

Ten fungal strains assembled in group II, inhibited mainly and strongly *Staphylococcus aureus* with mean inhibition zone of 20 mm. Table 3. *Mucor hiemalis* (14), *Ulocladium alternariae* (63), *Pythium nayoroense*

(38), *Alternaria pluriseptata* (43 and 21), *Fusarium oxysporum* (56) included in active subgroup against only *Staphylococcus aureus*. *Fusarium verticilloioides* (55) appeared in the same group but showed activity against *Candida albicanus* in addition to its activity against *Staphylococcus aureus.* The second subgroup represented by two isolates of *Alternaria pluriseptata* (12 and 15) and one isolate *of Sarcinomyces phaeomriformis* (20) was multiactive strains against *E. coli, Staphylococcus aureus* and *Candida albicans.*

Group III

Ten strains represented this group; seven isolate from leaf tissues, two other from stem and one from fruit tissues. Three different patterns of activity for these 10 endophytes with moderate inhibitory zones (main = 13 mm). *Alternaria pluriseptata* (8), *Alternaria chlamydospora* (13), *Aspergillus niger* (22), *Rhizopus stolonifer* (85) were similar in their activity against *Pseudomonas argenosa*, *Staphylococcus aureus* and *Aspergillus flavus*. While *Fusarium verticilloioides* (49) and *Alternaria pluriseptata* (61) isolated from *Asphodelus aestivus* and *Lotus polyphyllo* respectively showed high activity against *Pseudomonas argenosa, Staphylococcus aureus*, *Aspergillus flavus* and *Candida albicans* in a different pattern. *Cladosporium nigrellam*(58), *Alternaria alternaria* (44), *Phoma glomerata* (9), and *Spegazzinia pakeri* (27) isolated from leaf tissues of *Asphodelus aestivus*, *Pancratium maritimum*, *Fagonia cretica* and *Mellilotus indicus* respectively, mostly inhibited *Candida albicans* and *Aspergillus flavus* in third pattern.

Group IV

Fungal isolates in group IV characterized by distinct antimicrobial activity against *Pseudomonas argenosa* with main inhibition zone of 17 mm. table (3). Fifteen strains were incorporated into this group (graph 1) mostly isolated from investigated leaf tissues and assembled in two subgroups. *Rhizopus stolonifer* (19), *Penicillium raistrickii* (60), *Aspergillus niger* (66), *Nigrosora sphaeria* (71), *Rhizopus oryzae* (34), *Spegazzinia pakeri* (87), *Ulocladium atrum* (82), *Pythium elongutum* (37), *Alternaria pluriseptata* (57), *Aspergillus niger* and *Coccidioides immitis* (84) aggregated in a subset and behaved in a similar manner against one pathogen, *Pseudomonas argenosa*. The other subgroup, *Alternaria phragmospora* (26), *Ulocladium atrum* (75), *Penicillium expansum* (30) and *Penicillium marneffei* revealed high antimicrobial activity against two pathogens, *Candida glabrata* and *Pseudomonas argenosa*.

Group V (Inactive group)

Twenty-six strains assembled together in group V that gave negative effect against all tested pathogens. Plant species, six inactive strains obtained from *Lycium europaeum, four isolates from Carduus getulus*, and three inactive strains from both *Asphodelus aestivus* and *Nicotiana glauca*. Other plant species represented by at least one inactive strain. *Alternaria phragmospora* (4) and *Alternaria pluriseptata* (76) grouped as closely related to group V However these two strains aere active against *Candida glabrata* and *Penicillium expansum*, respectively graph (1).

Group VI

This group contained 18 strains isolated from all plant species, shared the ability of inhibiting *Bacillus subtilis* with inhibition zone (main = 21mm) table (3).and differentiated from each other in terms of antimicrobial activity against other tested pathogens. Group VI divided into smaller six subsets based on the number of pathogens affected by its endophytic fungi. Four isolates, *Aspergillus niger* (100), *Sarcinomyces phaeomriformis* (28*), Rhizopus stolonifer* (59) *Cladosporium nigrellam* (101) were active against four different pathogens. While, *Rhizopus stolonifer* (73), *Nigrosora sphaeria* (81*), Pythium nayoroense* (45) revealed Antimicrobial activity against three pathogens. *Phoma dennissii* (2), *Cladosporium nigrellam* (46), *Ulocladium atrum* (99) and *Alternaria pluriseptata* (65) gave positive effect against one pathogen while the rest of the isolates in group VI activated positively against two pathogens.

