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Biological and Chemical Evaluation of Secondary Metabolites from Endophytic Fungi Isolated from Egyptian Ornamental Plants

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Abstract

Endophytic microorganisms are considered as a promising source for the production of bioactive secondary metabolites to treat the drug resistant microbes. Fifteen fungal isolates were isolated from four ornamental plants grown in Egypt namely; *Ricinus communis, Hibiscus rosa-sinensis, Morus alba* and *Ficus nitida*. The fungal isolates were screened for their ability to produce bioactive secondary metabolites by cultivating them on rice medium for 14 days. The metabolites were extracted by ethyl acetate followed by acetone. The obtained extracts were tested for their antimicrobial activities. Scale-up fermentation was performed for three promising fungal strains isolated from *Ricinus communis* and *Hibiscus rosa-sinensis*. Antioxidant activities, total phenolic content, anticancer activities as well as GC/Mass studies were carried out for their extracts. The isolated fungal strains under investigation were identified using the molecular technique 18SrRNA. The fungal isolates were identified as *Alternaria tenuissima* isolate ANAS-9, *Phoma herbarum* isolate ANAS-10 and *Aspergillus tamarii* ANAS-13 with accession numbers MW662645, MW662651 and MW662652, respectively. High total phenolic content, potent antioxidant and Gram+/- antimicrobial as well as antifungal activities have been evidenced for the three new isolates. Both ANAS-9 and ANAS-10 had very strong anticancer effect on liver (HepG2) and breast (MCF-7) cancer cell lines, while ANAS-13 reported moderate cytotoxicity against both of them. This study concluded that these fungal strains have potent biological activities for the treatment of multidrug resistance pathogenic bacteria, as well as anticancer and anti-inflammatory agents' production.

Keywords: Ornamental plants; Endophytes; Fungi; Antimicrobial; Antioxidant; Anticancer; GC/MS; 18SrRNA.

1. Introduction

Nature is known to have a lot of beneficial bioactive molecules that their usage led to drug discovery. From ancient times, these bioactive molecules have been used by a lot of people in the form of traditional medicine systems as herbal remedies in order to treat and prevent diseases without really knowing their real value. These molecules are now known as natural products (NPs) [1]. Natural products are defined as any chemical substance that is characterized by its pharmacological and biological importance and is produced by a living organism and could be synthetically prepared as well [2]. Drug discovery and development of medicine are very complicated processes. They are not the same as ancient times, they are totally different from traditional medical prescriptions, herbal remedies and dietary supplements. Nowadays, in order to discover a successful and effective drug from nature, a long series of different steps have to be followed, which include not only extraction, isolation and purification of the active ingredient, but also characterization beside other steps.[3] Breaking down proteins, fats, carbohydrates and nucleic acid, which are known as primary metabolites, are crucial for the living

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organisms in order to perform vital processes necessary for growth and development. Meanwhile, the metabolites that are synthesized by the living organism itself are referred to as secondary metabolites (natural products). These secondary metabolites are distinctive to the organism itself or special to specific species. The release of secondary metabolites is not related to neither growth nor development of the organism but they are produced for other reasons, for instance, as a defence mechanism against some predators or to adapt to new environmental conditions [4]. A remarkable number of drugs were derived from plants that were originally used for thousands of years in traditional medicine. One way that led to the discovery of natural products and their usage was self-medication by animals. It was thought that wild animals fed on certain types of plants for medical reasons rather than nutrition, like recovering from parasitic infections [5].

Plant essential oils are common kinds of natural products that have been used since centuries by ancient civilizations (e.g., Egyptian, Romanian and Greek) not only as diet spices but also as effective therapeutic tools for certain medical disorders [6]. Natural products took part in treatment regimens for digestion and mental dysfunctions as well as diabetes, arthritis, fever and inflammation [7-9]. Research never stopped revealing health secrets of natural products treasure, especially in our era in which change of human life modes has introduced new surrounding environmental oxidant and carcinogenic pollutants [10-11]. Recent studies reported that natural products exerted antioxidant activity that can balance the uncontrolled production of reactive oxygen species, and hence resulted in organ recovery from oxidative stress [12-15]. Natural antioxidants are needed to replace synthetic ones which themselves caused nucleic acid damage and cell toxification [16]. Additionally, natural products ability to fight cancer cells (e.g., hepatocellular carcinoma) has been also proved [17-19]. In fact, natural products could also act as in vivo preventive agents against a vast number of diseases (e.g., stomach ulcers, kidney cytotoxicity and hepatic induced malfunction) [15, 20-23].

Microorganisms have always been an essential part of the human's life as well as they were subjected to investigation throughout the years. Previously, bacteria and fungi provided new chemical entities and valuable natural products. Foremost, Alexander Fleming discovered the first antibiotic, Penicillin, from the fungus *Penicillium notatum* in 1929 [24]. This was followed by the discovery of a vast number of different structures that were helpful candidates in drug industry [2]. Fungi are found in nature as eukaryotic heterotrophic microorganisms and their relationship with humankind is strong and they have a close connection since they were used for ages in food production as in beers, bread and soy food in addition to treatments [25]. Obviously, the fact that fungi can act as both harmful and beneficial microorganisms was exposed by the aflatoxin poisoning event Turkey X disease in the 1960s. Aflatoxin is a member of mycotoxin, a large group of toxins produced from certain types of fungi. On the contrary, the discovery of the first broad-spectrum antibiotic, penicillin (known as the wonder drug which was isolated also from a fungus named *Penicillium notatum*) emphasized the importance of fungi in therapeutics industry. Additionally, these bioactive compounds, or secondary metabolites, are released by certain fungal taxa mostly filamentous fungi that belong to the Pezizomycotina Ascomycete class, and several Basidiomycete classes [26]. Though, the process of producing secondary metabolites usually begins after fungal growth termination due to nutrients deprivation, however, an abundant carbon source encourages the initiation of secondary metabolites formation process. Many widely used therapeutic agents are derived from fungal secondary metabolites as cyclosporine and mycophenolic acid that have immunosuppressive activity. Furthermore, recent studies on cyclosporine revealed that it can be used to develop Debio025 drug which exhibited potent antiviral activity [27]. The antimicrobial agents derived from fungal secondary metabolites include fusidic acid and griseofulvin [28].

Endophytes can be defined as microorganisms which are often bacteria or fungi that live within the tissues of different plants. They usually express mutual relationships with the plants and do not cause any harm or morphological variations to them. Endophytes form an obviously miscellaneous group of microorganisms ubiquitous in plants and maintain a clear association with their hosts for at least a part of their life cycle [29-31]. Endophytic fungi were arranged into two extensive groups based on their taxonomy; The clavicipitaceous endophytes, which inhabit some grasses and this class is limited to cool regions, and the non-clavicipitaceous endophytes, which are isolated from asymptomatic tissues of non-vascular plants and they are confined to Ascomycota or Basidiomycota group [32]. This work aims to isolate endophytic fungal strains to be used as a source of biologically active secondary metabolites. The isolation, biological and chemical evaluation could also be considered.

