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Establishment the Biological Activity of Some Endophytic Fungi Isolated from Fig Trees (Ficus Carica) in Basrah Province

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Abstract

The study aimed to find biologically active contents from endophytic fungi with evaluate their effectiveness as antibacterial and antifungal agents.

Leaves and stems samples were collected from *Ficus carica* from Al-Qurna groves in Basrah province of Iraq from December 2019 to February 2020. After that, fungal isolates were cultivated on semi-solid flour rice medium as fermentation medium for 40 days at 28^{o}C to yield secondary metabolites.

The results showed the isolation of 12 fungal isolates, *Alternaria* sp. appeared with the highest percentage followed by *Aspergillus* sp. Furthermore, the biological activity of fungal crude extracts showed an inhibition effect against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* with different effectiveness at 20 mg/ml. The bacterial isolates of *S.aureus* seen high susceptibility to crude extract of *A. flavus* and *A. niger* with inhibition zone 20mm for each one in comparison with gentamicin (12mm), as well as, *E. coli* revealed sensitivity toward raw material of *Penicillium chrysogenum* (35mm). On the other hand, the result appeared the bioproduct of *A. niger* with the highest activity against *C. albicans* (24mm) in compassion with ketoconazole (15mm).

Keywords: endophytic fungi, *Ficus carica*, biological activity

Introduction

Fungal endophytes are microscopic fungi that survive and grow in healthy plant tissues without causing disease symptoms (Petrini,1991). Due to the fact that they have been found in all main plant lineages and over a wide range of terrestrial habitats, these organisms are assumed to be common among terrestrial plants (Müller *et.al.*, 2001). Endophytes may produce a variety of compounds that have protective and survival value for their host plants, including substances that increase resistance to stress-, insect invasion-, and disease, enhance productivity, and have herbicide activity (Arnold *et.al.*, 2007). It is crucial to find new antimicrobials due to the rise in antibiotic resistance among microorganisms (Payne *et al*., 2007). However, after exploration for decades, it is become harder to discover novel bioactive metabolites from usual environments (e.g., soils) (Lopes *et al*., 2016:Jiang *et al*., 2018and Zhang *et al.*, 2018). This scenario has been promoted for a variety of reasons, including the excessive and frequently improper use of antibiotics, unsanitary living circumstances, constant travel, an increase in patients with immunocompromised states, and a delay in the detection of infections (von Nussbaum *et al.*, 2006). Exploring fresh niches and habitats makes it easier to conduct a thorough search for new, powerful antimicrobial drugs, which is important as a result (Xing *et al*., 2011 and Zhao *et al*., 2011).

The earlier investigations about entophytic fungi from different plants especially fig trees were limited, so that, the objective of current study is target to isolation and identification of endophytic fungi from fig trees *Ficus carica* in the Qurna district of Basrah, then test the biological activities of their crud extracts against some pathogenic isolates of bacteria and fungi.

Materials and Methods

Collection of plant's parts

The parts of sanitary trees of fig *F. carica* (leaves and stems) were collected from Al-Qurna groves in Basrah province of Iraq (30°53′7″N 47°17′27″E) in December 2019 (fig. 1). Each sample was placed in a sterile, clean, and labeled plastic bag, then transferred to the clinical and physiological laboratory research of fungi in the Department of Biology, College of Sciences, University of Basrah, and stored at 4^{o}C in the frig until further studies, while, the plant samples were classified by a staff of plant taxonomy laboratory.

figure (1): *Ficus carica* tree collected from Al-Qurna groves in Basrah province/Iraq

Isolation and purification of Endophytic Fungi

The isolation of entophytic fungi from plant samples was carried out according to Rimbawan *et al.* (2018) as follows: the parts of plants were washed in tap water and left to dry, then severed to small parts (2-3 cm), after that, the segments of the plant were superficially sterilized by being immersed two minutes in a 5.25% sodium hypochlorite (NaOCl) solution, followed by washing with sterile distal water, then transferred to sunken in 70% ethanol for two minutes, finally, another rinse with sterile distal water was done for all parts of plants. By using a sterile blade, the samples were divided into smaller parts (1x1 cm^2) and each three or four parts were distributed on petri dishes containing Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar SDA with duplicate for each medium. After the incubation period (5–14 days) at 28°C, the fungal colonies of endophytic fungi were purified by transmitting tiny portion of each colony into another PDA or SDA medium and cultured under the same conditions as before to obtain the single isolate of entophytic fungi

Identification of Endophytic Fungi

All fungal isolates were identified relying on morphological features such as shape, color, size of colonies, texture, exudates, odor, and others, as well as, the microscopic appearance was examined under a light microscope. Furthermore, the final identification was achieved by molecular approach (Degruyter *et al.*,2013; Spatafora *et al*., 2016; Wanasinghe *et al*., 2017).