Group VII (Multi-active group)

six isolates classified into two subgroups identified multivariate responses against all pathogens with wide inhibition zones (main = 20 mm). Two strains of *Alternaria pluriseptata* (6 and 7) isolated from *Scorpiurus muricatu* leaf tissues and one strain of *Rhizopus stolonifer* isolated from the leaf tissues of *Nicotiana glauca* revealed high antimicrobial activity against all tested pathogens except for *C. albicanus.* On the other hand; *Penicillium marneffei* (24) isolated from *Mellilotus indicus*, *Alternaria pluriseptata* (32) isolated from *Carduus getulus* and *Spegazzinia pakeri* (92) isolated *from Nicotiana glauca* revealed strong activity against all tested pathogens except *E. coli.*

Discussion

Endophytes are a big hidden world of unique fungal isolates which have different biological activities and produce bioactive compounds with a high level of structural diversity. It can be considered as major reservoirs of natural bioactive metabolites which are very efficient in industrial, agricultural and medicinal applications (Selim et al. 2016). In the present work, the selection of medicinal plants in Northwestern coast of Egypt based on the little previous work on isolation and identification of endophytes from these plants growing in this area. Our isolation strategy was based on each part of the plant because the endophytes isolates depend not only on the plant, but also on the tissue of the plant (Golinska et al. 2015). From 11 medicinal plant species 101 fungal isolates belonging to 18 genera and 35 fungal species were isolated and the most frequent endophytic fungal species is *Alternaria pluriseptata*, To discuss these results, no isolation or identification trails have been done in fungal endophytes of these plants of these area, but in comparison with other published studies these findings is more diverse than that recorded By Hassanein et al. (2016) who isolated Thirty endophytic fungal species from green onion, basil, green pepper, roselle, mint, watercress, tagetes and white radish and more also than that isolated by Ebrahimia et al. (2010) who isolated only 8 endophytic fungi from random leaves and branches samples of five native medicinal plants in iran namely, *Stachys lavandulifolia, Rumex pulcher, Hypericum scabrum, Starja bachteriarica* and *Achillea kellalensis*. And more than that recorded by Raviraja (2005) who

isolated eighteen species of endophytic fungi from bark, stem and leaf segments of five medicinal plant species growing within the Kudremukh range in the Western Ghats of India. In the other hand Son (2002) obtained 121 fungal isolates from 62 different types of medicinal plants.

The present study declares that fungal endophytes showed significant differences in their presence, absence and abundance in the study plants. These results were consistent with the previous reports (Hassanein et al. 2016). In addition the endophytic fungi have been found in different tissues of a single plant which is a tissue specificity reflection and might be also due to other factors like, degree of exposure to sun, air, rain, wind, moisture and aeration conditions and nutrients availability (Andreote et al. 2014), this explain that endophytic fungal species and their population structure of even the same host plant species from different regions normally presented very low similarity degree (Jiang et al.2010).

The variation in number and type of isolated fungal endophytes can be explained by some medicinal plants harbored more endophytic fungi than others and some of the common endophytes not only existed in more plant hosts but also had higher relative frequencies within each of the hosts. In contrast, some other endophytic fungi were detected in only one given plant host (Huang et al. 2008) moreover some studies have shown that endophytes colonization can be stimulated by host plant species, geographic location, seasonality and different tissues of the same plant (Yao et al. 2017).

In our study the most dominant and most active in its microbial activity is *Alternaria pluriseptata*, to the pest of our knowledge this is the first report about this species as endophytes. Generally *alternaria* genus is one of active and common endophytes, Tonial et al. (2016) reported it on *Schinus terebinthifolius* endophytes collected in an unspecified location in South America. The endophytic isolates were identified as *Alternaria spp*. Sect. *Alternate* and from 11 different medicinal plants collected from Tamil Nadu, India, the most frequently isolated fungi were *Alternaria* sp. (Palanichamy et al.2018) parallel to this study Mani et al. (2015) isolated *Alternaria citrimacularis* and *Alternaria alternate* from the medicinal tree *Aegle marmelos*, collected in India. Most of the *Alternaria sp* produces many of biologically active secondary metabolites such as Hexahydroaltersetin, Altersetin, Dihydroaltersetin, Equisetin,, Zinnimide, Deprenylzinnimide, DesmethyldestruxinB, Cichorine, HomodestruxinB, Alternariol, Alternariol 5-O-sulfate, and Macrosporin (Lou et al.2013). Elgorban et al (2019) isolated Thirty-seven bioactive chemical compounds were recorded in the crude extracts of *Alternaria sp*. (A8) using GC-MS Thirteen major bioactive compounds were recorded namely: 1,2-Benzenedicarboxylic acid, bis (2-ethyhexyl) ester representing 51.15% of the crude extract, 6,8-dimethoxy-4-methyl-4H- chromene (10.87), 2,5-Cyclohexadien-1-one, 2,6-bis (1,1-dimethylethyl)-4-ethylidene- (3.94%), Cetene (3.32%), 1,2-Benzenedicarboxylic acid, dibutyl ester (3.23%), 1-Octadecene (2.64%), Benzene ethanol (2.47%), 1-Octdecene (2.21%), Cycloe- icosane (1.96%), 1-Butanol, 3- methyl-, acetate sopentyl alcohol, acetate (1.91%), 1-Tetradecene (1.77%), Naphthalene (1.69%), Phenol, 2,4-di-t-butyl-6-nitro (1.59%). These compounds showed strong antibacterial activity in combination Johann et al. (2012) recorded the biphenyl derivative compound altenusin, produced by the endophytes *Alternaria sp*. This compound is 50-fold more active against *Paracoccidioides brasiliensis* than the reference trimethroprim/sulfamethoxazole compounds. It was suggested that altenusin possibly affects cell wall

