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Evaluation of Antimicrobial bioactive compounds from Endophytic Fungi Isolated from *Moringa oleifera*

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Abstract

Endophytic fungi are microorganisms that inhabit the living tissues of their host plants without causing any host loss. They are considered as a continuous natural source of novel bioactive secondary metabolites with potential application in medicine, which are almost same to their host plant. In this study a total of nine endophytic fungal isolates were collected from leaves and stems of ***Moringa oleifera***. Based on the colonization frequency (CF) results, the highest number of isolates was obtained from plant stem, while the least was from leaves. The nine isolates were identified by keeping track of morphological and microscopic observations. Identification of the two antimicrobial potent strains was confirmed by 18S rDNA-based molecular analysis. The nine isolates were found belonging to ***Chaetomium, Alternaria, Fusarium, Aspergillus, Mycelia, Penicillium*** and ***Nigrospora*** taxa. Among them, ***Chaetomium*** taxon was included the highest CF (40%) . Evaluation of antimicrobial activity documented ethyl acetate fungal extract as the highest effective inhibitor against Gram-negative and Gram-positive bacteria, and ***Aspergillus fumigatus***. Minimum inhibitory concentration (MIC) was examined for the two most potent antimicrobial effective extracts, from ***Chaetomium laterale*** and ***Chaetomium interruptum***; it was ranged from 12.5 to 0.39 mg/ml.

Keywords

Endophytic fungi, fungi identification, fungal isolation, 18S rDNA, *Chaetomium* Sp.

1. Introduction

Many microorganisms are embedded in the aerial and subsurface sections of plants, which may be beneficial or damaging to the plant's health. Endophytes are fungi or bacteria that live in plant tissues at intercellular or intracellular regions and do not cause disease symptoms [1]. Endophytes help plants develop, produce biomass, and boost their resistance to biotic and abiotic challenges [2] by maintaining a healthy balance inside the host tissues [3].

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Endophytes have recently been identified as a secondary metabolite source for plants. Many endophytic fungal secondary metabolites have anticancer, antibacterial, antitumor, antidiabetic, and antioxidant properties [4]. Endophytes generally modulate plant growth and development. However, mechanism of metabolite secretion by endophyte and its systemic colonization during diseases management at plant-endophyte interface remains elusive. Many endophytic fungal secondary metabolites have a various biological activity established therapeutic applications such as anticancer, antimicrobials, antitumor and antidiabetics, and cholesterol inhibitors and antioxidant agents. Endophytic fungal metabolites can also be used effectively in weed control.

Endophytic fungi are thought to interact mutualistically with their host plants mainly by increasing host resistance to herbivores and have been termed (acquired plant defence). The wide ranges of fungal diversity are potential source of novel natural products for exploitation in medicine , agriculture and industry as microbial diversity imply chemical diversity due to the constant chemical innovation that exists in ecosystem moreover , due to the world ‘s urgent need for new antibiotics , chemotherapeutic agents and agro chemicals to cope with the growing medicinal and environmental problems facing mankind , growing interest is taken into the research on the chemistry of endophytic fungi[5,6]

Moringa oleifera Lam (synonym: ***Moringa pterygosperma Gaertner***) is a well-known fast-growing, medicinal plant that belongs to the ***Moringaceae*** family, widely grown in other parts of the world and one of the most useful trees in the world; because of its medicinal and nutritional properties, it has been dubbed a "miracle tree" or "tree of life" [7]; leaves, roots, flowers, pod ***Moringa oleifera*** has been tested for antimicrobial, anticancer, and anti-inflammatory qualities all over the world [8,9,10].

Chaetomium is a fungus that belongs to the ***Pyrenomycetes*** (***Ascomycotina***) class and the ***Chaetomiaceae*** family, with approximately 95 species worldwide. ***Chaetomium*** species are heterothallic fungus that can be found in organic compost, soil, and some plants, as well as in marine algae and soft coral. Chaetoglobosins, terpenoids, steroids, tetramic acids, xanthones, benzoquinones, diketopiperazines, bis (3-indolyl)-azaphilones, anthraquinones, and orsellides are only a few of the more than 200 secondary metabolites isolated and identified from ***Chaetomium***. Some of these metabolites reported various bioactivities such as antimicrobial, cytotoxic, antiviral, and anticancer activities [11]. ***Chaetomium*** has been reported as a good antimicrobial activity and the presence of various bioactive compounds was assessed by chromatographic techniques which might be responsible for such activities [12].

The aim of this study was to investigate microbiologically the endophytic fungi associated with *Moringa oleifera*, a commonly used medicinal plant; survey and isolation of antimicrobial effective secondary metabolites generated by endophytic fungi were also targeted.

2. Materials and Methods

2.1 Collection of samples

The Botanical Garden, Faculty of Science, Ain Shams University was the source of gathering and documenting the fresh healthy leaves and stems of medicinal plant ***Moringa oleifera*** (Photo 1). After removing excess moisture by blot drying, leaves and lateral stem branches were clipped from the plants and placed in sterile plastic bags. On the day of collection, all sample materials were stored in an ice-packed bucket for the experimental process [13].

Photo (1)

2.1.1 Preparation of samples

To remove any soil pollutants, the samples were properly rinsed under tap water. Surface sterilisation of samples was carried out under laminar air flow by immersion in 70% ethanol for 2 minutes, then in aqueous sodium hypochlorite (4%) for 2 minutes, rinsed with 70% ethanol for 10 seconds, then washed with sterilised distilled water for 30 seconds. To remove excess moisture, the samples were wiped on sterile filter paper and each plant sample was chopped into small segments under sterile condition. [14]

2.2 Isolation of endophytic fungi

Endophytic fungi were isolated according to modified approach reported in [15]. Freshly prepared Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), and Sabouraud Dextrose Agar (SDA) media were treated with a total of 30 plant segments (15 from the stem

as well as from the leaves). Each medium was supplemented with streptomycin (500 mg/L) to prevent the bacterial growth and tested in three petri dishes. Any Petri plate was provided with 5 segments of the same plant part and incubated for 10 days at 28°C ± 2°C. Fungal development was tracked daily and purified [16]. Purified fungal endophytes were preserved at 4 °C on PDA slants for further research [17].

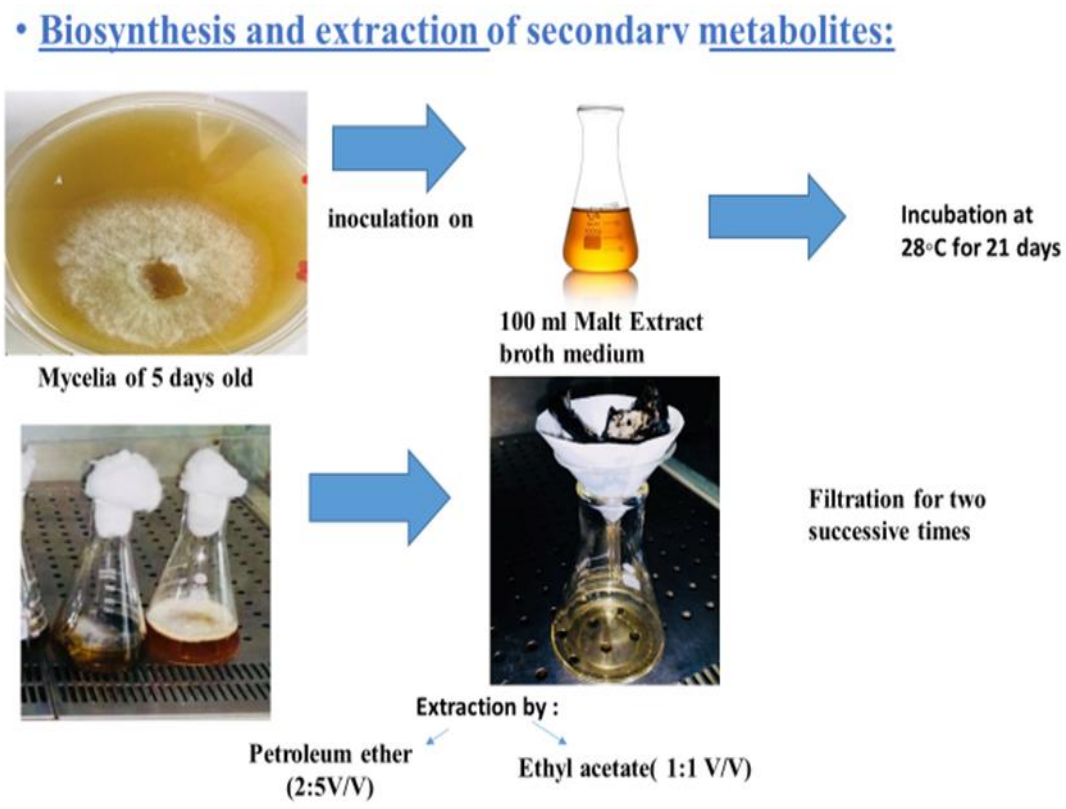
Determination of colonizing frequency (CF)