2. Experimental

2.1. Fungal isolation

The isolation of fungi was carried out according to the method described by Torres *et al.* [33]. Samples were collected from four different plants: *Hibiscus rosa-sinensis*, Ricinus *communis*, Ficus *nitida*, and Morus *alba* from the agriculture field in Dekernis city Dakahlia governorate, Egypt, in December 2019. Different plant parts (leaves and stems) were exposed to surface sterilization protocol. Fungal strains were isolated from leaves and stems. Samples were washed with tap water, followed by washing with distilled water. Surface sterilization was done sequentially by soaking the plants in 70% ethanol for 1 min, then they were immersed in 0.5% sodium hypochlorite for 30 seconds. The samples were rinsed again by ethanol followed by sterile distilled water; this step was repeated three times. Next, the samples were dried using sterilized filter papers [33-34]. Leaves and stems were cut into small pieces and inoculated in Petri dishes (15cm diameter). Petri dishes were divided into two groups; each had potato-dextrose agar (PDA) supplemented with neomycin (50mg/l) as antibacterial agent, the second group had PDA supplemented with neomycin in addition to Rose Bengal (30mg/l) to prevent fungal overgrowth [35]. The dishes were sealed with parafilm and incubated at 27°C for 3–6 days, growth was observed after two days. The incubation period for each fungus was recorded. This was taken when the first visual growth was observed from the plating date and considered as an incubation period of growth.

2.2. Fungal cultivation, scale-up fermentation and extraction

Scale-up fermentation and extraction were carried out according to Hamed et al. [36]. Scale-up fermentation was maintained using 15 Erlenmeyer flasks (1 L volume); each contains 100 g rice and 100 ml distilled water, sterilized at 121 °C (15lb) for 20 min. Each flask was inoculated with spore suspension from 1 slant (10 days old), duplicate flasks were prepared for each fungus. After incubation at 30°C for 15 days, the medium was extracted with ethyl acetate several times till exhaustion. Ethyl acetate was evaporated by rotary evaporation. This step was repeated by exchanging ethyl acetate with acetone. Finally, after evaporation, the 30 extracts obtained from ethyl acetate and acetone were subjected to biological evaluation.

2.3. Evaluation of antimicrobial activity

The antimicrobial activity of ethyl acetate and acetone extracts of different fungal strains isolated from the previously mentioned plants was carried out by the agar disc diffusion technique. Staphylococcus aureus (G+ve), Escherichia coli (G-ve), Candida albicans (yeast) and Aspergillus niger (fungus) were used as representative test microbes. Nutrient agar plates were inoculated homogeneously with 0.1 ml of 105-106 cells/ml in case of bacteria and yeast. Potato dextrose agar plates inoculated with 0.1 ml of the fungal spores were used to evaluate the antifungal activities. Filter paper discs (0.5 cm in diameter) loaded with about 0.5 mg from each extract were placed over the surface of inoculated plates. Then plates were kept at low temperature (4 °C) for 2-4 hours to allow maximum diffusion. The plates were then incubated at the proper temperature and time for each test microbe (37 °C for 24 h for bacteria and at 30 °C for 48 h for fungi). The antimicrobial activity was considered as the diameter of the zone of inhibition expressed in millimeters (mm). Neomycin is used as positive control against bacterial whereas cycloheximide is used as positive control for fungal test strains [37].

2.4. Total antioxidant capacity

The antioxidant activity of each sample was determined according to the phosphomolybdenum method using ascorbic acid as standard. This assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green-colored [phosphate=Mo (V)] complex at acidic pH with maximal absorption at 695 nm. In this method, 0.5 ml of each extract (200 µg /ml) in methanol were combined in dried vials with 5 ml of reagent solution. The vials containing the reaction mixture were appropriately sealed and shaken to ensure that they were mixed well together. The vials were incubated in a thermal block at 95 °C for 90 min. After the samples had cooled at room temperature, the absorbance was measured at 695 nm against a blank. The blank consisted of all reagents and solvents without the sample, and it was incubated under the same conditions. All experiments were carried out in triplicates. The antioxidant activity of the sample was expressed as the number of ascorbic acid equivalent (AAE) [38-39].

2.5. Total phenolic content

In this method, the reaction mixture was composed of (100 µl) of the sample (200 µg/ml), 500 µl of the Folin-Ciocalteu's reagent, and 1.5 ml of sodium carbonate (20 %). The mixture was shaken and made up to 10 ml using distilled water. The mixture was allowed to stand for 2 h, and then the absorbance was measured at 765 nm; gallic acid was used as standard. All determinations were carried out in triplicates. The total phenolic content was expressed as mg gallic acid equivalent (GAE) per g extract [40-41].

2.6. Cytotoxicity activity of the endophytic fungi against two cell lines (HepG2 and MCF7)

The cells were cultured according to the protocol proposed by Mosmann [42]. Cell lines were cultured in RPMI-1640 medium with 10 % fetal bovine serum. Antibiotics were added 100 units/ml penicillin and 100 µg/ml streptomycin at 37 ℃ and incubated in a 5 % CO2 incubator. The cell lines were seeded in 96-well plates at a density of 1.0x104 cells/well at 37 ℃ for 48 h under 5 % CO2. After incubation, the cells were treated with different concentrations of compounds and incubated for 24 h. Doxorubicin was used as a standard anti-cancer drug for comparison. After 24 h of drug treatment, 20 µl of MTT solution at 5 mg/ml was added and incubated for 4 h. Dimethyl sulfoxide (DMSO) in volume of 100 µl was added into each well to dissolve the purple formazan formed. The colorimetric assay was measured and recorded at absorbance of 570 nm using a plate reader. The relative cell viability in percentage was calculated as (A570 of treated samples / A570 of untreated sample X 100).

2.7. Gas chromatography-mass spectrometric (GC-MS) analysis

A Thermo Scientific, Trace GC Ultra/ISQ Single Quadrupole MS, TG-5MS fused silica capillary column with 30 m, 0.251 mm and 0.1 mm film thickness and an electron ionization system with ionization energy of 70 eV were utilized. Helium gas was used as the carrier gas at a constant flow rate of 1 ml/min. The injector and MS transfer line temperature was 280 °C. The oven temperature was programmed to an initial temperature of 50 °C (hold 2 min) to 150 °C at an increasing rate of 7 °C/min. then to 270 °C at an increasing rate of 5 °C/min (hold 2 min) then to 310 °C as a final temperature at an increasing rate of 3.5 °C/min (hold 10 min). Samples were prepared according to the method of Madkour et al. [43]. Twenty-µl pyridine mixed with 30 µl N,O, bis (trimethylsilyl) trifluoroacetamide (BSTFA) were added to 2.5 mg dried extract for each sample. Samples were incubated for 30 min at 85 °C and then analyzed by GC/MS. The quantification of all of the identified components was investigated using a percent relative peak area. A tentative identification of the compounds was performed based on the comparison of the irrelative retention time and mass spectra with those of the NIST (National Institute of Standards and Technology) and WILLY library data of the GC/MS system.

