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Antimicrobial Activities of Endophytic Fungi of Red Sea Aquatic Plant *Avicennia marina*

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MEDICINAL plants endophytes are a promising trend to meet the increasing threat of drug-resistant strains of human pathogens. Mangrove plants (*Avicennia marina*) have been used in folklore medicines where extracts from mangrove species have proven inhibitory activity against human, animal and plant pathogens. This study focuses particularly on testing the antimicrobial activity of mangrove endophytic fungi isolated from South Safaga and Wadi Abu Hamrah mangrove along the Red Sea against different human pathogens. A total of 35 endophytic fungi were isolated from mangroves leaves at two study areas and were identified. Crude extracts of the endophytic isolated fungi were screened for their antimicrobial activity using a well diffusion method against the following pathogenic microorganisms; *S. aureus*, *S. pyogenes*, *P. vulgaris*, *K. pneumoniae*, *B. subtilus*, *C. albicans*, *P. chrysogenum* and *A.niger*. The most effective extracts which exhibited significant activity against most of the tested pathogens were from *A. aculeatus*, *A. niger*, *A. terreus*, *E. amstelodami*, *E. rostratum* and *M. racemosus*.

Keywords: Mangroves, *Avicennia marina*, The Red Sea, Endophytic fungi, Antimicrobial activity.

Introduction

Mangrove plants are intertidal forest wetlands established at the interface between land and sea in tropical and sub-tropical latitudes. They are unique for their adaptation to extreme environmental conditions of high salinity, changes in sea level, high temperatures and anaerobic soils (Maria & Sridhar, 2004). Mangroves extend into temperate regions between 30° north and 30° south of the equator.

In Egypt, mangroves occupying many sites along the Sinai Peninsula and the Red Sea shoreline are represented by two species; *Avicennia marina* (black mangrove) and *Rhizophora mucronata* (red mangrove). Mangrove is a source of traditional medicine that contains bioactive compounds with antimicrobial, antifungal, antiviral, antitumor, insecticide and antileukemia activities (Tarman et al., 2013). Mangroves serve as nurseries and refuge for many marine organisms that are of potential commercial value, including endophytic fungi (Kairo & Hegazy, 2003).

Approximately 300.000 plant species growing in unexplored areas of the earth are host to one or more endophytes. High diversity is an important characteristic of endophytic mycobiota, as depicted by the fact that it is quite common for endophyte surveys to find assemblages consisting of more than 30 fungal species per host plant species (Pimentel et al., 2011). Endophytes play a key role in habitat adaptation of plants, resulting in improved plant performance and plant protection against biotic and abiotic stresses (Lugtenberg et al., 2016). The exponential increase in the use of endophytes for improving plant adaption, which has been featured in recent reviews (Porras-Alfaro & Bayman, 2011; Kivlin et al., 2013; Johnson et al., 2013; Hardoim et al., 2015 and Card et al., 2016), indicates a growing recognition by the scientific community of the potential to exploit novel endophytes. A compelling feature of fungal endophytes is their exceptional diversity both at a global scale and at the scale of individual leaves, plants and locations (Arnold, 2008). These fungi have been shown to contain a broad variety of bioactive secondary metabolites with unique structure, including alkaloids, benzopyranones,

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chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones, and others. Such bioactive metabolites find wide-ranging application as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants, and anticancer agents (Pimentel et al., 2011).

The objectives of the present work, therefore, were to isolate the endophytic fungi associated with *Avicennia marina* and to investigate the antibacterial and antifungal properties of their crude extracts against some important human pathogens.

The study area

Two sites of mangrove stands along the Red Sea were selected for this study. The two study areas (Fig. 1) are South Safaga Stand and North Quseir (Wadi Abu Hamrah) Stand. South Safaga stand is located 17km south of Safaga, forming a long series of compact patches where mangroves were clumped and the clumps had sandy areas in between. Wadi Abu Hamrah Stand is located 35km north of Quseir. Plants were clumped and showed vigorous growth.