The genomic fungal DNA was extracted using a Presto ^{TM} Mini gDNA Yeast kit, (Geneaid, New Taipei City, Taiwan). An appropriate amount of activated fungal colonies on PDA were collected for the extraction process, whilst, the region of 28S large subunit of rDNA was utilized for the amplification step that was applied by the general primers Null1(NL1) F-5'- GCA TAT CAA TAA GCG GAG GAA AAG -3' and Null4 (NL4) R-5'- GGT CCG TGT TTC AAG ACG G -3 under the following conditions: 94°C for 5 min, then 30 cycles of 94°C for 30 sec, 56°C for 45 sec, 72°C for 1 min, and final extension at 72°C for 5 min, however, the total volume of PCR composition was 25 µl consisted of 12.5 µl Master mix (Bioneer, Korea), 1.5 µl of individual primer, 1.0 µl of fungal DNA, and 9 µl of nuclease-free water (Mirhendi *et al*. 2008)

Sequencing

PCR products of fungal isolates were sent to Macrogen company (Seoul, South Korea) for sequencing, then after, the sequences were treated by Chromas 2.6.6 and deposited in Basic Local Alignment Search Tool

(BLAST) in comparable with reference isolates of National Center of Biotechnology Information (NCBI)and compared with those available in Gen Bank via BLAST searches.

Phylogenetic tree

Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA11 (Tamura *et al.*, 2021)

Fermentation and extraction

After activation on PDA at 28 °C for seven days, the fungal isolates were grown on the semisolid flour rice. The fermentation process was beginning by transferring five blocks of 6 mm in diameter of activated isolates to Erlenmeyer flasks (250 ml) containing autoclaved fermentation medium which provided by weighting 12.5 gm of flour rice that added to 3‰ of saline water (3gm of NaCl was dissolved in 1000 ml of distilled water) and incubated for 40 days at 28°C. After the incubation duration, the fungal growth and flour rice substrate were extracted three times by adding 250 ml EtOAc for each once, then, the organic solvent was collected and left to evaporate entirely at room temperature.

Phytochemical Analysis

Following Kokate (1994) and Harborne's (1998) approach, phytochemical analytical tests were carried out to determine the presence of phenols, flavonoids, sugars, glycosides, alkaloids, proteins, steroids, saponins, tannins, coumarin, peptides and free amino acids groups. The evaluations were based on the visual observation of a color change or precipitate development following the addition of particular chemicals.

Antimicrobial Assay

The crude extracts of thirteen fungal isolates were tested to evaluate biological activity against three pathogenic microorganisms, including *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*, using agar well diffusion assay according to Akpotu *et al*., (2017). All fungal extracts were dissolved in dimethyl sulphoxide (DMSO 100% v/v) to provide working concentrations (20 mg/mL) of the extracts.

The Mueller Hinton Agar (MHA) was used for the determination of the antibacterial effect against *E. coli* and *S. aureus*, while, SDA was employed for antifungal examination toward *C. albicans*. 100 µl of 1×10^8 of activated bacterial culture were spread on MHA by sterile glass L-shape rod, whereas, 100 µl of 1×10^6 of *C. albicans* was diffused on SDA, then all culture plates were left to dry and the 6 mm in diameter well was created in the center of each plate by using a sterile cork borer. After that, 100 µl of the fungal extracts were loaded into these wells. Subsequently, the cultures were abandoned to allow the absorption of agents by agar medium, later, the plates were incubated at 37 ° C for 24 hrs. for bacterial isolates and 48-72 hrs. for *C. albicans*. After an incubation period, the actual inhibition zone diameters (IZDs) were determined. This assay was achieved in triplicate for all fungal extracts in addition to positive controls of antibacterial and antifungal agents (200 µg/ml of gentamicin and 1mg/ml of ketoconazole respectively), in addition to a negative control (100% v/v DMSO).

Statically analysis

The static analysis was employed by using SPSS Statistical Packages of Social Sciences (Version 26; USA) to determine means ± SD for all data. Also, the least significant difference test (LSD) was used to test the difference between means; Statistical significance was considered significant at $p < 0.05$.