2.8. Molecular identification of fungi

Molecular identification has been recognized by DNA extraction that was performed using Qiagen DNeasy Mini Kit followed by PCR and sequencing (18SrRNA). The amplification reactions were performed using two primers ITS1 (5`-TCCGTAGGTGAACCTGCG-3`)/ ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) and the following PCR thermal profiles: denaturation step at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 90 sec, and a final extension step of 72 °C for 5 min. The PCR products purification was done to remove separate PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). Sequencing was achieved by means of Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). To determine the sequencing products, the applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) was used. ITS1 (5'- TCC GTA GGT GAA CCT GCG G-'3) and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-'3) were the Primer sequences used for the identification in the current study

3. Results and Discussion

3.1. Antimicrobial activity studies

Both produced ethyl acetate and acetone extracts of the selected 15 endophytic fungi were firstly evaluated by measuring their antimicrobial activities. The antimicrobial activity was measured by disc agar plate diffusion. Results in Table 1 and Figure 1 revealed the antimicrobial activities of the ethyl acetate and acetone extracts of 15 fungal endophytes against Gram-positive bacteria (S. aureus), Gram-negative bacteria (E. coli), yeast (C. albicans) and the fungus (A. niger). It has been found that extracts 1, 3, 5, 9, 10, 13 and 14 exhibited the highest activities against S. aureus with inhibition values of 17, 15, 15, 16, 18, 20 and 16 mm, respectively. Whereas, extracts 4, 7, 11 and 15 showed moderate activities with inhibition values of 12, 10, 10 and 14 mm, respectively. But, extracts 6 and 12 had lowest activities (9 and 8 mm, respectively). For E. coli, all extracts had moderate to low activities. The effect of different extracts was also tested against C. albicans and it has been found that extracts 1, 2, 3, 5, 8, 9, 10, 13 and 14 exerted higher activities with inhibition values of 18, 16, 16, 17, 19, 18, 16 and 17 mm, respectively. Samples 4, 6, 7, 11, 12 and 15 showed moderate activities against E. coli with inhibition values of 11, 10, 11, 10, 11 and 11 mm, respectively. For A. niger, extracts 1 and 14 had moderate activities (10 and 10 mm, respectively). Whereas samples 3, 8, 9, 10, 13 and 15 exhibited low antifungal activities (9, 7, 7, 7, 8 and 8 mm,

respectively) and the other extracts did not exhibit antifungal activities. Acetone extracts were also subjected to antimicrobial studies. It has been found that extract 1 exhibited highest anti-Gram-positive activity against S. aureus (16 mm). Whereas samples 3, 9, 11 and 14 when tested against the same test microbe (S. aureus) had moderated activities (9, 9, 10 and 10 mm, respectively). The other extracts (2, 4, 5, 8, 10, 12, 13 and 15) showed low or no anti-Gram-positive activities (8, 8, 0, 6, and 8, 8 and 7 mm, respectively). When the extracts were tested against the Gram-negative bacteria (E. coli), all extracts did not show activities except extract 1 (8 mm). But, for C. albicans, extract 1 exerted higher activity (17 mm), whereas extracts 4, 5, 7, 11 and 14 showed moderate activities with C. albicans (10, 9, 9, 9 and 9 mm, respectively). The other extracts (7, 12 and 15) showed low anti-candida activities (11, 11 and 11 mm, respectively). Results of A. niger revealed that all extracts did not have antifungal activities except for extracts 2, 9, 10 and 15 that showed considerable antifungal activities (8, 9, 8 and 8 mm, respectively). Many recent studies have reported the medicinal importance of endophytic fungi and how they can be the untapped sources of bioactive secondary metabolites. However, few of them were isolated and identified. Kuo et al. [44] reported that ethyl acetate crude extract of 23 isolates which represented 20.35 % of the total isolated endophytic fungi expressed antimicrobial effect, these were isolated from Zanthoxylum simulans. Additionally, some medicinal plants such as Indigofera suffruticosa, Cephalotaxus hainanensis, and Melastoma malabathricum exhibited antimicrobial activity by 27.69 %, 34.74 %, and 26.37 % respectively against several Gram-positive and Gram-negative bacteria.

Likewise, Sandhu et al. [45] mentioned that endophytic fungi isolated from *Ricinus communis* expressed antimicrobial effect against several bacterial strains. The maximum zone of inhibition was expressed by genera *Phomopsis* and *Aspergillus*. They showed zone of inhibition against *B. subtilis* (25 mm), *K. pneumoniae* (25 mm), *S. aureus* (24 mm), *E. coli* (22 mm), furthermore, *Alternaria tenuissima* expressed antimicrobial effect against *B. subtilis* (22 mm), *E. coli* (20 mm), *K. pneumoniae* (25 mm), *S. aureus* (23 mm), but it exhibited least activity against *S. typhimurium* (18 mm) compared to the previously mentioned zone of inhibition. Kuo et al. [44] explained these variations in antimicrobial activities, where endophytic fungi extracts expressed relatively high effect against Gram-positive bacterial strains compared to their activity against Gram-negative strains, due to the fact that Gram-positive bacterial cell wall composition differs from Gram-negative bacterial cell wall. The outer layer of Gram-negative bacterial cell wall composes of lipopolysaccharides, alternatively the outer layer of Gram-positive bacteria is a thick layer of peptidoglycan. These differences in cell wall compositions affects the activity of bioactive compounds towards them [46]. Besides, Chi et al. [47] mentioned that ethyl acetate extracts are generally less polar metabolites compared to other used solvents, whereas polarity might be a reason for antimicrobial activity against Gram-positive strains.

Table 1: The antimicrobial activities of different fungal ethyl acetate and acetone extracts against *Escherichia coli, staphylococcus aureus, Candida albicans* and *Aspergillus niger*

Serial no	Clear zone (ϕmm)							
	Ethyl acetate fractions				Acetone fractions			
	S. aureus	*E. coli*	*C. albicans*	*A. niger*	*S. aureus*	*E. coli*	*C. albicans*	*A. niger*
1	17	6	18	10	16	8	17	0
2	7	6	16	0	8	0	7	8
3	15	0	16	9	9	0	7	0
4	12	0	11	0	8	0	10	0
5	15	6	16	0	8	0	9	0
6	9	9	10	0	0	0	0	0
7	10	6	11	0	8	0	9	0
8	10	9	17	7	0	0	0	0
9	16	8	19	7	9	0	8	9
10	18	0	18	7	6	0	7	8
11	10	6	10	0	10	0	9	0
12	8	0	11	0	8	0	7	0
13	20	7	16	8	8	0	7	0
14	16	6	17	10	10	0	9	8
15	14	0	11	8	7	0	6	8
Control 1	19	17	22	0				
Control 2	0	0	0	32				

Control 1(neomycin) as antibacterial, while control 2 (cycloheximide) as antifungal