Materials and Methods

Collection of the plant materials

Healthy Mangrove (*Avicennia marina*) leaves were collected from ten sites at South Safaga stand and Abu Hamrah stand along the Red Sea which are located between 26°37'7.17"N to 26°23'48.70"N latitude and 34°0'28.24"E to 34°7'18.10"E. The samples were collected from mangrove clumps (Fig. 2) and the collection was conducted four times between November 2016 and August 2017, as following: Autumn (November 2016), Winter (February 2017), Spring (June 2017) and Summer (August 2017). Healthy leaves (forty samples) of *Avicennia marina* were collected for isolation of endophytic fungi. The samples were immediately placed on ice in sterilized bags and brought to the laboratory where they were kept in a refrigerator at 4°C until they were processed. The materials were analyzed within 24-48h.

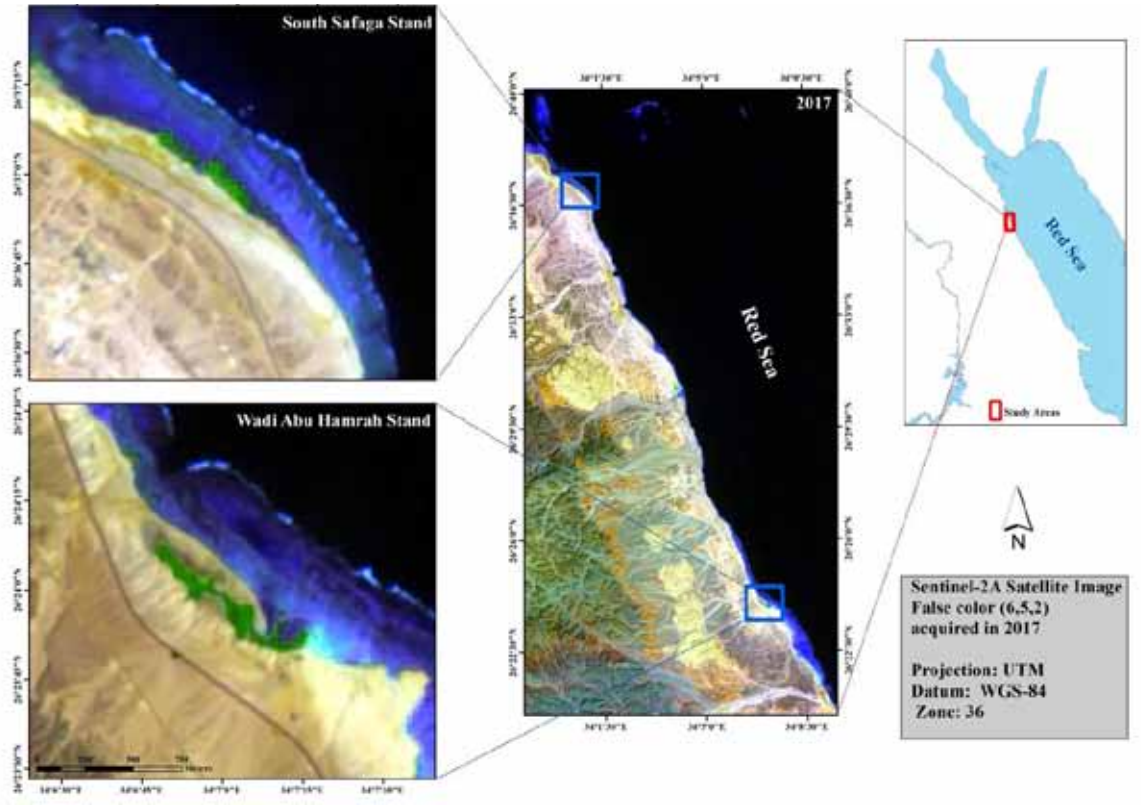

Fig. 1. Satellite image of the two study areas.

Fig. 2. Photos of mangroves at the two study areas during samples collection.

Isolation of fungal endophytes

The collected plant materials were rinsed under running tap water for 10min, surface sterilized by immersing them sequentially in 70% ethanol solution for 3min and 0.5% sodium hypochlorite for one minute and then air-dried. Leaves were cut aseptically into small segments (1cm long and 1cm broad segments) with a sterile knife and were surface-sterilized. After surface drying under sterile conditions in a laminar air flow chamber to remove the excess water, segments were inoculated on plates containing Malt Extract Agar (MEA) medium. The plates were incubated at 28°C for one week until mycelium appeared surrounding the segments. Plates were checked every other day continuously for 2 weeks and fungal colonies were transferred onto other plates with MEA for pure culture and pure cultures were maintained on MEA slants.