The frequency of colonization (CF) was assessed for each isolation plate daily. At the end of incubation time, the colonizing frequency was calculated using the method described by [18] to determine the endophytic fungal abundance in each plant part as:

$$CF\% = (Number\ of\ species\ isolated / total\ Number of\ segments\ screened)\ X100$$

Where: CF = Colonization frequency

2.3 Secondary metabolite biosynthesis and extraction



Five days old fungal mycelia of each of the nine isolates were inoculated separately in 100 ml Malt Extract Broth (MEB) per 250 ml Erlenmeyer flasks and cultured for 21 days under static conditions at 28°C ± 2°C. Each fungal broth culture was filtered twice using cheese cloth and Whatman filter paper No. 1 to eliminate any fungal mycelium, according to the method described by [19,20]. The broth filtrate was extracted into 1000 ml separating

funnel with two successive solvents, petroleum ether (20 ml/100 ml broth) followed by ethyl acetate 1:1 (v/v) [21]. The two obtained organic extracts and residual broth extract (aqueous extract) were concentrated using a rotary evaporator at 45 °C, to obtain crude fungal extracts. Each crude extract was weighted (50mg each) and dissolved in 1 mg/ml (DMSO) then screened for its antimicrobial activities [22,23] as well as the extract treated with petroleum ether separately.

2.4 Antimicrobial activity evaluation of endophytic fungal extracts

All gained concentrated crude extracts were evaluated for their antimicrobial activity by well diffusion technique regarding the inhibition zone [24]. Two gram-positive bacteria, ***Staphylococcus aureus*** (ATCC25923) and ***Bacillus subtilis*** (RCMB 015), and two gram-negative bacteria, ***Escherichia coli*** (ATCC 25955) and ***Proteus vulgaris*** (ATCC13315) were used as test microorganisms, as well as three fungal strains, ***Aspergillus fumigatus*** (RCMB002008), ***Aspergillus flavus*** (RCMB002002), and ***Candida albicans*** (ATCC10231); all test microorganisms were obtained from Regional Center of Mycology and Biotechnology (RCMB). Bacterial and fungal strains were inoculated on NA (Nutrient Agar) and PDA plates, respectively and wells (6mm diameter) were pitted in the plate's agar. Each bacterial and fungal strain was administered to the plates in full loop suspension. All crude extracts were diluted to a concentration of 50 mg/ml in 0.5% dimethyl sulfoxide (DMSO).

For bacteria, Gentamycin (1mg/100ml) was applied as a positive control, however Ketoconazole (1mg/100ml) was used for fungi; 100 µl of the tested fungal extract was applied to each well. The plates were left in the refrigerator for 1 hour to allow the extracts diffusion before microorganisms' proliferation. They were then placed in the incubator for 24 hours at 37°C for bacteria and yeast and at 28°C for filamentous. Using a ruler, the diameters of inhibition zones were measured in (mm) and recorded. The fungal extracts that showed substantial antibacterial or antifungal activity against the test pathogenic microorganisms examined were chosen for further investigations.

2.5- Identification of the most potent endophytic fungi

2.5.1- Morphological identification

Endophytic fungi were cultivated for 10 days at 28 ± 2 °C, Fungal isolates were identified morphologically with microscopic examination at Regional Centre of Mycology and Biotechnology's (RCMB), Identification Unit, Al-Azhar University. Colony growth rate and appearance, along with its microscopic features like hypha characteristics, conidium formation, and other cellular bodies such as fruiting bodies (asexual or sexual spores) or structures, were observed using an image analysis system (Olympus BX 40) and Soft-Imaging GmbH software (analysis Pro ver. 3.0).

2.5.2- Genetic identification

The study of the 18S rRNA gene was used to identify most antimicrobial potent endophytic fungal isolate(s). The Qiagen DNeasy Mini Kit technique was used to extract fungal DNA. Using universal primers (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), the isolated DNA was subjected to polymerase chain reaction [25].

Amplified DNA was sequenced at Macrogen Companies, Seoul, South Korea [26]. The results were found to be identical to the DNA sequence in the NCBI GenBank database. The gene sequence was then submitted to the NCBI GenBank database and assigned an accession number. http://www.ncbi.nlm.nih.gov.blast

3-Determination of the minimum inhibitory concentration (MIC)

Microliter broth dilution approach was performed to estimate MIC values of the most antimicrobial bioactive fungal extract(s) [27]. Serial dilution method was proceeded [28]. The crude extracts were diluted in dimethyl sulfoxide (DMSO) with 14 concentrations (50, 25, 12.5, 6.25, 3.125, 1.56, 0.781, 0.39, 0.195, 0.0976, 0.0488, 0.02441, 0.0122, and 0.0061 mg/ml) for screening test addressing the inhibitory zone as mentioned before. After incubation periods, the inhibition zone around each well was measured. The lowest crude extract concentration at which the growth of test organism was inhibited is expressed as MIC.

Analytical statistics

According to SPSS software [29], a one-way analysis of variance (ANOVA 1) was used after testing the data for normality to assess the variation in the responses of the investigated microbes to each fungal extract, as well as the variation in the responses of each organism to different fungal extracts **using Duncan test**.

3. Results

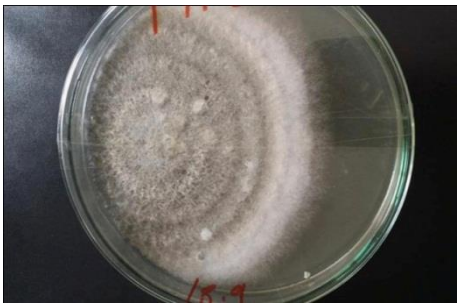
3.1 Isolation of *Moringa oleifera* endophytic fungi

As shown in table (1), a total of nine endophytic filamentous fungal isolates were collected from ***Moringa oleifera***. The brief morphological and microscopic description of the nine isolates was illustrated in table (1) and figure (1). The nine isolates were found belonging to eight taxa: ***Chaetomium, Alternaria, Fusarium, Aspergillus, Mycelia, Penicillium*** and ***Nigrospora***. Leave parts documented 4 taxa while the stem showed 5 (Table, 1). The occurrence of endophytes in each plant part was assessed according to colonization frequency (CF) equation (Table, 2). The (CF) of endophytic fungi found in *Moringa oleifera* stems was 60% exceptional when compared to those found in the leaves

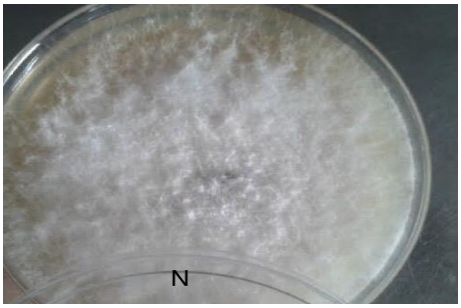
(40%). It is also worth noting that Potato Dextrose Agar is a good substrate for isolated fungi to thrive on (Figure, 1).