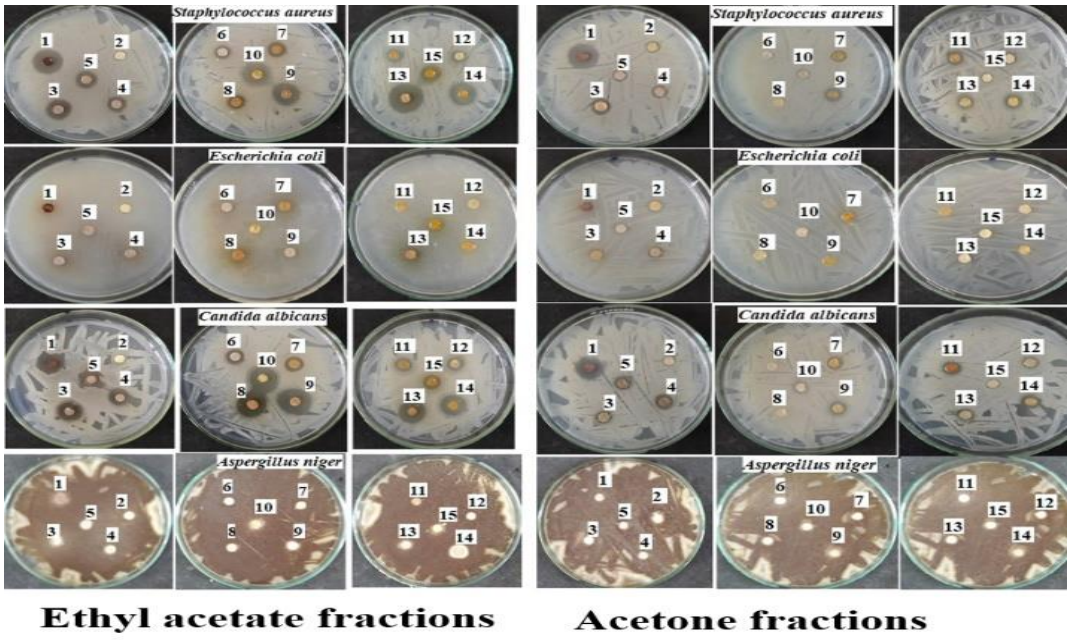

Figure. 1: Antimicrobial activities of different fungal ethyl acetate extracts against *Escherichia coli*, *staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*

3.2. Total antioxidant capacity

In this study, the total antioxidant capacity (TAC) was measured for both ethyl acetate and acetone extracts (Table 2) using the phosphomolybdenum method. In this test, the presence of antioxidants causes phosphomolybedic acid reduction to phosphomolybdenum, as a result a change in solution color occurs which was assessed by recording the absorbance at 695 nm. The results illustrated potent antioxidant capacity of 444.54, 362.0 and 268.0 mg AAE/g dry extract for ethyl acetate extracts of isolates 9, 10 and 13, respectively. On the other hand, acetone extracts of isolates 9, 10 and 13 exhibited antioxidant activities of 330.73, 211 and 588.81, respectively. Our results are in consistence with Hamed et al. [36], they studied total antioxidant capacity (TAC) of different extracts from isolated endophytic fungi, where, fungal crude extracts of eight different genera were evaluated for their TAC. The results confirmed that fungal extracts showed potent antioxidant activity with different TAC value varied between 813.5 to 150 mg AAE/g extract (mg of ascorbic acid equivalents/g extract).

Table 2: Total antioxidant contents of both ethyl acetate and acetone fractions of selected endophytic fungi

Isolate extract	Total antioxidant capacity (mg AAE/g dry extract)[1,2]	
	Ethyl acetate fractions	Acetone fractions
9	444.54 ± 1.15	330.73 ± 5.03
10	362.0 ± 2.0	211.0 ± 4.0
13	268.0 ± 6.92	588.81 ± 3.11

[1]Results are (means ± S.D.) (n = 3). [2]AAE (ascorbic acid equivalent).

3.3. Total phenolic content

Total phenolic content (TPC) is a quantitative assay used to quantify active metabolites present in samples [48]. In this work, total phenolic content was assessed for both ethyl acetate and acetone fungal extracts, where the results showed that extracts 9, 10 and, 13 exhibited high phenolic contents with values of 379.04, 341.71, and 287.61 mg GAE/ g dry extract, respectively (Table 3). Similarly, acetone extracts 9 and 13, 14 and 15 showed the highest phenolic

contents (302.37 and 338.09 mg GAE mg/ g dry extract, respectively). Moderate phenolic content was observed with extract 10 with value of 192.51 mg GAE/ g dry extract. Phenolics are important compounds that are considered as free radicals scavengers, in addition to their vital biological activities as anti-cancers and antimicrobial candidates [49]. Previous studies conducted on medicinal plants and their endophytes revealed that TAC and TPC are co-related to each other, however this co-relation between fungal metabolites was investigated by Huang et al. [50], the results confirmed this co-relation between total antioxidant capacity of endophytic fungi metabolites and their phenolic content. A linear co-relation was noticed, since endophytic fungi which exhibited strong antioxidant capacity possessed high phenolic content, and fungi that expressed low antioxidant activity had lower levels of phenolic content.

Table 3: Total phenolic contents of both ethyl acetate and acetone fractions of selected endophytic fungi

Isolate extract	Total phenolic contents (mg GAE/ g dry extract)[1,2]	
	Ethyl acetate fractions	Acetone fractions
9	379.04 ± 5.25	302.37 ± 4.36
10	341.71 ± 3.85	192.51 ± 5.94
13	287.61 ± 4.36	338.09 ± 5.94

[1]Results are (means ± S.D.) (n = 3). [2]GAE: Gallic acid equivalent.

3.4. Anticancer studies of the endophytic fungal extracts

In this research, in vitro cytotoxicity was evaluated for the three extracts from three endophytic fungi grown on rice and extracted by ethyl acetate and acetone. These extracts were tested against HePG2 and MCF-7 cell lines. The results showed that fungal extracts 9 and 10 exhibited very strong cytotoxic activity against both cell lines (HePG2 and MCF-7) with IC_{50} values of (6.74 and 7.45 µg/ml) and (9.30 and 8.66 µg/ml) for extracts 9 and 10 against HePG2 and MCF-7, respectively. Meanwhile, extract 13 exhibited moderate cytotoxicity against both cell lines with IC_{50} values of 25.88 and 29.42 µg/ml, respectively (Table 4 and Figure 2). Previous studies have proved endophytic fungi anticancer effects against leukemia, hepatoma, lung, colorectal, breast, ovarian and cervical cancer [51-52]. Ramírez et al. [52] evaluated the antitumor potential of L. mariginatus endophytic fungi against cancer cell lines using MTT reduction assay in addition to other several methods. The 3 isolated genera were Penicillium, Aspergillus, and Cladosporium. Different cell lines were treated with fungal extracts with concentration ranging from 31 µg/ml to 250 µg/ml. Cladosporium fungal extracts showed high growth inhibition percentage (42.5 %) on human colorectal adenocarcinoma cell line. As well as another fungal extract showed the highest growth inhibition percentage (55.8 %) at of concentration 250 µg/ml against breast cancer cell line. Another study was performed on Aspergillus species to assess cytotoxicity activity on hepatocellular carcinoma (HePG2) and breast adenocarcinoma (MCF-7) cell lines. the observed results showed that HePG2 cell line is more sensitive to treatment rather than MCF-7 cell line. Generally, cytotoxicity activity on HePG2 varied between moderate with IC_{50} values of 161.81, 232.07 and 265.20, and weak activity with IC_{50} > 400. Likewise, cytotoxic effect on MCF-7 cell line exhibited best activity at IC_{50} of 225.21, and the other values showed weak effect, where IC_{50} >350 [53].