Results and discussion

Isolation and identification of endophytic fungi

During the current study, twelve fungal isolates were obtained from the leaves and stem of *Ficus carica* (fig. 2), be classed into five different genera including *Alternaria* sp. with high frequency (38.46%), followed by

Aspergillus spp. that appeared with 23.07%and 15.38% for *Rhodotorula* sp., while *Stemphylium eturmiunum* and *Talaromyces atroroseus* were seen by lowest percent (7.69%) as one isolate for each one. Table (1) was showed the lists of fungal isolates that were deposited in GenBank with their accession numbers depending on the alignment of BLAST. Furthermore, figures (3and4) represented the electrophoresis findings of DNA and amplification of endophytic fungal isolates.

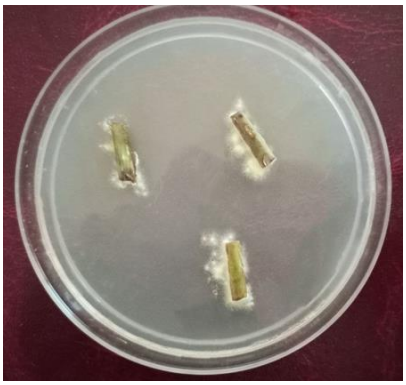

Figure (2): Isolation of endophytic fungal isolate from leaves of *Ficus carica*

Table (1): Fungal isolates that registered in gen bank with their accession number

No.	Homolog sequence	Accession Number	Sequence Identity %	Closest Accession Number
1.	*Alternaria alternata*	OM420234.1	100%	MT533836.1
2.	*Alternaria alternata*	OM420235.1	100%	MT533836.1
3.	*Alternaria alternata*	OM420236.1	100%	MT533836.1
4.	*Aspergillus costaricensis*	OM420244.1	99.66%	NG_069877.1
5.	*Alternaria alternata*	OM420238.1	99.82%	MT533836.1
6.	*Stemphylium eturmiunum*	OM420553.1	100%	NG_069866.1
7.	*Alternaria alternata*	OM420239.1	99.31%	MT533836.1
8.	*Aspergillus fumigatus*	OM420257.1	98.12%	MN134347.1
9.	*Rhodotorula mucilaginosa*	OM420494.1	100%	MG833306.1
10.	*Talaromyces atroroseus*	OM420714.1	99.64%	MH869518.1
11.	*Rhodotorula mucilaginosa*	OM420495.1	100%	KY109112.1
12.	*Aspergillus flavus*	OM420252.1	100%	MT252035.1

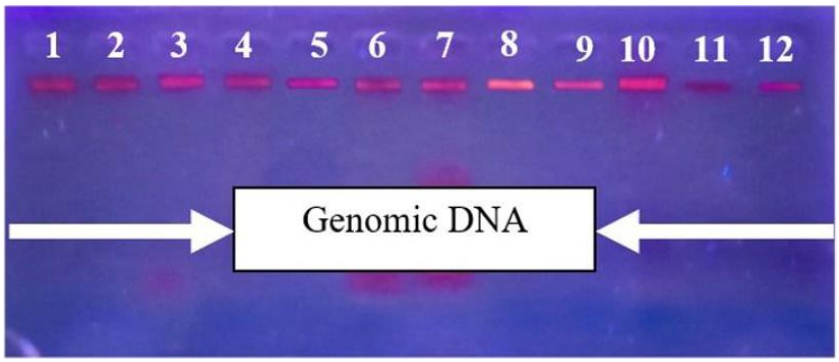

Figure (3): 1% agarose gel electrophoresis of genomic DNA for endophytic fungal isolates: (1-12) genomic DNA of fungal isolates.

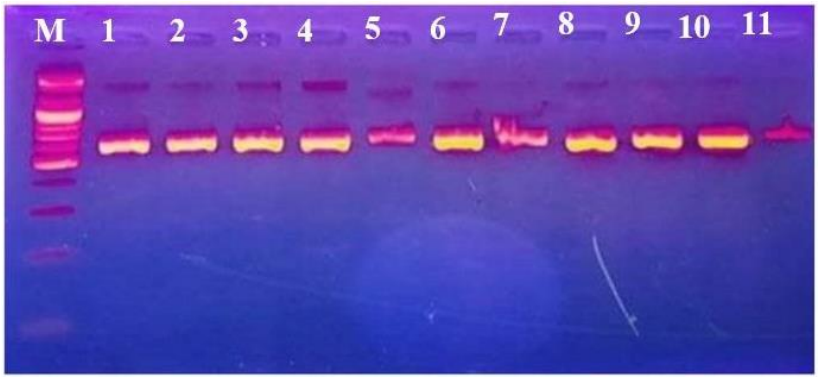

Figure (4): 2% agarose gel electrophoresis analysis for PCR assay with two primers NL1, NL4, the size of gene was approximately (550-600) bp, (1-11) PCR products, (M) DNA marker.