Table (1): Isolation and brief morphological and microscopic description of endophytic fungi isolated from *Moringa oleifera*

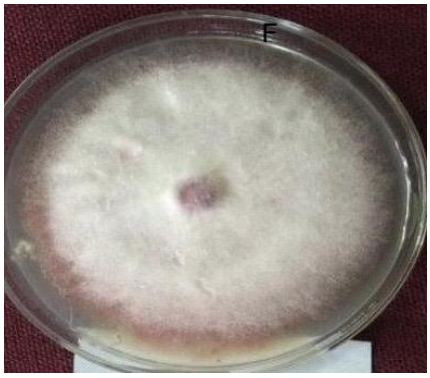
Plant Part	Isolates	Description
Leaves	**1-*Chaetomium* spp.**	olivaceous-buff to greyish-sepia with sparse aerial mycelium; reverse similar in colour to colony surface. Mycelium composed of hyaline, septate, smooth hyphae, Ascomata superficial to immersed.
	***2-Alternaria* sp.**	Olivaceous-black to black, dark brown conidia, pale brown smooth-walled conidiophores.
	3- *Fusarium* sp.	Oval to kidney shaped conidia, thin-walled conidiophores, terminal chlamydospore.
	4- *Mycelia* sp.	Do not produce any recognizable sexual/asexual conidia state in culture.
Stem	***1-Aspergillus* sp**.	Buff to sand brown in color, conidial heads are compact and biseriate, conidiophores are hyaline and smooth-walled.
	***2-Penicillium* sp.**	Green with white periphery, long dry chains conidia, hyaline, rough-walled conidiophores.
	***3-Nigrospora* sp.**	White at first, turns black later, with black shining smooth conidia, micronematous branched, brown conidiophores.
	***4-Chaetomium* spp.**	olivaceous in colour. Perithecia were flask-shaped, huge, dark brown to black fungi. Perithecia had filamentous, hair-like, brown-to-black appendages (setae) on their surface.
	5-*Mycelia* sp.	Do not produce any recognizable sexual/asexual conidia state in culture.



***Aspergillus* sp.**

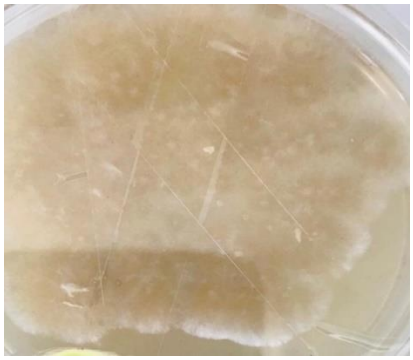


***Nigrospora* sp.**



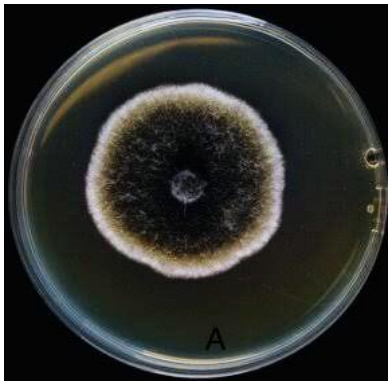
***Fusarium* sp.**

***Penicillium* sp.**

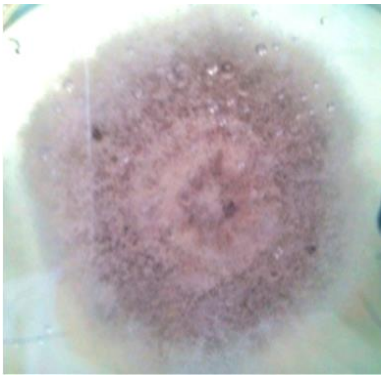


***Chaetomium* spp.**

***Chaetomium* spp.**



***Alternaria* sp.**



***Mycelia* sp.**

Figure (1): Endophytes isolated on Potato Dextrose Agar from *Moringa oleifera* stem and leaves

Table (2) Colonization frequency of endophytic fungi isolated from leaves and stem of *Moringa oleifera*

Isolate No.	Name of the isolates	Leaf	Stem	CF%
Ascomycetes				
1	***Chaetomium* spp.**	**-**	**3**	**20**
2	***Chaetomium* spp.**	**3**	**-**	**20**
Hyphomycetes				
3	***Alternaria* sp.**	**1**	**-**	**6.66**
4	***Fusarium* sp.**	**1**	**-**	**6.66**
5	***Aspergillus sp.***	**-**	**2**	**13**
6	***Penicillium* sp.**	**-**	**1**	**6.66**
7	***Nigrospora* sp.**	**-**	**1**	**6.66**
Sterile form				
8	***Mycelia* sp.**		**2**	**13**
9	***Mycelia* sp.**	**1**	**6**	
			40	**60**

3.2 Antimicrobial activity evaluation of endophytes broth crude extracts

Tables (3 & 4) demonstrated the antimicrobial activity screening results of the discharged fungal crude metabolites extracted respectively by petroleum ether separately then by ethyl acetate. It was observed that largest inhibition zone diameters were achieved on applying both successive solvents one by one. The two ***Chaetomium*** isolates crude metabolites recorded (32.07, 30.47, 29.80, 27.49, 21.60 mm) and (28.80, 27.07, 26.60, 21.60, 21.40 mm) against most test microorganisms and lack fungal activities against resistant ***A. flavus*** and ***C. albicans.*** The other endophyte isolates showed weak to non-effective antimicrobial activities against the test organisms. Regarding to these results the two isolates of ***Chaetomium*** were chosen as the most antimicrobial potent isolates and subjected to the further studies.

Table (3): Antimicrobial activities of petroleum ether crude extracts of fungal endophytes isolated from *Moringa oleifera* stem and leaves

Endophytic strain / Test organisms	Inhibition zone diameter (mm)								
	***Chaetomium* spp.**	***Chaetomium* spp.**	***Alternaria* sp.**	***Aspergillus* sp.**	***Nigrospora* sp.**	***Fusarium* sp.**	***Penicillium* sp.**	**M sterilia**	**Control[1]**
A. fumigatus	19.1±1.10	13.11±0.45	0.00	0.00	0.00	0.00	4.71±0.16	0.00	21.31±1.45
A. flavus	0.00	0.00	0.00	1.58±0.20	0.00	0.00	0.00	0.00	18.62±0.58
C. albicans	0.00	0.00	0.00	1.67±0.19	7.00±0.20	5.03±1.28	0.00	0.00	23.83±0.89
S. aureus	0.00	17.25±0.62	0.00	0.00	8.13±0.20	6.62±0.27	9.89±1.82	5.25±0.49	24.95±1.97
B. subtilis	0.0	24.26±0.91	0.00	0.00	7.05±0.28	8.41±0.32	0.00	4.11±0.62	28.92±1.24
E. coli	13.42±0.71	13.43±0.76	1.46±0.27	2.37±0.20	0.00	0.00	0.00	0.00	27.34±1.27
P. vulgaris	10.12±0.83	16.93±1.32	0.00	0.00	3.64±0.13	6.25±0.20	0.00	4.85±0.23	29.64±1.82

F-value[2]	606.3***	490.3***	196.0***	357.3***	1537.0***	1714.0***	9018.0***	564.4***	67.4***

[1]Gentamycin and Ketoconazole were used as positive control against bacteria and fungi, respectively

[2] F value was performed; ***means significant difference at $P < 0.001$

Table (4): Antimicrobial activities of ethyl acetate crude extracts of fungal endophytes isolated from *Moringa oleifera* stem and leaves

Endophytic strain / Test organisms	**Inhibition zone diameter (mm)**								
	***Chaetomium* spp.**	***Chaetomium* spp.**	***Alternaria* sp.**	***Aspergillus* sp.**	***Nigrospora* sp.**	***Fusarium* sp.**	***Penicillium* sp.**	**M sterilia**	**Control[1]**
A. fumigatus	29.80±1.11	26.60±0.92	0.00	0.00	0.00	0.00	10.09±0.37	0.00	21.31±1.45
A. flavus	0.00	0.00	5.00±0.20	4.30±0.46	0.00	0.00	0.00	0.00	18.62±0.58
C. albicans	0.00	0.00	4.03±0.15	1.47±0.23	8.70±0.30	10.14±0.42	0.00	0.00	23.83±0.89
S. aureus	27.49±0.87	21.40±0.60	3.20±0.30	3.90±0.36	10.83±0.35	8.22±0.46	18.92±0.36	12.08±0.43	24.95±1.97