Identification of isolates

The fungal endophytes were identified in the Regional Centre for Mycology and Biotechnology (RCMB), Al-Azhar University based on their morphological and reproductive characters using universal identification manuals of Nagamani et al. (2006) and by using image analyser (OLYMPUS BX 40) supported by Panasonic camera (WVGP240) for microscopic examination.

Production of crude metabolites

All the isolates were cultivated to produce crude metabolites according to the protocols of Trisuwan et al. (2008). Endophytic fungal isolates were grown in 250ml Erlenmeyer flasks containing 100ml Malt Extract Broth (MEB) medium and incubated at 28°C for 2 weeks under a stationary condition. The crude fermentation broth was filtered using cheesecloth to separate the fungal mat from the filtrate. Ethanol (70%) was used as a solvent for the extraction of metabolites. The resulting extracts (filtrates) were examined for antimicrobial activity against some selected human pathogens.

Antimicrobial activity assay

Media and cultivation conditions

Both NA and MEA media were used for antimicrobial activity test. Nutrient agar medium contains (Beef Extract, 3.0g; Bacteriological Peptone, 5.0g; Agar, 20.0g), the pH was adjusted at 6.2±0.2 at 25(±2)°C for bacterial growth. Malt Extract Agar (MEA) medium contains (Malt extract, 20.0g; Bacteriological Peptone, 1.0g; glucose, 20g; Agar, 20.0g). The pH of the medium was adjusted to 5.4±0.2 at 25(±2)°C for fungal growth. All the media were prepared by dissolving the solid components in 1000ml of cold distilled water and then heating to 60-70°C with stirring. Media were sterilized by autoclaving at 121°C (1.5atm.) for 15-20min. For maintenance of stock cultures, agar slant tubes were used.

Crude extracts from fungal endophytes were tested at a concentration against 8 human

pathogenic microorganisms obtained from the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University namely; *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pyogenes* as Gram-positive bacteria, *Klebsiella pneumoniae,* a Gram-negative bacterium, *Penicillium chrysogenum*, *Aspergillus niger* as pathogenic fungi and *Candida albicans* as a yeast.

Antimicrobial activity

Antimicrobial activity was assayed by the well diffusion method (Holder & Boyce, 1994). Activity was evaluated by measuring the inhibition zones with the diameter was calculated in millimetres. Eight holes were made in agar plates using a sterile cork borer, and then filled with 100µL of each extract. One well was filled with chloramphenicol or griseofulvin as a positive control, and another one with the corresponding solvent as a negative control. Plates were left at 4 (±2)°C for one hour and then incubated for 24-48h at 37(±2)°C for bacteria and 28(±2)°C for fungi. Diameters of inhibition zones developed were measured after 24-48h of incubation (Mekawey et al., 2009). Three replicates were maintained in each treatment. All procedures were done under aseptic conditions in a sterile laminar air flow according to good laboratory practice.

Results

Isolated endophytic fungi

Thirty five isolates from seven genera were obtained from leaf samples of *Avicennia marina*. The occurrence of Ascomycota was the highest as compared to Zygomycota (Fig. 3). Species of *Aspergillus* and *Penicillium* were the most frequently isolated endophytes during the present investigation along with *Alternaria*, *Exserohilum*, *Eurotium*, *Rhizopus* and *Mucor* (Table 1). The colony morphology and microphotograph of active endophytic fungal isolates are shown in Fig. 3.

Antimicrobial activity of crude extracts against some pathogenic microorganisms

Crude extracts of seven isolates in 70% methanol showed activity against all test human bacterial pathogens (Table 2). Endophytic fungal isolate *Eurotium amstelodami* (isolated from South Safaga) showed the highest zone of inhibition of 28±0.0, 28±0.0, 26±0.06, 27±0.13, and 24±0.2mm diameter, against *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Streptococcus pyogenes*, respectively (Table 2).

TABLE 1. Occurrence and frequency of dominance (%) of endophytic fungi isolated from leaves of *Avicennia marina*.