Species-level identification of fungi may be challenging due to the tremendous variety of their ecology and anatomy; therefore, it looks difficult to design an accurate species identification for all fungi, while, choosing the possible DNA barcode region (s) essential for identifying biological data and attributing them to certain species (Tekpinar and Kalmer, 2019; Alfartosy *et al.*, 2021).

The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-4062.63) is shown (fig. 5). This analysis involved 12 nucleotide sequences. There were a total of 584 positions in the final dataset

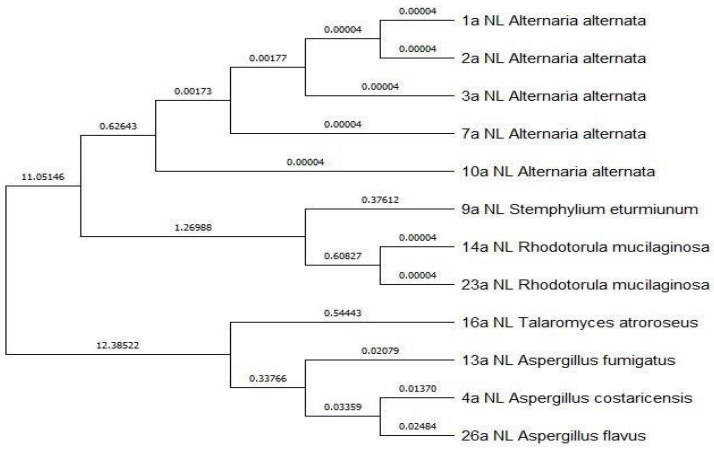

Figure 5: The maximum likelihood tree to determine that estimated the pairwise distances among 12 nucleotide sequences of this study. The tree presented the clustering of the related isolates in the same clusters.

Fermentation and extraction

Many previous studies used a solid medium of rice as a fermentation source to produce different secondary metabolites from fungi, whereas, the present investigation revealed that semi-solid medium yields equivalent to biomass and bioproducts.

The extraction processes by using ethyl acetate appeared that *Aspergillus* spp. and *Alternaria* sp. provided a huge amount of crude extract than other fungal endophytic isolates, however, table (2), presented the mass of extracts that ranged 50-100 mg, and this result accepted with the finding of Song and co-authors (2013).

Moreover, ethyl acetate was used in the current study as an organic solvent for its poorly miscible with water (miscibility ratio was 33), as well as, has low boiling points of 77°C, which assist in its fast evaporation (El-Naggar *et. al*. 2001).

Table 2: the weight of fungal crude extracts after drying

NO	Fungal isolates	Crude extract code	Weight
1	*Alternaria alternata*	Alt 1	79.357mg
2	*Alternaria alternata*	Alt2	84.453mg
3	*Alternaria alternata*	Alt3	53.406mg
4	*Aspergillus costaricensis*	Asp1	97,839mg
5	*Alternaria alternata*	Alt4	83.748gm
6	*Stemphylium eturmiunum*	Ste1	59.804mg
7	*Alternaria alternata*	Alt5	77.345mg
8	*Aspergillus fumigatus*	Asp2	54.01mg
9	*Rhodotorula mucilaginosa*	Rho1	51.848mg
10	*Talaromyces atroroseus*	Tal1	53.409mg
11	*Rhodotorula mucilaginosa*	Rho2	51.84mg
12	*Aspergillus flavus*	Asp3	52.993mg

Phytochemical assay

The results of table (3) showed that endophytic fungal isolates comprised different chemical groups involving phenols, flavonoids, carbohydrates, alkaloids, proteins, and tannins, all of the compounds have properties that make them able to use in different medical and pharmaceutical approaches (Subban *et al.* 2012). This result is similar to the study of Yin and Chen (2008) and diaz *et. al.* (2012) which pointed to the appearance of various chemical compounds in fungal extracts.

Table (3): Chemical contents of endophytic fungal crude extracts

Name of test	Crude extracts											
	Alt1	Alt2	Alt3	Asp1	Alt4	Ste1	Alt5	Asp2	Rho1	Tal1	Rho2	Asp3
Phenols test	+	+	+	+	+	-	-	-	-	+	+	+
Flavonoids test	-	-	-	-	-	-	-	+	-	-	-	+
Carbohydrates test	+	+	+	+	+	+	+	+	+	+	+	+
Saponins test	-	-	-	+	-	-	-	+	+	+	+	-
Glycosides test	+	+	+	+	-	-	-	+	+	+	+	-
Alkaloids test	+	+	+	+	+	+	+	+	+	+	-	-
peptides and free amino groups test	-	-	+	+	-	+	-	+	+	+	+	-
Proteins test	+	+	+	+	-	-	-	-	-	+	+	-
Tannins test	+	+	+	-	+	+	-	-	+	+	+	+
Coumarin test	-	+	-	-	-	-	-	-	-	-	-	-
Steroids test	-	-	-	-	+	-	-	-	+	-	-	-