B. subtilis	32.07±0.12	27.07±1.40	0.00	2.00±0.20	9.93±0.31	12.14±0.35	4.08±0.32	10.93±0.31	28.92±1.24
E. coli	21.60±1.11	21.60±1.11	3.00±0.62	4.07±0.21	0.00	0.00	0.00	0.00	27.34±1.27
P. vulgaris	30.47±0.84	28.80±1.08	2.20±0.26	2.07±0.31	9.03±0.25	10.71±0.37	0.00	8.62±0.74	29.64±1.82
F-value[2]	1069.00	593.63***	125.2***	94.5***	1528.0***	1386.0***	3542.0***	1013.0***	67.4***

[1]Gentamycin and Ketoconazole were used as positive control against bacteria and fungi, respectively

[2] F value was performed; ***means significant difference at $P < 0.001$

Ethyl acetate was applied successively after petroleum ether treatment.

3.2.1 Antimicrobial testing of the most potent fungal crude metabolites from *Moringa oleifera*

Figure (2) clarified the antimicrobial activity screening results of ethyl acetate extracted crude metabolites by (***Chaetomium* spp.**) isolate which was derived from ***Moringa oleifera*** leaves. ***Bacillus subtilis*** had the widest antimicrobial inhibitory zone diameter (32.07 mm) followed by ***Proteus vulgaris*** (30.47mm), ***Aspergillus fumigatus*** (29.80 mm), ***Staphylococcus aureus*** (27.49), then ***Escherichia coli*** (21.60). The studied extract was extremely resistant to ***A. flavus*** and ***C. albicans***. Whereas ethyl acetate crude extract of ***Chaetomium* spp.** which inhabits ***Moringa oleifera*** stem exhibited highly significant antimicrobial activity. It was (28.80) mm with ***Proteus vulgaris*** followed by (27.07) mm for ***Bacillus subtilis***, (26.60) mm with ***Aspergillus fumigatus***, (21.60) with ***Escherichia coli***, and finally (21.40) with ***Staphylococcus aureus*** (Figure, 3). These remarkable results were achieved when compared to petroleum ether extract, residual aqueous broth, and the +ve control. Statistical analysis (ANOVA-1) revealed that the responses of each microbe to the varied extracts were extremely significant.

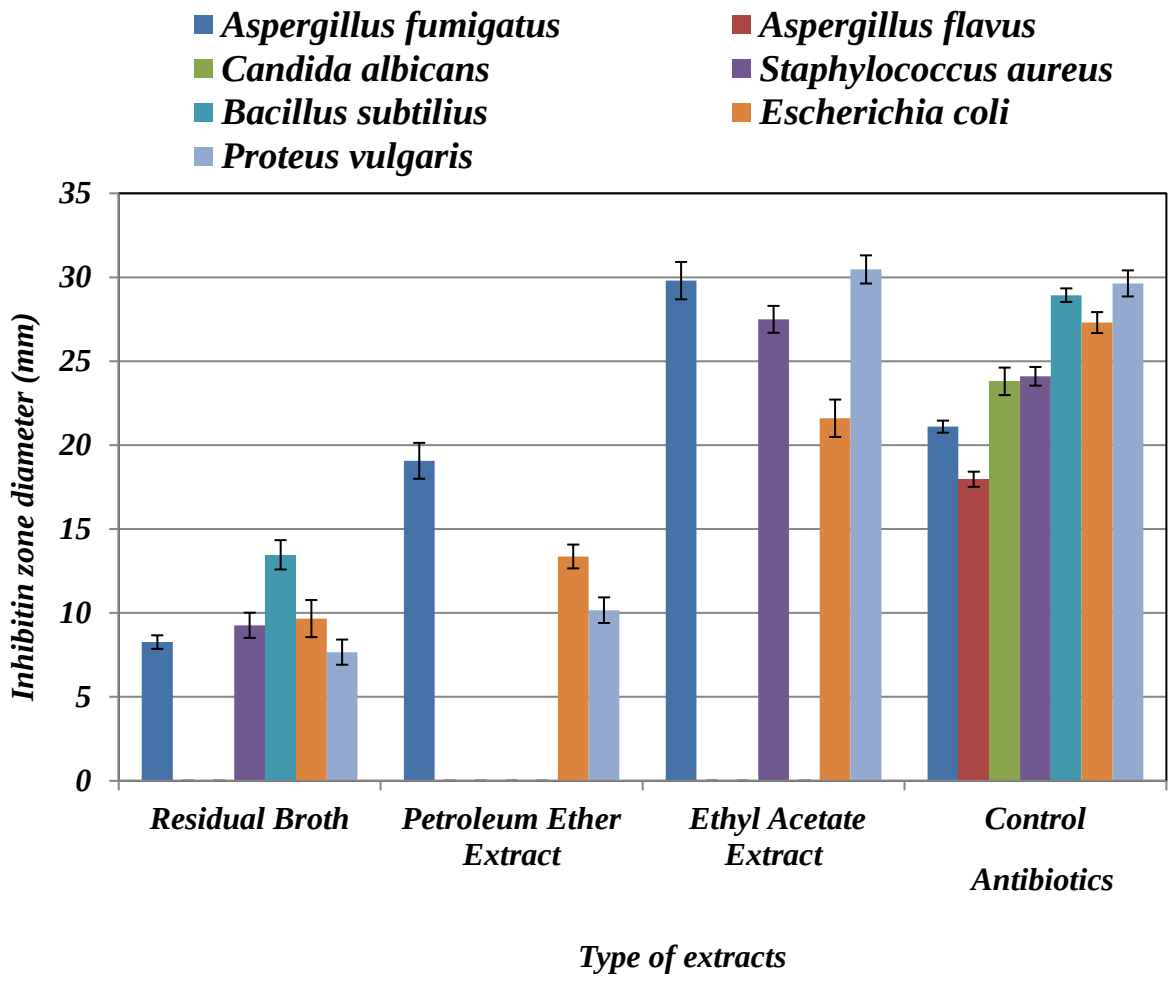


Figure (2): Antimicrobial activity of *Chaetomium laterale* crude extracts from ***Moringa oleifera*** leaves