Endophytic fungi	Isolation Date	Total No. of isolates	Frequency of dominant endophytes (%)	Endophytic fungi	Isolation date	Total No. of isolates	Frequency of dominant endophytes (%)
A. parasiticus		2	21.43	*P. fellutanum*		1	21.43
P.citrinum		1	14.29	*A.parasiticus*	February	1	35.71
R. oryzae		2	14.29	*A.phoenicis*	2017	1	25
A. fischeri		1	10.72	*A. fischeri*		1	21.43
A.aculeatus		2	21.43	*A. flavus*		*1*	39.26
M.racemosus	November	1	3.57	*A. phoenicis*		*1*	21.43
A. niger	2016	1	28.57	*A. awamori*		*2*	28.57
A.chlamydospora		1	7.14	*A. aculeatus*	June 2017	*1*	28.57
E. rostratum		1	7.14	*P.canescens*		*1*	14.29
A. fumigatus		1	10.72	*A.fumigatus*		*1*	17.86
A. pseudo-niger		1	17.86	*A.tamarii*		*1*	7.14
A. awamori		1	14.29	*A. fischeri*	August	2	35.71
E. amstelodami		2	21.43	*A. japonicus*		1	17.86
A. awamori	February 2017	1	17.86	*A.terreus*	2017	1	21.43
A. niger		1	32.14				