synthesis or assembly (Johann et al.2012). In 2009, Gu (2009) reported on the compounds altechromone A and herbarin A produced by *Alternaria brassicicola* ML-P08, an endophyte of Chinese *Malus halliana*. Altechromone A showed activity against *B. subtilis*, *E. coli, C. albicans* and *Pseudomonas fluorescens* Herbarin A showed activity against *C. albicans* and *Trichophyton rubrum*. The habitats of the Egyptian flora possess a large number of potential aromatic and medicinal plant species that accumulate and concentrate secondary metabolites as response of the environmental stresses.

The present study shed the light on the promising sources of the Egyptian medicinal plant regarding secondary metabolic contents. The habitats of the Egyptian flora possess a large number of potential aromatic and medicinal plant species that accumulate and concentrate secondary metabolites as response of the environmental stress. In conclusion, this study showed the diversity of species and activity of isolated fungi. Which opens endless horizons to more and more research in medicinal plants endophytes.

Declarations

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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Figures

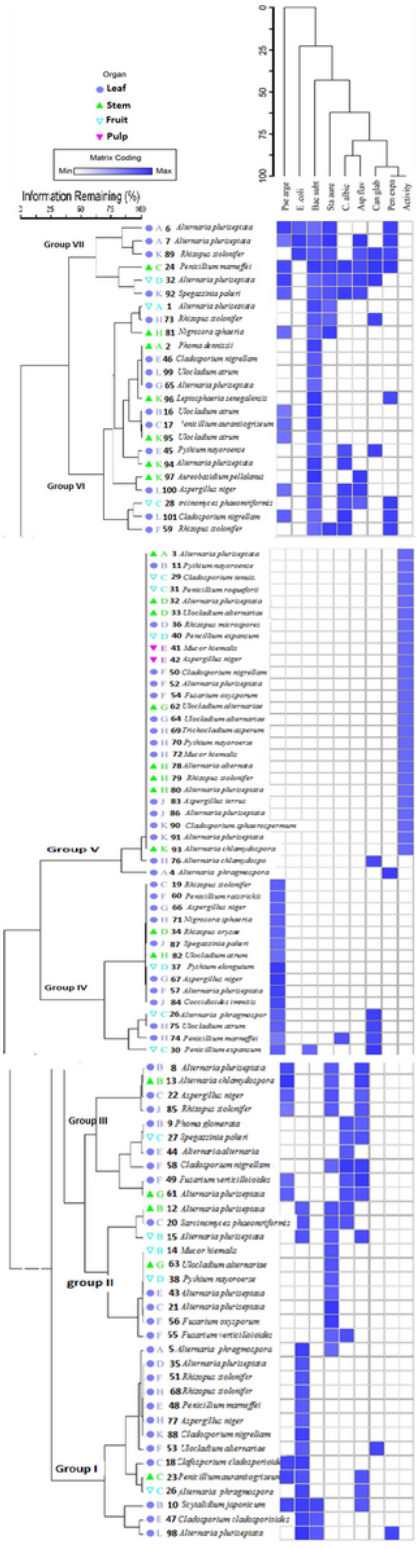

Figure 1

Two-way cluster analysis dendrogram showing the analysis of the 101isolated endophytes against 8 pathogens. Groups (I - VII) are presented on the hierarchal classification of the isolates. Antimicrobial activity (%) for each fungi is plotted as a colored squares ranged from the white (no antimicrobial activity) to the blue (the highest activity %). Intensity of the blue color reflects an isolate activity % in relation to other isolates. Eleven Plant species are symbolic (A to L).