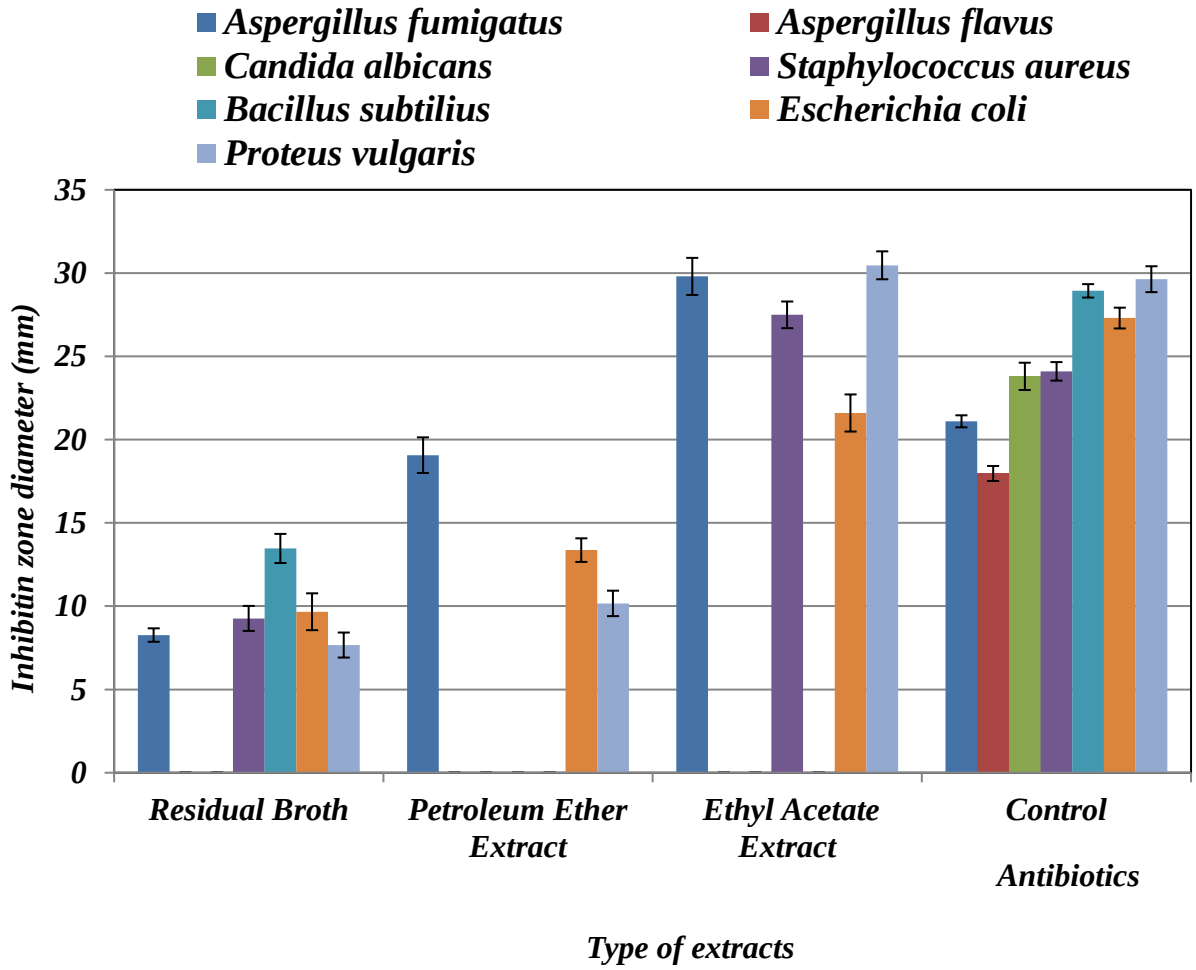
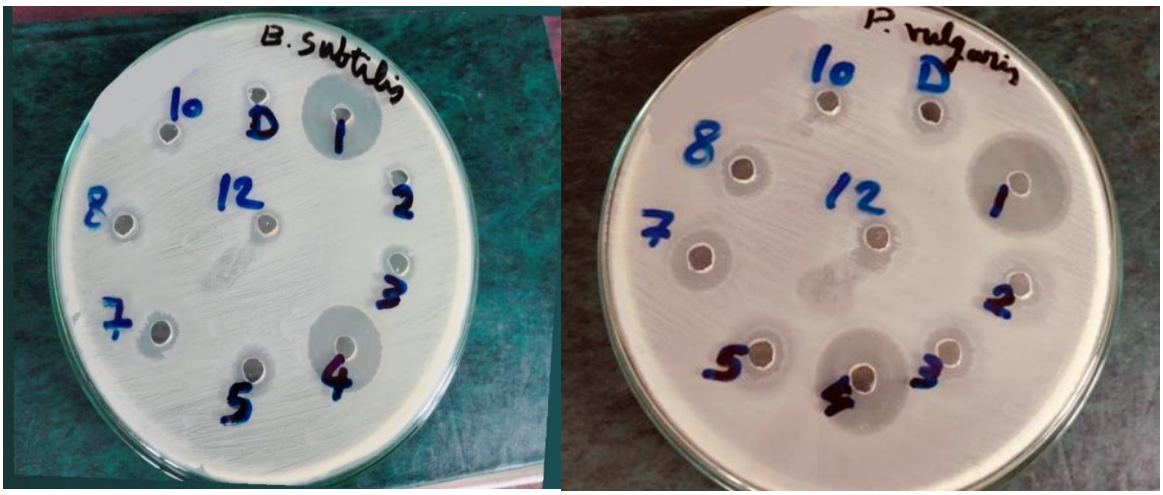
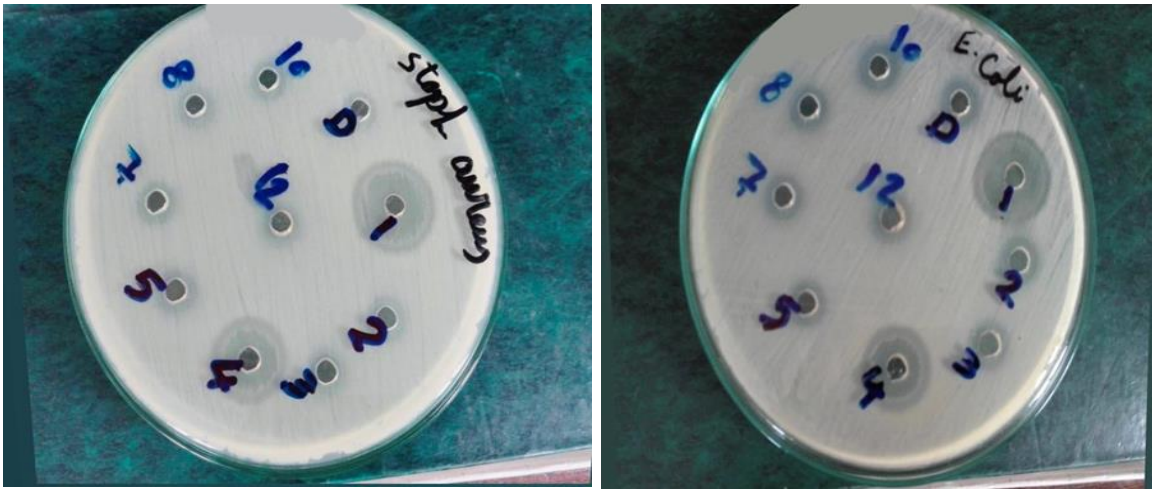


Figure (3): Antimicrobial activity of *Chaetomium interruptum* crude extracts from *Moringa oleifera* stem





1 *Chaetomium laterale ***4 *Chaetomium interruptum***

Figure (4) Antimicrobial activity of Chaetomium *laterale* and *Chaetomium interruptum*

3.3.1 Identification of the most active endophytic fungal isolate based on morphology

According to Regional Centre of Mycology and Biotechnology's (RCMB), Identification Unit, Al-Azhar University's, ***Chaetomium* spp.** from ***Moringa oleifera*** leaves is attaining 62 mm growth diameter on MEA after 7 days at 28°C. It is olivaceous-buff to greyish-sepia with sparse aerial mycelium, reverse colony colour is as well as surface, as shown in figure (5). Mycelium is of 9.2 µm diameter with hyaline, septate, smooth hyphae. Ascomata is superficial to immersed, scattered, oscillating, subglobose, 188.9 x 158.4 µm in size, brown rhizoids, weakly linked to mycelium. Terminal hairs are of various lengths, 315.4 µm long; Lateral hairs are of 58.3 µm long and 3.4 µm wide at the base, straight, short, coarsely roughened. Ascospores are one-celled, brown, fusiform, symmetrical, and thick-walled, measuring 12.6 x 7.8 µm. Consequently, as stated by isolate characteristics, it belongs to ***Chaetomium* spp**.