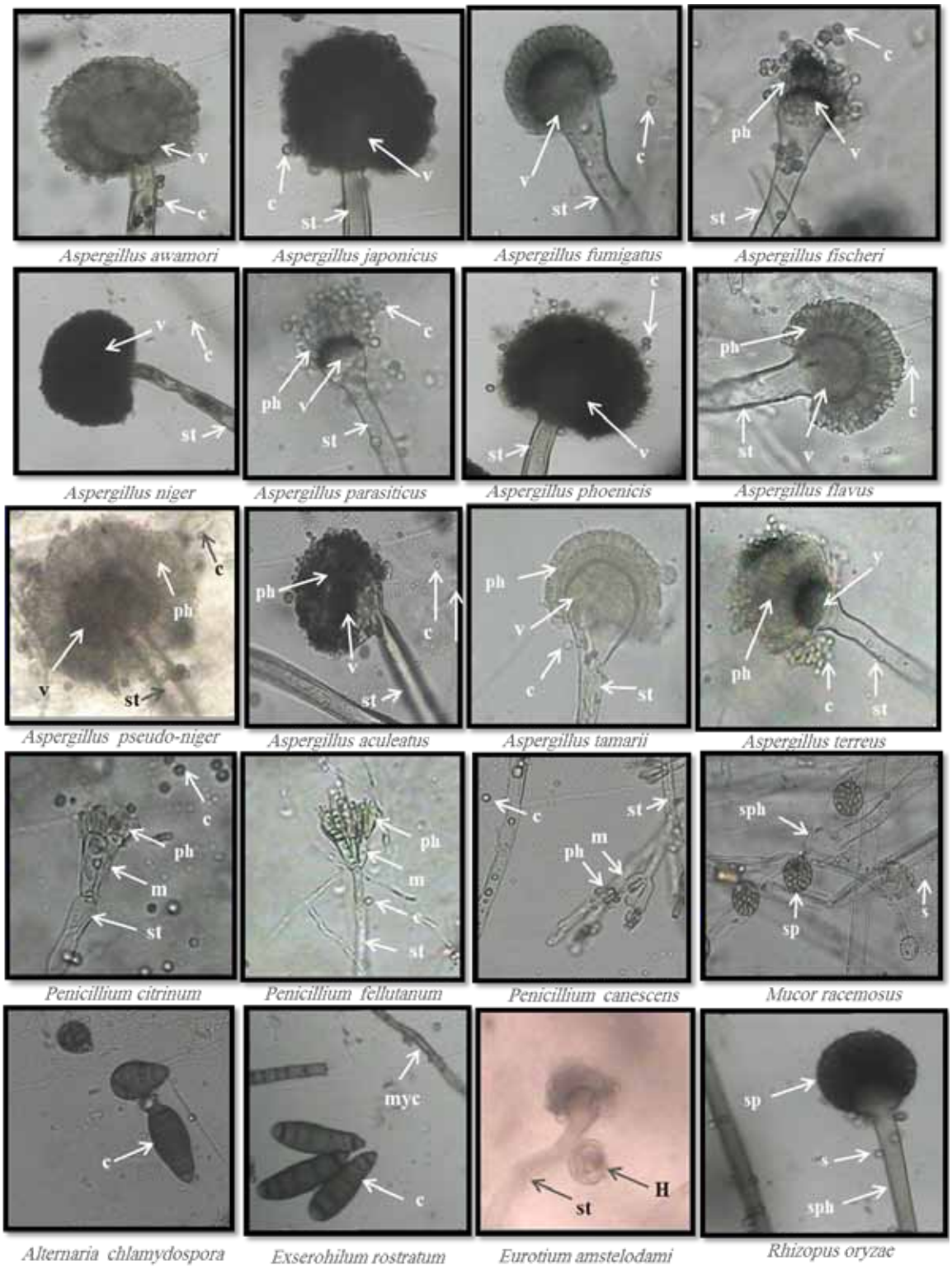

Fig. 3. Microscopic images of some active endophytic fungal isolates from the Red Sea Mangrove, (c: Conidia, st: Stipe, v: Vesicle, ph: Phialide, m: Metulae, sph: Sporangiophore, sp: Sporangium, s: Sporangiospore, myc: Mycellium and H: Hull cell).

TABLE 2. Zone of inhibition of crude extracts obtained from different endophytic fungi isolated from *Avicennia marina* against some Gram+ and Gram- bacteria (#: South Safaga isolates).

Isolation Date	Endophytic fungi	Antibacterial activity (mm)				
		B. subtilis	***S. aureus***	***K. pneumoniae***	***P. vulgaris***	***S. pyogenes***
November 2016(Autumn)	*Aspergillus parasiticus#*	-	-	-	-	-
	Penicillium citrinum#	12±0.1	-	-	-	17±0.3
	Rhizopus oryzae#	-	-	-	-	-
	Aspergillus fischeri#	-	-	-	-	-
	Aspergillus aculeatus#	-	12±0.13	11±0.1	18±0.06	11±0.2
	Mucor racemosus#	15±0.03	13±0.0	15±0.1	20±0.2	13±0.13
	Aspergillus niger#	-	10±0.0	9±0.0	16±0.13	10±0.0
	Rhizopus oryzae	-	-	-	12±0.03	12±0.13
	Aspergillus aculeatus	17±0.0	16±0.26	22±0.0	17±0.33	16±0.25
	Alternaria chlamydospora	-	-	-	17±0.23	-
November 2016(Autumn)	*Exserohilum rostratum*	16±0.06	14±0.0	12±0.03	-	20±0.13
	Aspergillus fumigatus	-	-	-	-	-
	Aspergillus pseudo-niger	-	-	12±0.2	13±0.13	10±0.1
	Aspergillus parasiticus	-	-	-	-	10±0.06
	Aspergillus awamori	-	-	-	-	-
February 2017(Winter)	*Eurotium amstelodami#*	28±0.0	28±0.0	26±0.06	27±0.13	24±0.2
	Aspergillus awamori#	-	-	-	14±0.13	-
	Aspergillus niger#	13±0.0	16±0.1	10±0.03	17±0.16	13±0.1
	Eurotium amstelodami	15±0.0	15±0.03	15±0.1	15±0.1	17±0.35
February 2017(Winter)	*Penicillium fellutanum*	-	-	-	-	-
	Aspergillus parasiticus	-	-	-	-	-
	Aspergillus phoenicis	-	12±0.03	13±0.2	16±0.1	17±0.23
	Aspergillus fischeri	-	-	-	-	-
June 2017(Spring)	*Aspergillus flavus#*	-	-	-	-	-
	Aspergillus phoenicis#	19±0.03	19±0.1	15±0.2	20±0.13	14±0.23
	Aspergillus awamori#	-	-	-	-	-
	Aspergillus aculeatus#	21±0.0	13±0.06	19±0.13	16±0.2	18±0.16
	Aspergillus awamori	-	-	-	-	-
	Penicillium canescens	-	-	-	-	-
	Aspergillus fumigatus	-	-	-	-	-
	Aspergillus tamarii	-	-	-	-	-
August 2017(Summer)	*Aspergillus fischeri#*	-	-	-	-	-
	Aspergillus japonicus#	-	-	-	-	-
	Aspergillus fischeri	-	-	-	-	-
	Aspergillus terreus	14±0.03	16±0.23	21±0.23	20±0.16	-

Table 2. Cont.