Table 4: In vitro cytotoxicity IC_{50} (µg/ml) of the produced extracts against the two cell lines HePG2 and MCF-7

Isolate extract	In vitro Cytotoxicity IC_{50} (µg/ml)*	
	HePG2	MCF-7
DOX	4.50±0.2	4.17±0.2
9	6.74±0.5	7.45±0.8
10	9.30±0.8	8.66±0.8
13	25.88±2.0	29.42±2.4

***** IC_{50} (µg/ml): 1 – 10 (very strong). 11 – 20 (strong). 21 – 50 (moderate). 51 – 100 (weak) and above 100 (non-cytotoxic**)**

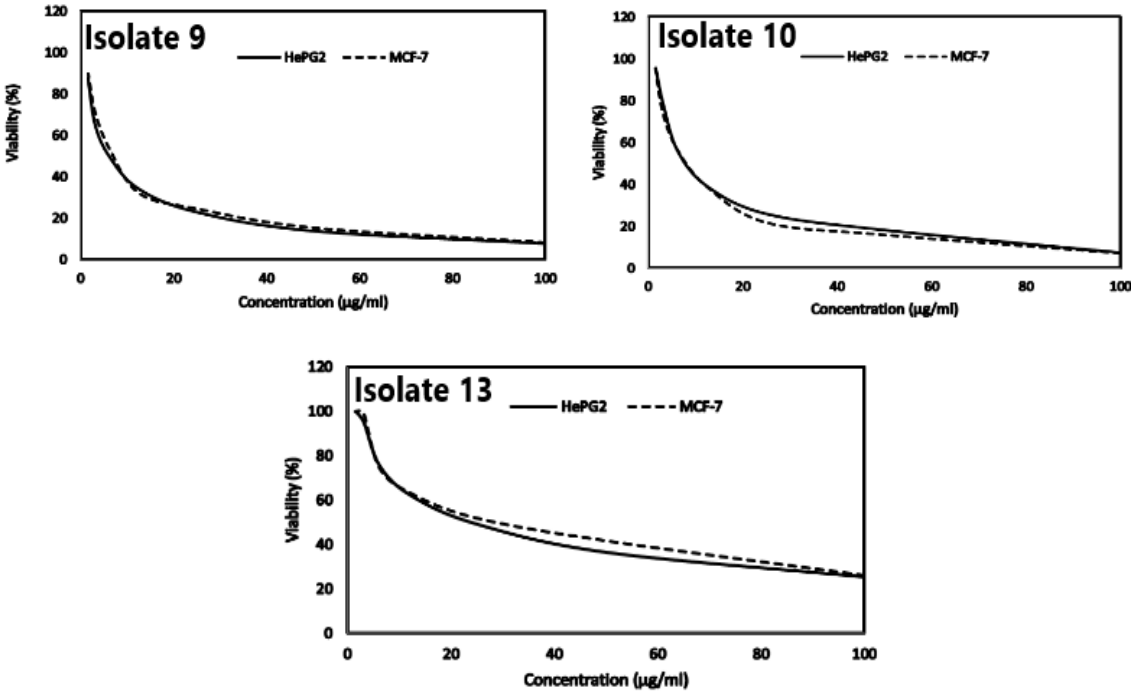

Figure. 2: Average of relative viability of cells (%) of different extracts from different endophytic fungi gown on rice medium against HepG2 cell line

3.5. Gas chromatography with mass spectroscopy (GC/MS) of total endophytic fungal extracts

Figure 3 represents the total peak areas of the identified ingredients of extracts 9, 10 and 13. The compounds which exhibited an area (%) equal to 2 or more were selected to simplify the data. The prospects of the chemical structures of the identified compounds of the total fungal extract 9 are presented in Table 5: The main detected compounds are Z-8-methyl-9-tetradecenoic acid (10.54 %), 2-hexadecen-1-ol, 3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]] (6.04 %), nonadecane (3.41 %), octadecane, 3-ethyl-5-(2-ethylbutyl) (3.36 %), docosane (3.26 %), 1-dodecanol(3.07 %), heptadecane (3.06 %), ethyl linoleate (2.83 %), 1,1-dihydroperoxycyclopentane (2.59 %), fumaric acid, cis-hex-3-enyl pentadecyl ester (2.47 %) and E-2-octadecadecen-1-ol (2.46 %), for which represented 43.09 % of the overall peak areas. The expectations of the chemical structures of the identified compounds from extract 10 are illustrated in Table 6, and the main detected compounds of this extract are 9,12-octadecadienoic acid, methyl ester (18.80 %), hexadecanoic acid, methyl ester (9.83%), 9-octadecenoic acid (Z), methyl ester (8.73%), 1-decanol, 2-hexyl (3.74%), cyclotridecanone (2.94%), dodecanoic acid, methyl ester (2.94%), 1,2-benzenedicarboxylic acid, dibutyl ester (2.47%), cyclododecanol, 1-ethenyl (2.14 %), and cyclopentadecanone (2.03 %), for which represented 53.62 % of the overall peak areas.

Moreover, GC-MS analysis of the total extract of isolate 13 compounds are recorded in Table 7, and the main detected compounds are ethyl linoleate (16.02 %), hexadecanoic acid, ethylester (13.44 %), trans-13-octadecenoic acid, methyl ester (12.85 %), mevalonolactone (7.25 %), 9,12-octadecadienoic acid, methyl ester, (E,E) (4.88 %), hexadecanoic acid, 1-methylethyl ester (4.70 %), dodecane, 2,2,4,9,11,11-hexamethyl (3.32 %), 6,7-benzotricyclo[3.2.2.0(2,4)]-6-nonen-8-one (2.91 %), 9-octadecenoic acid (Z), methyl ester (2.77 %) and hexadecanoic acid, methyl ester (2.55 %), representing 70.69 % of the overall peak areas. Since endophytic fungi active metabolites have expressed antimicrobial, antioxidant and cytotoxic activities, separating and identifying these compounds was a crucial step in order to investigate the chemical composition of these purified compounds, in addition to validating the previous results. GC/MS gas chromatography/mass spectrometry technique was used. Several studies reported GC/MS is a very useful assay to identify low molecular weight (volatile or semi volatile) organic compounds / molecules [54].

The isolated fungal extracts have yielded many compounds as fatty acids, alcohols, esters, ethers and aldehydes. A number of 204 compounds (Tables 5-7) were identified from all the extracts. The main

detected compounds were 9,12- octadecadienoic acid (Z,Z), methyl ester, E,E,Z-1,3,12-nonadecatriene-5,14-diol, ethyl linoleate, hexadecanoic acid, Z-8-methyl-9-tetradecenoic acid, 2-hexadecen-1-ol, 3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]], 1,2-benzenedicarboxylic acid, dibutyl ester, hexadecanoic acid, methyl ester, trans-13-octadecenoic acid, methyl ester, mevalonolactone, 9,12,15-octadecatrienoic acid, (Z,Z,Z) and 9-octadecenoic acid (Z), ethyl ester. Jayakumar et al. [55] reported that the 9,12-octadecadienoic acid (Z,Z), methyl ester compound has expressed anti-cancer, hepatoprotective, antihistaminic, hypocholesterolaemic, and antieczemic effects. Furthermore, dodecanoic acid has antibacterial, antifungal, anticancer and antioxidant activity. Likewise, 9-octadecenoic acid (Z) has proved cytotoxic effects against several cancer cell lines. Significantly, Elgorban et al. [56] studies on endophytic fungi, especially on *Alternaria* sp, revealed that 1,2-benzenedicarboxylic acid has been isolated and identified using GC/MS assay. 1,2-benzenedicarboxylic acid, dibutyl ester has expressed antioxidant and strong antimicrobial activity against Gram +ve bacteria. As well as hexadecanoic acid, methyl ester showed anti-inflammatory, antioxidant, cancer preventive and hepatoprotective activities. Tyagi and Agarwal [57] research carried on *Pistia stratiotes* L. and *Eichhornia crassipe* showed that 9,12,15-octadecatrienoic acid, (Z,Z,Z) has antimicrobial, anticancer, hepatoprotective, and anti-arthritic activity. Ultimately, ethyl linoleate has showed anti-inflammatory and anti-bacterial properties, moreover recent clinical studies have proved its effect as anti-acne agent [58]. A study was carried out by Ko and Cho [59] experimenting the activity of ethyl linoleate against melanogenesis, the results confirmed its activity in inhibiting melanogenesis through interfering with AKt /GSK3β/β-catenin signal pathway.