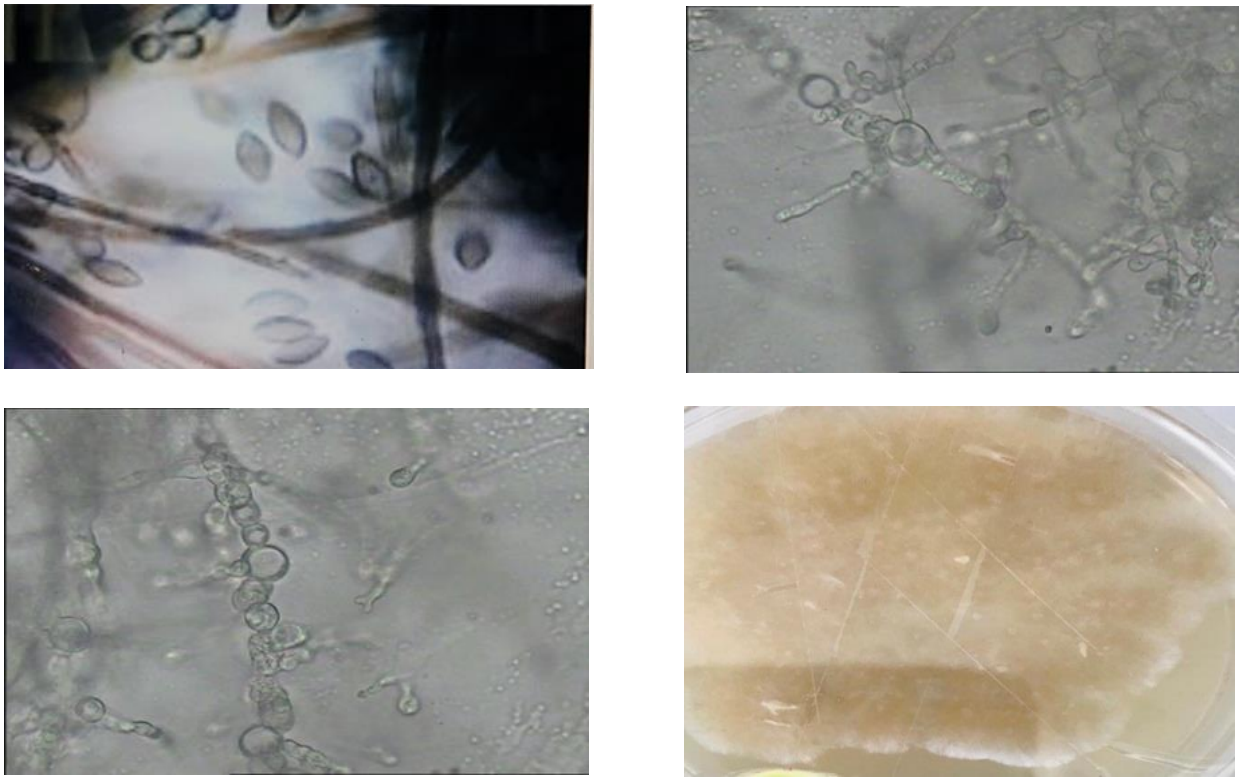


Figure (5): Hyphae and microscopic observations under light microscope of *Chaetomium* sp from *Moringa oleifera* leaves

Figure (6) indicated that the fungus recovered from ***Moringa oleifera*** stem has a striking resemblance to ***Chaetomium* spp.** according to Regional Centre of Mycology and Biotechnology's (RCMB), Identification Unit, Al-Azhar University's. Colonies are formed rapidly on MEA, reaching 76 mm in diameter after 7 days at 28°C in darkness. Septate hyphae, perithecia, asci, and ascospores are found. Ascomata is a surface ascomata that oscillates and is often covered with aerial hyphae. It is olivaceous in colour. Perithecia are flask-shaped, huge, dark brown. Perithecia have filamentous, hair-like, brown appendages (setae) on their surface (186 µ m) and hold asci and ascospores inside. The hairs on the ascoma are abundant, flexuous, coiled, septate, and brownish in colour. Asci are clavate with eight biseriate ascospores, with spore-bearing parts measuring 34.1 x 23.4 µ m and stalks measuring 20.4 m. One-celled, olive brown, limoniform shaped ascospores with a diameter of 10.5 µ m.

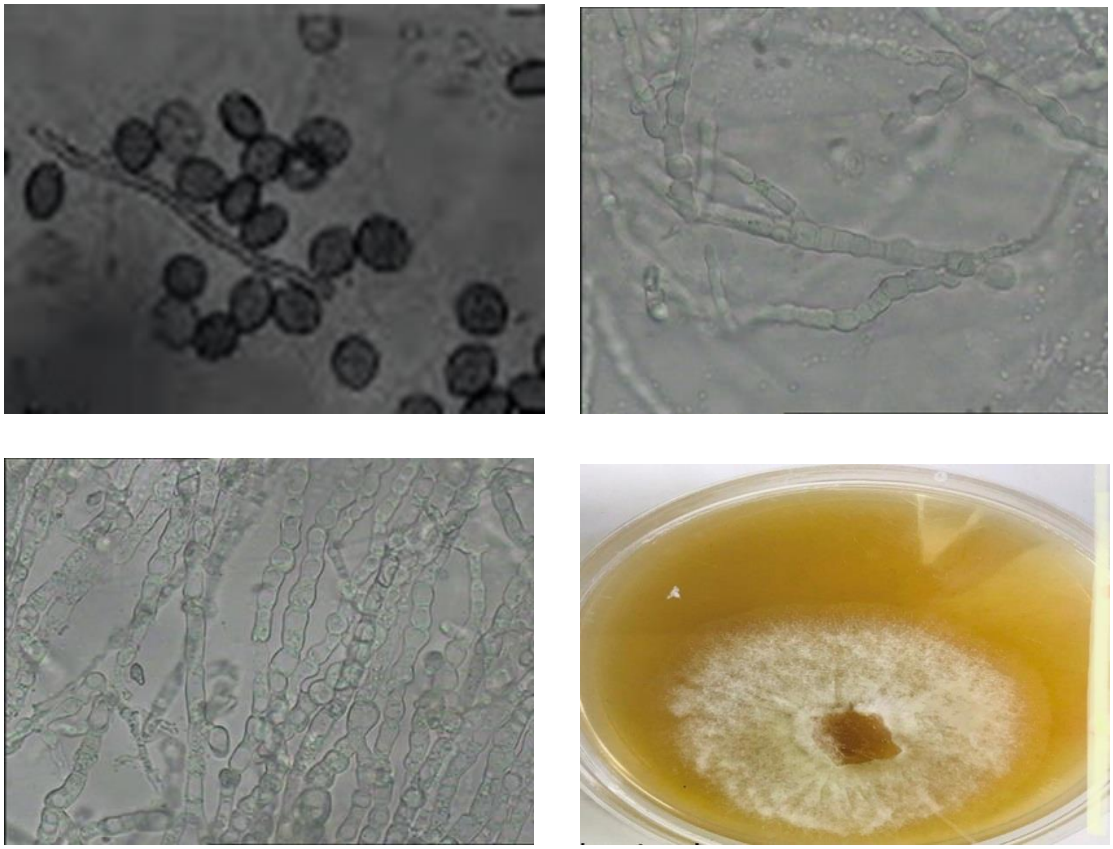


Figure (6): Hyphae and microscopic observation under light microscope of *Chaetomium* spp. from *Moringa oleifera* stem

3.3.2 Identification of the most active endophytic fungal isolate by molecular analysis

The 18S rRNA gene sequence was used to identify the two ***Chaetomium*** species and compare other sequences found in the GenBank database, using BLAST (http://www.blast.ncbi.nlm.nih.gov/Blast) to determine score similarity, the two fungal species were deposited in Gene Bank under accession number (ON782293) (ON778729) respectively.

Accordingly, the fungus isolated from ***Moringa oleifera*** leaves had a resemblance of 96.4 % to ***Chaetomium laterale* LC4146** (Figure, 7).