Isolation date	Endophytic fungi	Antifungal activity (mm)		
		C. albicans	*P. chrysogenum*	*A. niger*
November 2016(Autumn)	*Exserohilum rostratum*	-	-	-
	Aspergillus fumigatus	-	-	-
	Aspergillus pseudo-niger	-	-	-
	Aspergillus parasiticus	-	-	-
	Aspergillus awamori	-	-	-
February 2017(Winter)	*Eurotium amstelodami#*	-	-	-
	Aspergillus awamori#	-	-	-
	Aspergillus niger#	-	-	-
	Eurotium amstelodami	-	-	-
	Penicillium fellutanum	-	-	-
	Aspergillus parasiticus	-	-	-
	Aspergillus phoenicis	-	-	-
	Aspergillus fischeri	-	-	-
June 2017(Spring)	*Aspergillus flavus#*	-	-	-
	Aspergillus phoenicis#	-	-	-
	Aspergillus awamori#	-	-	-
	Aspergillus aculeatus#	-	-	-
	Aspergillus awamori	-	-	-
	Penicillium canescens	-	-	-
	Aspergillus fumigatus	-	-	-
	Aspergillus tamarii	-	-	-
August 2017(Summer)	*Aspergillus fischeri#*	-	-	-
	Aspergillus japonicus#	-	-	-
	Aspergillus fischeri	-	-	-
	Aspergillus terreus	-	-	-

Seven isolates of endophytic fungi namely; *Aspergillus aculeatus, Eurotium amstelodami* (isolated from Wadi Abu Hamrah), *Aspergillus niger, Mucor racemosus* and *Aspergillus phoenicis* extracts were effective against all tested pathogenic bacteria, the other tested isolates have activity against only one, two or three of pathogenic bacterial species (Table 2).

Ten fungal extracts inhibited the growth of *Bacillus subtilis*; *Eurotium amstelodami* showed the highest effect with inhibition zone of 28±0.0 mm diameter, while the lowest one was obtained by *P. citrinum* with a diameter of only 12±0.1.

Twelve isolates inhibited the growth of *Staphylococcus aureus*, the highest effect was also obtained by *Eurotium amstelodami* with 28±0.0mm diameter of the inhibition zone while the lowest inhibition zone was recorded for *Aspergillus niger* with 10±0.0mm diameter.

Thirteen isolates produced substances that inhibited the growth of *Klebsiella pneumoniae, Eurotium amstelodami* gave the highest antibacterial activity with 26±06mm diameter while the least activity was that recorded for *Aspergillus niger* isolate with a zone of inhibition less than 10mm diameter (9±0.0mm). The growth of *Proteus vulgaris* was affected by fifteen isolates, the highest activity was recorded for *Eurotium amstelodami* (27±0.13mm diameter) and the lowest was obtained by *Rhizopus oryzae* (12mm±0.3mm diameter). Fifteen isolates also inhibited the growth of *Streptococcus pyogenes*. *Eurotium amstelodami* gave an inhibition zone of 24±0.2mm diameter whereas *Aspergillus parasiticus, Aspergillus pseudo-niger* and *Aspergillus niger* gave small inhibition zone of 10±0.1mm.

Discussion

Endophytic fungi that invading plants tissues during their life cycle without causing disease symptoms have biotechnological importance; they produce a variety of secondary metabolites with a potential medical application and hence provide a chance for discovery of new antibiotics (Costa et al., 2012).

The results of occurrence and frequency for the endophytic fungi isolated from leaves of *Avicennia marina* show that *Aspergillus* was the most common isolated genus. Antimicrobial activity of the isolates indicates that most of the isolated endophytic fungi have an antibacterial effect but with varying levels whereas no isolates affected growth of test fungal pathogens. These results are consistent with the results of Shebany (2012) who detected the antimicrobial abilities of mangrove endophytic fungi isolated from *Avicennia marina* from Egypt against *Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, Escherichia coli, Aspergillus niger* and *Alternaria alternate* and also found some isolates of the same species showed different behavior against the test bacteria and didn't record any antimicrobial effect against the test fungi.

Hamzah et al. (2018) tested the antimicrobial activity of different endophytic fungi isolated from the Malaysian mangrove *Rhizophora mucronata* against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* and found that *Fusarium lateritium* and *Xylaria* sp. showed antibacterial activities against the pathogenic bacteria.

Similar results were also obtained by Prihanto et al. (2011), who tested the antibacterial activity of endophytic fungi isolated from mangrove (*Rhizopora mucronata*) from Porong River Bank on *Staphylococcus aureus* and *Escherichia coli*. Handayani et al. (2017) in consistence with the results of this study proved the antimicrobial effect of the endophytic fungi isolated from mangrove (*Sonneratia grifithii* Kurz), collected from Bungus, West Sumatra, Indonesia against *Staphylococcus aureus* and *Escherichia coli,* but recorded the absence of such activity against *Candida albicans*.