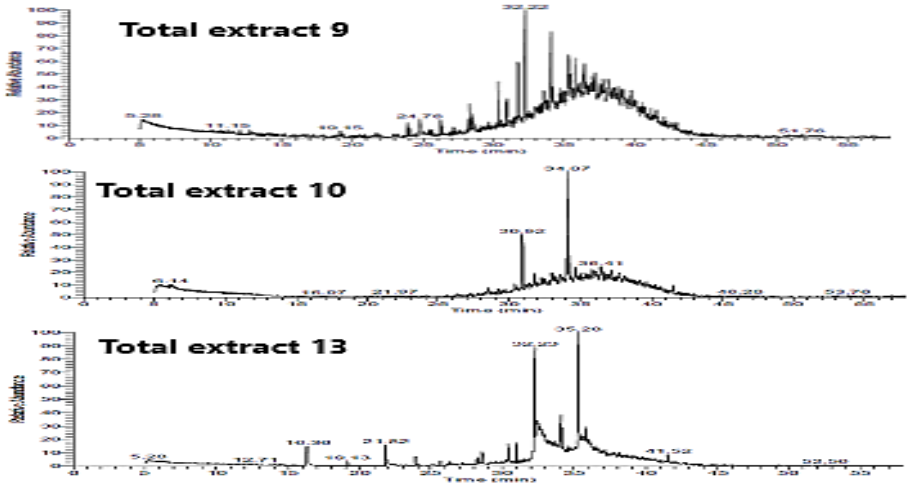

Figure. 3: GC-MS chromatogram of extract 9, 10 and 13

Table 5: Chemical composition of extract 9

Area%[a]	M.W.	M.F.	Main Fragments	Identified compounds	Class/Category
2.59	134	$C_5H_{10}O_4$	84, 101, 134	1,1-Dihydroperoxycyclopentane	Cyclic alkane derivatives
2.33	142	$C_8H_{18}N_2$	57, 69, 84, 98, 123, 141	1,3-Diethyl-2-methyl-1,3-diazolidine	Heterocyclic compound
1.03	408	$C_{29}H_{60}$	57, 71, 85, 99, 141, 211, 275, 381	Nonacosane	A straight-chain alkane
3.41	268	$C_{19}H_{40}$	57, 71, 85, 113, 141, 169, 233, 268	Nonadecane	A straight-chain alkane
2.09	326	$C_{21}H_{42}O_2$	57, 74, 87, 97, 143, 185, 227, 276	Eicosanoic acid, methyl ester	A fatty acid methyl ester

10.54	240	$C_{15}H_{28}O_2$	55, 69, 97, 113, 141, 185, 227	Z-8-Methyl-9-tetradecenoic acid	A long-chain fatty acid derivatives
2.47	408	$C_{25}H_{44}O_4$	55, 67, 82, 97, 125, 153, 222, 280, 349	Fumaric acid, cis-hex-3-enyl pentadecyl ester	Organic acid derivatives
6.04	296	$C_{20}H_{40}O$	56, 71, 81, 97, 110, 141, 168, 263, 296	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]]	Acyclic diterpene alcohol
2.31	296	$C_{19}H_{36}O_2$	55, 69, 97, 125, 166, 203, 249, 280	9-Octadecenoic acid (Z), methyl ester	A fatty acid methyl ester
2.83	308	$C_{20}H_{36}O_2$	54, 67, 79, 95, 110, 219, 262, 308	Ethyl linoleate	A long-chain fatty acid ethyl ester
3.07	186	$C_{12}H_{26}O$	57, 68, 83, 95, 110, 125, 152, 179	1-Dodecanol	A fatty alcohol
2.46	268	$C_{18}H_{36}O$	54, 67, 81, 96, 108, 169, 210, 245, 266	E-2-Octadecadecen-1-ol	A fatty alcohol
3.06	240	$C_{17}H_{36}$	57, 71, 85, 98, 127, 155, 183, 210	Heptadecane	Alkane
3.26	310	$C_{22}H_{46}$	55, 69, 85, 96, 111, 154, 196, 235, 255, 309	Docosane	A straight-chain alkane
3.36	366	$C_{26}H_{54}$	57, 71, 85, 97, 110, 141, 163, 197, 309, 348	Octadecane, 3-ethyl-5-(2-ethylbutyl)	Branched alkanes
2.27	270	$C_{18}H_{38}O$	55, 85, 96, 111, 126, 196, 225, 255	1-Octadecanol	A fatty alcohol
2.21	254	$C_{18}H_{38}$	57, 71, 85, 96, 112, 125, 139, 169, 225	Hexadecane, 7,9-dimethyl	Branched alkanes

Rt: Retention time; M.W.: Molecular weight; M.F.: Molecular formula.

Table 6: Chemical composition of extract 10

Area%[a]	M.W.	M.F.	Main Fragments	Identified compounds	Class/Category
9.83	270	$C_{17}H_{34}O_2$	74, 85, 97, 128, 171, 199, 227, 270	Hexadecanoic acid, methyl ester	A fatty acid methyl ester
2.47	278	$C_{16}H_{22}O_4$	65, 93, 121, 149, 155, 205, 235, 264	1,2-Benzenedicarboxylic acid, dibutyl ester	A phthalate ester
2.94	196	$C_{13}H_{24}O$	54, 71, 84, 109, 125, 150, 165, 178	Cyclotridecanone	Cyclic ketones
2.03	224	$C_{15}H_{28}O$	55, 71, 97, 109, 139, 165, 177, 222	Cyclopentadecanone	Cyclic ketones
18.80	294	$C_{19}H_{34}O_2$	55, 68, 81, 95, 109, 149, 263, 294	9,12-Octadecadienoic acid, methyl ester	A fatty acid methyl ester
8.73	296	$C_{19}H_{36}O_2$	55, 74, 83, 96, 137, 180, 222, 265, 296	9-Octadecenoic acid (Z), methyl ester	A fatty acid methyl ester
2.94	214	$C_{13}H_{26}O_2$	55, 74, 87, 111, 149, 177, 207	Dodecanoic acid, methyl ester	A fatty acid methyl ester
3.74	242	$C_{16}H_{34}O$	57, 69, 85, 97, 111, 125, 154, 183, 205	1-Decanol, 2-hexyl	Fatty alcohols
2.14	210	$C_{14}H_{26}O$	57, 70, 83, 110, 137, 150, 167, 192	Cyclododecanol, 1-ethenyl	Tertiary alcohols