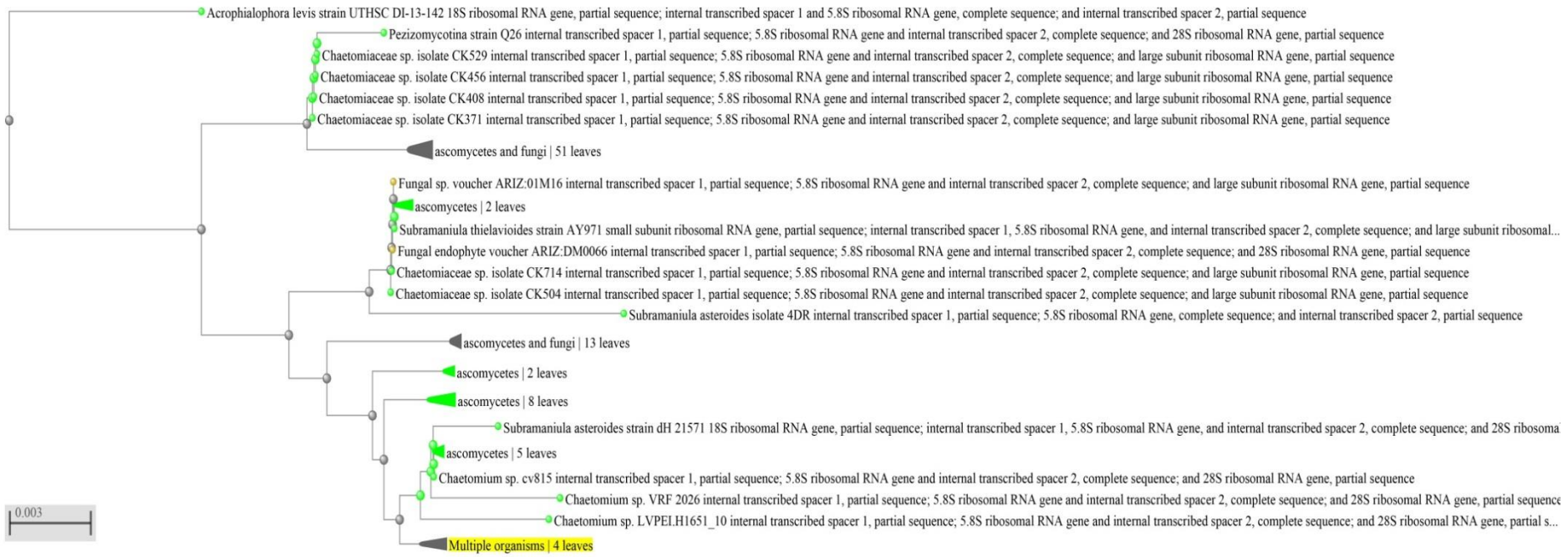


Figure (7): Phylogenetic tree of the DNA sequence of the fungus isolated from *Moringa oleifera* leaves.

As well, the study of 18S rRNA gene sequence of the fungus isolated from ***Moringa oleifera*** stem and according to the Gene Bank database revealed a higher homology of 99.6% with sequences of ***Chaetomium interruptum*** **CBS126660** (Figure, 8).

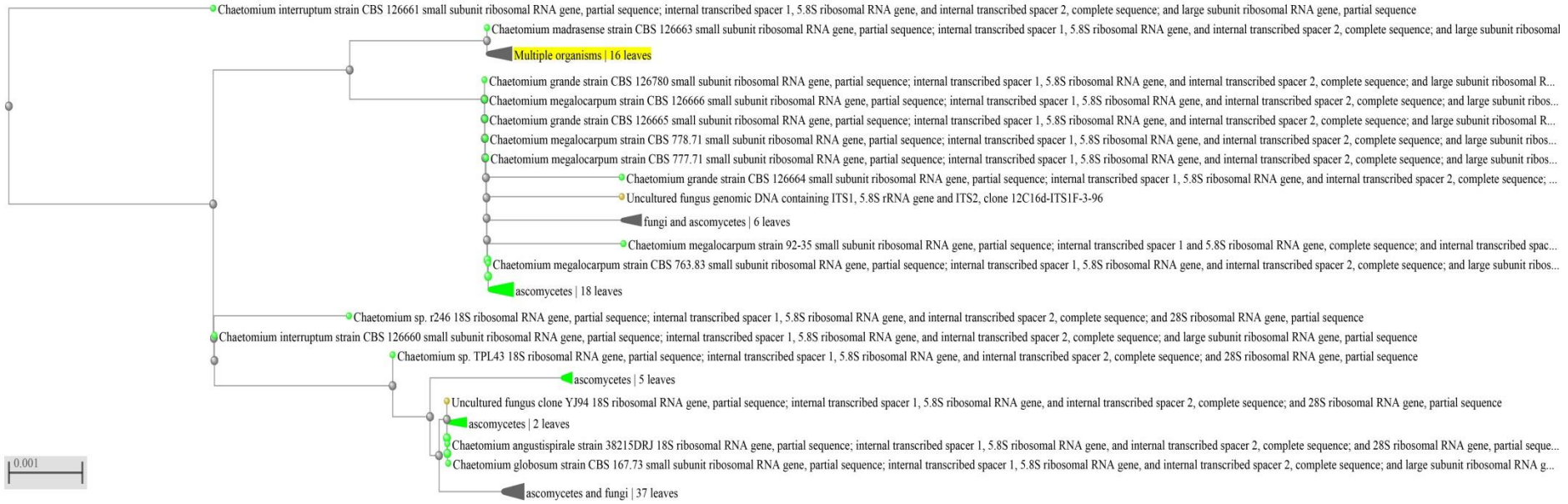


Figure (8): Phylogenetic tree of the DNA sequence of the fungus isolated from *Moringa oleifera* stem.

Minimum inhibitory concentration (MIC) of fungal extracts

The minimum inhibitory concentration (MIC) of ethyl acetate crude extract of ***Chaetomium laterale*** was found ranged from 12.5 to 0.39 mg/ml showing its high activity against ***Proteus vulgaris***, followed by both ***Staphylococcus aureus*** and ***Bacillus subtilis***, then ***Aspergillus fumigatus*** and ***Escherichia coli*** (Figure, 8). It generally exhibited a higher antimicrobial activity than ***Chaetomium interruptum*** ethyl acetate crude extract, whose MIC was ranged from 12.5 to 0.781 mg/ml against ***Proteus vulgaris*** followed by ***Staphylococcus aureus*** and ***Aspergillus fumigatus*** then ***Escherichia coli***, and finally ***Bacillus subtilis*** as illustrated in figure, 9.

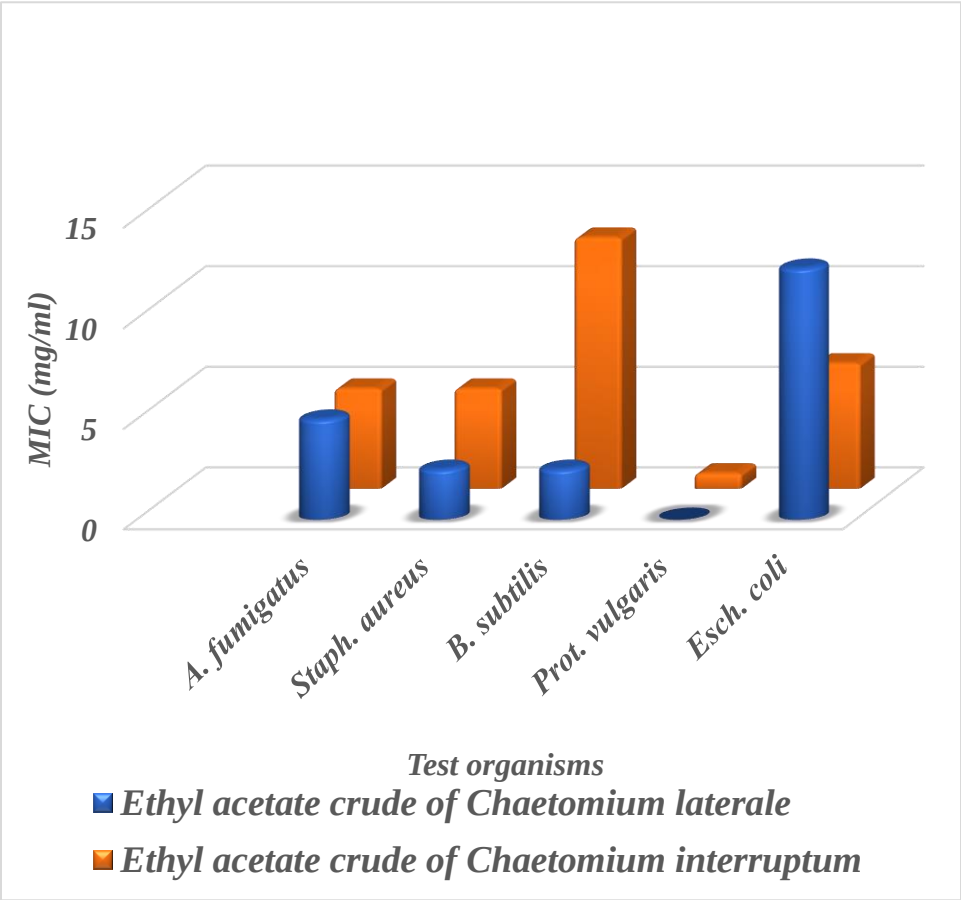


Figure (9): Minimum inhibitory concentration (MIC) in mg/ml against test microorganisms