It is revealed from the results that *P. vulgaris* and *S. pyogenes* were more sensitive than the other pathogens (inhibited by fifteen isolates), while *B. subtilis* was the more resistant which inhibited by only ten isolates.

Results also indicate the diversity of mangrove endophytic fungi during autumn and winter than compared with summer and spring. The most effective extracts which exhibited significant activity against most of the tested pathogens were obtained from *Aspergillus aculeatus, Aspergillus niger, Aspergillus terreus, Eurotium amstelodami, Exserohilum rostratum, Mucor racemosus,* while *Penicillium citrinum, Rhizopus oryzae, Alternaria chlamydospora, Aspergillus pseudo-niger, Aspergillus parasiticus,* and *Aspergillus awamori* gave less active extracts.

Conclusion

The present study reveals antibiotic potential from extracts of a total of 35 isolates obtained from *Avicennia marina* leaf samples collected from Red Sea. *Aspergillus aculeatus, Aspergillus niger, Aspergillus terreus, Eurotium amstelodami, Exserohilum rostratum* and *Mucor racemosus* were the most dominant and potent endophytes showing highest antimicrobial activity against *S. aureus, S. pyogenes, P. vulgaris, K. pneumoniae, B. subtilus, C. albicans, P. chrysogenum* and *A. niger*. All the isolates showed antimicrobial activity against some of the test bacterial pathogens while none of them proved to have any activity against pathogenic fungi. Such results suggest that *Avicennia marina* harbour some endophytic fungi producing antimicrobial secondary metabolite which may have novel compounds. These endophytes may be sources of new antibacterials that could be used as therapeutic agents in pharmaceutical industries. However, further investigation is needed for the characterization of these endophytes within the host plant, proper establishment of their role and chemical characterization of secondary metabolites produced by them for their future applications.

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الأنشطة الضد ميكروبية للفطريات الداخلية لنباتات البحر الاحمر المائية *Avicennia marina*

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هناك أهمية متزايدة يجب أن توضع على الجهود الدولية من أجل تقليل المقاومة للمضادات الحيوية. أصبحت المقاومة المكتسبة في جزء واحد من العالم الآن حرة في الإنتشار على نطاق واسع، بالإضافة إلى ذلك، فإن ظهور الكائنات الدقيقة المقاومة للمضادات الحيوية يتطلب استراتيجيات بحث وتطوير ابتكارية. تثبيط هذه الكائنات الدقيقة المسببة للأمراض قد يكون نهجا علاجيا واعدا. يعتبر فحص المركبات المضادة للميكروبات من الفطريات الداخلية للنباتات الطبية طريقة واعدة لمواجهة التهديد المتزايد للسلالات المقاومة للكائنات الممرضة للإنسان. استخدمت نباتات المانجروف (*Avicennia marina*) في الأدوية التقليدية حيث أثبتت مستخلصات من أنواع المانجروف فعالية مثبطة ضد الكائنات الممرضة للإنسان والحيوان والنبات. تعتبرالفطريات الداخلية البحرية مصدر مهم وجديد للمركبات الطبيعية النشطة بيولوجيا مع إمكانية تطبيقها في الزراعة والطب والصناعات الغذائية. تركز هذه الدراسة بشكل خاص على اختبار النشاط ضد الميكروبي للفطريات الداخلية للمانجروف المعزول من غابات جنوب سفاجا ووادي أبو حمرة على طول البحر الأحمر ضد الكائنات المسببة لأمراض الإنسان المختلفة. تم عزل 35 نوعًا من الفطريات الداخلية من أوراق أشجار المانجروف وتم تحديدهما. تم فحص المستخلصات الخام للفطريات الداخلية لإثبات نشاطها ضد الميكروبي باستخدام طريقة well diffusion ضد الكائنات الدقيقة التالية المسببة للأمراض ، *K. pneumoniae*، *P. vulgaris*، *S. pyogenes*، *S. aureus*، *P. chrysogenum*، *C. albicans*، *B. subtilus* و *A.niger* ، كان أكثر المستخلصات فعالية هو مستخلص *E. rostratum*، *E. amstelodami*، *A. terreus*، *A. niger*، *A. aculeatus*، و *M. racemosus*.