Rt: Retention time; M.W.: Molecular weight; M.F.: Molecular formula

Table 7: Chemical composition of extract 13

Area%[a]	M.W.	M.F.	Main Fragments	Identified compounds	Class/Category
7.25	130	$C_6H_{10}O_3$	58, 71, 84, 103, 130	Mevalonolactone	A delta-lactone
2.91	184	$C_{13}H_{12}O$	55, 69, 85, 125, 142, 155, 184	6,7-Benzotricyclo[3.2.2.0(2,4)]-6-nonen-8-one	Aromatic derivatives
2.18	590	$C_{42}H_{86}$	57, 69, 83, 156, 198, 239, 376, 430, 511	Octadecane, 2,2,4,15,17,17-hexamethyl-7,12-bis(3,5,5-trimethylhexyl)	Branched alkanes
3.32	254	$C_{18}H_{38}$	56, 85, 97, 129, 155, 200, 213, 241	Dodecane, 2,2,4,9,11,11-hexamethyl	Branched alkanes
2.55	270	$C_{17}H_{34}O_2$	55, 74, 87, 129, 143, 171, 227, 270	Hexadecanoic acid, methyl ester	A fatty acid methyl ester

13.44	284	$C_{18}H_{36}O_2$	55, 73, 88, 101, 157, 199, 241, 284	Hexadecanoic acid, ethylester	A fatty acid ethyl ester
4.70	298	$C_{19}H_{38}O_2$	57, 71, 82, 97, 125, 204, 274, 321, 392, 467	Hexadecanoic acid, 1-methylethyl ester	A fatty acid ester
4.88	294	$C_{19}H_{34}O_2$	55, 69, 82, 95, 110, 135, 150, 227, 256, 294	9,12-Octadecadienoic acid, methyl ester, (E,E)	A fatty acid methyl ester
2.77	296	$C_{19}H_{36}O_2$	55, 69, 96, 111, 157, 180, 264, 296	9-Octadecenoic acid (Z), methyl ester	A fatty acid methyl ester
16.02	308	$C_{20}H_{36}O_2$	55, 68, 82, 95, 135, 220, 263, 285, 307	Ethyl linoleate	A long-chain fatty acid ethyl ester
12.85	296	$C_{19}H_{36}O_2$	55, 67, 83, 97, 123, 180, 213, 265, 280	trans-13-Octadecenoic acid, methyl ester	A fatty acid methyl ester

Rt: Retention time; M.W.: Molecular weight; M.F.: Molecular formula.

3.6. Molecular identification of the endophytic fungal isolates

A nucleotide sequence of 1118, 1182 and 1113 bp of the whole 18S rRNA gene of the fungal isolates 9, 10 and 13 were determined in both strands. BLAST search revealed 99 % similarity to *Alternaria tenuissima* (accession number MN075309.1), 99 % similarity to *Phoma herbarum* (accession number AY337712.1) and 99 % similarity to *Aspergillus tamarii* (accession number AF516140.1) for isolates 9, 10 and 13, respectively. The endophytic fungal isolates 9, 10 and 13 were identified as *Alternaria tenuissima* isolate ANAS-9, *Phoma herbarum* isolate ANAS-10 and *Aspergillus tamarii* ANAS-13 with accession numbers MW662638.1, MW662651.1 and MW662651.1, respectively. A phylogenetic tree of each entophytic fungal isolate was also constructed in Figures 4-6. Morphological identification is a very complicated process and unreliable, since endophytic fungi do not usually sporulate in cultures, as a result they do not have specific phenotype to be morphologically identified [60]. As a consequence, 18s rRNA gene sequence and internal transcribed spacer (ITS) are widely used to identify and obtain information about fungal community, furthermore 18s rRNA and ITS are used to assess fungal diversity [61]. Molecular identification using sequencing is usually efficient and reliable, since genomic information is specific to certain individual. Generally, identification relies on the molecular differences found in the hereditary material. Moreover, ITS is used to improve the accuracy of the identification process. Many recent studies have reported ITS as the preferred nucleotide marker for including many variations which help in resolving the taxonomic issues and identifying closely related species besides, ITS are easy to amplify [62].

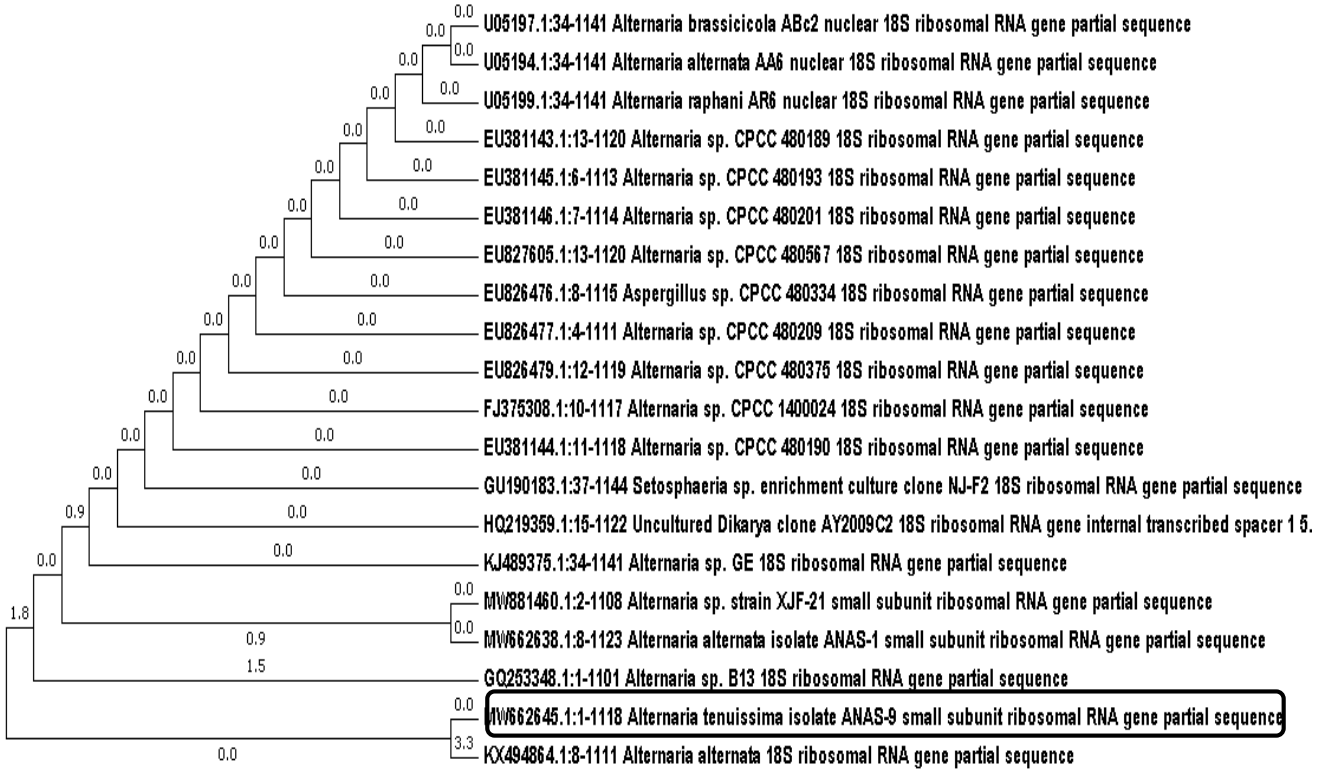

Figure. 4: Phylogenetic tree showing the relationship of the fungus Alternaria tenuissima strain ANAS-9 with other related fungal species retrieved from GenBank based on their sequence homologies of 18S rRNA.