4. Discussion

In the present study, 9 endophytic fungal isolates were isolated from ***Moringa olefiera*** leaves and stems. Potato Dextrose Agar (PDA) was found the best agar medium for fungal isolation; since potato infusion, potato starch, and dextrose supported fungal growth as established and explained by [30]. It comprises a high carbon and nutrient ratio, which enhance the development of a wide range of fungi and generation of several beneficial secondary metabolites. The data of this assay also elucidated that the secondary metabolites that were fermented by ***Moringa olefiera*** endophytes exhibited antibacterial and antifungal properties, which was in line with [31] findings.

In comparison to what has been reported in earlier studies [31, 32, 33], ***Moringa oleifera*** in this survey produced a comparatively low number of endophytic isolates. This could be due to a variety of reasons among many factors had been documented by [34], which influence endophyte diversity and abundance in host plants such as diverse microhabitats inside the tested plant, geographical locations, and seasonal fluctuations.

Another attempt examined the diversity of endophytic fungi of ***Moringa oleifera*** of Omalur region, India; fifteen different endophytic fungal isolates were collected, namely ***Aspergillus ochraceus***, ***Aspergillus niger***, ***Aspergillus flavus***, ***Aspergillus versicolor***, ***Aspergillus terreus***, **and** ***Bipolaris*** spp. as well as many unidentified sterile mycelial forms grouped under the class ***Mycelia sterilia*** [34].The colonization ratio (CF) for all fungal isolates in current study was comparatively lesser than that reported by [33 ,35] indicating that endophytes colonization may also varies due to the geographical distribution factor.

The results of this investigation affirmed that ethyl acetate crude extract derived from ***Moringa oleifera*** leaves endophytes isolated exhibited more antimicrobial activity than those obtained from ***Moringa oleifera*** stem. On the other hand, the residual broth extract (aqueous extract) demonstrated the lowest antimicrobial activity with all test microorganisms. Both ***Aspergillus flavus,*** and ***Candida albicans*** were found not sensitive in this assay, where no growth inhibition zones were formed when the two test strains were treated with all undertest fungal extracts.

Endophytic fungus belonging to genus ***Nigrospora*** was isolated by [36] from ***Moringa. oleifera*** roots and proved the production of secondary metabolites with antifungal activity. While ***Emericella*** sp., ***Aspergillus parasiticus***, ***Aspergillus tamari***, and ***Aspergillus***

sp. were isolated from the leaves of a ***Moringa*** plant from Democratic Republic of the Sudan and documented antibacterial activity against ***Escherichia coli***, ***Pseudomonas aeruginosa***, ***Klebsiella pneumonia***, ***Salmonella typhi,*** and ***Staphylococcus aureus*** [37]. Similarly, [38] isolated endophytic fungi from ***Moringa oleifera*** leaves, stem, and roots and confirmed their antibacterial activity. These findings were in agreed with most current screening outcomes.

Endophytes collected from ***Moringa oleifera*** leaves and stem were identified based on morphological characteristics and supported by molecular identification, both fungi belonged to family ***Chaetomiaceae*** under the genus ***Chaetomium***. The fungus isolated from ***Moringa oleifera*** leaves had 96.4% resemblance to ***Chaetomium laterale***, however the fungus isolated from ***Moringa oleifera*** stem had similarity to ***Chaetomium interruptum*** with 99.6%.

The endophytic fungus, ***Chaetomium globosum*** EF18, was isolated from ***Withania somnifera***; its ethyl acetate fungal extract displayed antifungal activity against ***Sclerotinia sclerotiorum*** [38]. It was found more effective than hexane extract, which was in accordance with this study findings.

It was interesting to notice that the secondary metabolites of undertest endophytic fungi isolated from the medicinal plant ***Moringa oleifera*** evinced some medicinal properties (antimicrobial activities); as well as reported by [39].

5. Conclusion

This study investigated antimicrobial activities of the secondary crude metabolites fermented by ***Moringa oleifera*** endophytic fungi. Both morphological and molecular methods delineated two important endophytic fungal species; ***Chaetomium laterale*** **and** ***Chaetomium interruptum*** from leaves and stem, respectively, of ***Moringa oleifera***. The ethyl acetate fungal crude extract of the two isolated ***Chaetomium*** species displayed effective antibacterial activities against pathogens of medical importance. It showed a wide spectrum, inhibiting both Gram-negative and Gram-positive organisms. The study outcomes support endophytic ***Chaetomium*** species as promising source of natural bioactive and novel metabolites and with great potential for further studies. As well, further consideration is needed to examine the antibacterial effectivity of fungal endophyte's secondary metabolites against a wide range of multi-drug resistant (MDR) bacterial pathogens, in addition to the antiviral and antifungal activities.

Subsequent studies are recommended to evaluate the secondary metabolites toxicity of endophytic fungi in paving a way for new therapeutic inventions.

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الملخص العربى

تقييم النشاط البيولوجى المضاد للميكروبات للمركبات النشطة المستخلصة من الفطريات المتعايشة داخل النبات المعزولة من نبات المورينجا اوليفيرا

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الملخص العربى

الفطريات المتعايشة داخليا هي تلك الكائنات الدقيقة التي تعيش داخل خلايا النبات العائل لها دون أن تتسبب في أي خسارة للنبات. تعتبر هذه الفطريات مصدر طبيعي متجدد لإنتاج المواد الأيضية الثانوية غير المألوفة، والتى قد تُستحدم فى بعض التطبيقات الدوائية والعلاجية. تناولت هذه الدراسة تجميع تسعة عزلات فطرية نقية من أوراق وسيقان نبات المورينجا. طبقاً لقانون تواتر تكوين المستعمرات (CF) وُجد أن أكبر عدد من العزلات تم الحصول عليه من ساق النبات، بينما كانت الأوراق هى الأقل عدداً في العزلات. تم تعريف العزلات التسعة بواسطة الفحص الظاهرى والمجهرى الدقيق. ثم أُجري تعريف تأكيدي على السلالتين الأقوى نشاطاً ضد ميكروبي بواسطة دراسة التحليل الجزيئي المبنية على 18S rDNA. وقد أظهرت التعريفات إلى أن التسعة فطريات المعزولة تنتمى إلى الأصناف التالية:

Chaetomium,* Alternaria, *Fusarium, Aspergillus terreus, Mycelia sterilia, Penicillium and ***Nigrospora***

كما تبين طبقاً لقانون (CF) أن الصنف ***Chaetomium*** له النسبة الأعلى بين الأصناف المعزولة (40 %). وثق تقييم النشاط ضد الميكروبي أن مستخلص الإيثيل أسيتات الفطري هو الأعلى تأثيراً مثبطاً ضد كل من البكتريا سالبة وموجبة لجرام وفطر ***Aspergillus fumigatus***. تم اختبار التركيز المثبط الأدنى (MIC) للمستخلص الفطري ضد ميكروبي الأقوى تأثيرا على الإطلاق، وذلك كان من السلالتين (***Chaetomium laterale*** and ***Chaetomium interruptum***) حيث لوحظ أنها تتراوح ما بين 0.39 – 12.5 ملج/مل.