(+) present, (-) not detected

Biological activity

The bacterial isolate of *S. aureus* seen that high susceptibility to crude extract of *A. flavus* and *A. niger* with an inhibition zone of 20mm for each one in comparison with the activity of gentamicin (12mm) which was used as a positive control, as well as, *E. coli* revealed that sensitivity toward extract of *P. chrysogenum* (35mm), while, the result of the table (4) showed that the bioproduct of *A. niger* presented the highest inhibition activity against

C. albicans (24mm) compared with the positive control (ketoconazole) that gave 15mm inhibition zone of *C. albicans* (fig. 6).

The current investigation accepted with the studies of Deshmukh *et al.,* (2014), Taufiq and Darah (2018), Jasim *et al.,* (2021), and Gu *et al.,* (2022) they reported the disparity in the activity of fungal metabolites is due to the difference in the chemical contents of the fungal crude extracts like alkaloids, terpenoids, steroids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenol, and lactones.

Table 3: inhibitory effect of fungal crude extracts against bacterial and yeast isolates.

NO	**Crude extract**	***S. aureus* 20mg/ml**	***E. coli* 20mg/ml**	***C. albicans* 20mg/ml**
1	Alt1	14.5 ± 2	22 ± 2.64575	16 ± 2
2	Alt2	16 ± 3	20 ± 1	16 ± 1
3	Alt3	13 ± 0.5	16 ± 3	17 ± 3
4	Asp1	11.5 ± 1	20 ± 1.80278	15 ± 2.64575
5	Alt4	13 ± 0	20 ± 3.60555	13 ± 1
6	Ste1	13 ± 2	18 ± 2.64575	14 ± 2.64575
7	Alt5	15 ± 2.64575	22 ± 1	15 ± 2
8	Asp2	12 ± 1	30 ± 2	23 ± 1.5
9	Rho1	15 ± 3	20 ± 3	22 ± 1.73205
10	Tal1	11 ± 1.73205	25 ± 3	14 ± 2.64575
11	Rho2	14 ± 2	18 ± 1	18 ± 1.32288
12	Asp3	20 ± 0.57735	25 ± 2.64575	12 ± 2
13	PC+	12.3333 ± 2.77612	22 ± 3	15 ± 2
14	Total	13.8718± 2	21.3846± 4.11586	16.1538 ± 3.5802

(PC+) positive control

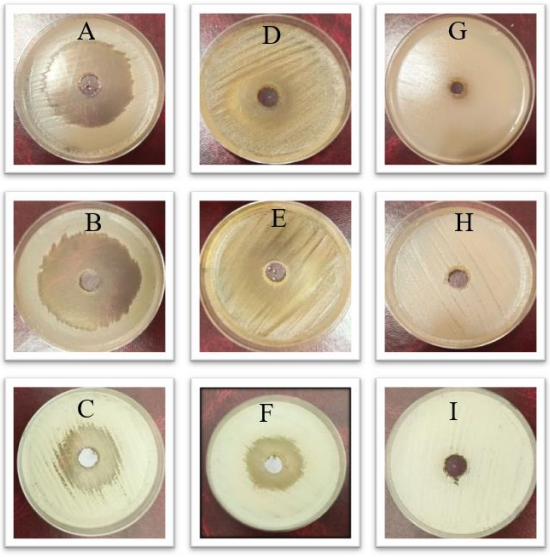

Figure (6): Inhibitory activity of fungal extracts against bacterial and yeast isolates using diffusion method, (A) *A. niger* activity against *S. aureus*, (B) *P.chrysogenum* activity against *E.coli*, (C) *A.niger* activity against *C. albicans*, (D and E) positive control (gentamicin) against *S. aureus* and *E.coli* respectively, (F) positive control

(ketoconazole) against *C. albicans*, (G, H, and I) negative control (DMSO) against *S. aureus*, *E.coli*, and *C.albicans* respectively

Conclusion

The present finding revealed the ability of endophytic fungal isolates to produce secondary metabolites in different amounts and various effects towards pathogenic microorganisms, therefore, this investigation indicated the ability of further studies to purify chemical compounds from crude extracts and evaluation their activity in medical and pharmaceutical applications.

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