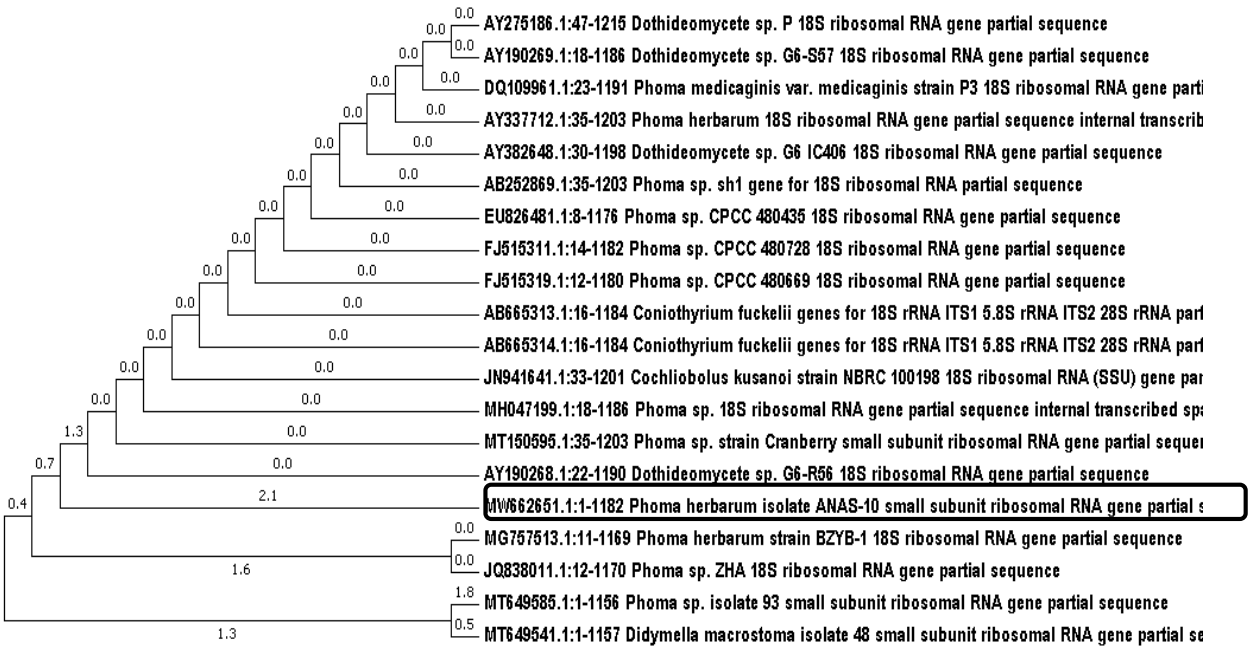

Figure. 5: Phylogenetic tree showing the relationship of the fungus *Phoma herbarum* strain ANAS-10 with other related fungal species retrieved from GenBank based on their sequence homologies of 18S rRNA.

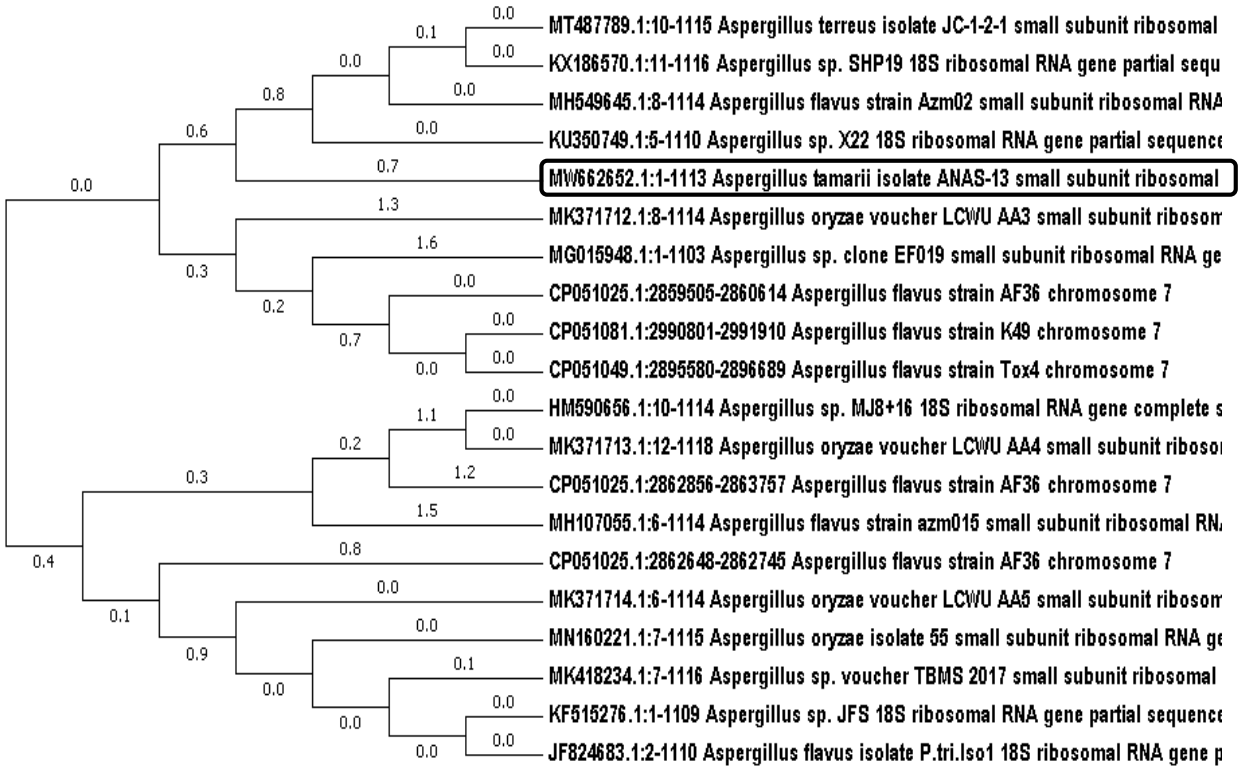

Figure. 6: Phylogenetic tree showing the relationship of the fungus *Aspergillus tamarii* strain ANAS-13 with other related fungal species retrieved from GenBank based on their sequence homologies of 18S rRNA

4. Conclusion

Endophytic fungi are considered a fruitful source for the production of bioactive secondary metabolites. Researchers focused on the isolation of potent fungal strains with robust biological activities for the treatment of multidrug resistance pathogenic bacteria, as well as anticancer and anti-inflammatory agents' production. This manuscript focused on the isolation, production and studying some biological and chemical criteria of ethyl acetate and acetone extracts from three entophytic fungi. All the obtained results were encouraging to plan for further work including isolation and structure elucidation of pure compounds as well as studying their biological activities.

5. Conflict of interest

The authors declare no conflict of interest**.**

6. Author contributions

All authors made significant contributions to idea and design, achievement of data, or analysis and explanation of data; took part in writing the article and revising it; accepted to submit to this journal

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