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Original Article

Bioactive Secondary Metabolite from Endophytic *Aspergillus Tubenginses* ASH4 Isolated from *Hyoscyamus muticus*: Antimicrobial, Antibiofilm, Antioxidant and Anticancer Activity

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ABSTRACT

Background: Endophytes are the richest sources of natural compounds, showing biological varieties and pharmacological activities. **Objective:** This study aims to isolate bioactive secondary metabolites from endophytic *Aspergillus tubenginses* with study the biological activity of the isolated bioactive compounds. **Materials and Methods:** Anofinic acid were obtained from *Aspergillus tubenginses* crude extract using chromatographic techniques and characterized by spectral analysis. **Results:** Nine endophytic fungi were isolated from *Hyoscyamus muticus* plant. The most efficient isolate was AF3 identified as *Aspergillus tubenginses* ASH4 by 18S rRNA gene sequencing. Anofinic acid is an isolated active metabolite biosynthesized by *A. tubenginsis* was extracted from ethyl acetate with UPAC name of 2,2-dimethyl-2H-1-benzopyran-6-carboxylic acid. It shows a strong antimicrobial activity against human pathogenic bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Bacillus subtilis*. Moreover, anofinic acid inhibits biofilm formation and has antioxidant activity, with strong activity against some carcinoma cells such as HCT-116, Hep-G2 and MCF-7. **Conclusion:** Anofinic acid was purified from the endophytic *Aspergillus tubenginses* crude extract and showed antimicrobial, antibiofilm, antioxidant, anticancer activities.

Key words: *Hyoscyamus muticus, Endophytes, Aspergillus tubenginses,* Bioactive secondary metabolite.

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INTRODUCTION

Scientists are tackling different diseases in our society due to rehabilitated environment and lifestyle. Several researchers are working on these emerging diseases to understand and cure them using different chemical and natural formulations. However, many areas are untouched due to poor knowledge and techniques.[1,2] Researchers are trying to isolate new bioactive compounds from new species of fungal endophytes for medicinal, agricultural and industrial applications as reported by Anisworth et al. (1973); Smith *et al.* (2008); Garg *et al.* (2011); Fahmy *et al.* (2017); Hamed *et al.* (2018); Nurunnabi *et al.* (2020).[3-8]

Endophytic fungi have a symbiotic relationship between fungi and their host plants.[6] Symbiosis refers to the living together of several species by which symbiont and host are beneficiaries.[9-11] The secondary endophytic fungal metabolites have a various biological activity. The multitude of secondary metabolites derived from fungal endophytes has established therapeutic applications such as anticancer, antimicrobials, antitumor and antidiabetics, and cholesterol inhibitors and antioxidant agents.[7,12-14]

Wang *et al.* (2007) showed that, endophytic fungal metabolites can also be used effectively in weed control. For example, *Cladosporium sp*. created the Brefeldin A. that suppresses the maturity of weed pollen tubes of *Picea meyeri*.[15] Khan *et al.* (2012) declared that, *Paraconiothyrium sp.* generates ascotoxin which influences seed germination of *Echinochloa crus-galli* and *Lactuca sativa.*[16] Fungal endophytes possess defensive mechanisms and greatly affect plant community growth.[17] Therefore, the aim of this study was to investigate the bioactive metabolites of endophytic *Aspergillus tubenginses* with biological evaluation of purified secondary metabolites.

EXPERIMENTAL

Collection of plant samples

Plant samples were collected from Wadi-Elnatrun Valley, labeled, transported in icebox to the Ecology laboratory and identified by Dr. Albaraa Salaheldin, Faculty of Science, Al-Azhar University. The identified specimens were launched in the Biotechnology Lab., Faculty of Science (Girls Branch), Al-Azhar University, Cairo, Egypt.

Endophytic fungal isolation

Endophytic fungi were isolated from plant parts according to the method of Kyeremeh *et al.* (2017).[18] To make ensure that, isolated fungi are endophytes, the plant part surfaces were sterilized by rinsing with sterile distilled water (SDW), followed by dipping in to 70% ethanol for 1 min. the pieces were again rinsed

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in SDW, immersed in 2% sodium hypochlorite for 1 min. followed by rinsing with SDW triply. The sterilized plant parts were allowed to dry in laminar flow, and a healthy leaf was cut into small pieces of 1 cm^2 and placed on a PDA plate. To check the efficiency of surface sterilization procedure, 4-5 water drops from the final rinse were inoculated on a PDA medium, held for approximately 5-6 days to investigate the growth of any endophytic fungi. The most potent obtained fungal endophyte was identified using 18S rRNA analysis, Macrogen Company, South Korea.

Genetic identification of endophytic fungus

Here, 18S rRNA analysis was performed on the endophytic fungus by biosynthesizing the major compound. In the laboratory, fungal DNA was extracted using the Qiagen DNeasy Mini Kit protocol. The extracted DNA was subjected to polymerase chain reaction using (5′-TCCGTAGGTGAACCTGCG-3′)/ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) universal primers. In Macrogen Companies, Seoul, South Korea. Amplified DNA was subjected to DNA sequencing and the gained DNA sequence was parallel to the DNA sequence presented at NCBI GenBank *. The resulting gene sequence was submitted to the NCBI GenBank database and an accession number was attained. http://www.ncbi.nlm.nih.gov.blast

Production of bioactive compounds

Rice in the solid state was used as a fermentation media, 100g rice was soaked in 100mL distilled sea water (Mediterranean Sea), and autoclaved, and 1ml of endophytic fungus was introduced to each medium flask. The inoculated flasks were incubated under static conditions at 30°C for 15 days. The extraction was performed using ethyl acetate, and then the organic phase was gathered, stored, and dried at 37°C.[19-21]

Antimicrobial activity

The antimicrobial activity of the obtained crude extracts was performed using agar diffusion method according to Abdel-Aziz *et al.* (2019).[22] Each fungal crude extract was dissolved in MeOH at 500 µg/ml and Aliquots of 50 µl were loaded on disks (Whattman No. 1 filter paper, 5 mm). The inhibition zone diameter was measured against various of pathogenic microorganisms comprising: Gram+ve bacteria; (*Staphylococcus aureus* ATCC6538-P) and *Bacillus subtilis*. Gram–ve bacteria (*Pseudomonas aeruginosa* ATCC27853) and *E. coli.* and Yeasts (*Candida albicans* ATCC10231). On the other hand, the antibacterial activity of the pure compound isolated from selected fungus was measured using Antimicrobial assay and MIC were performed as described by Ingebrigtsen et al. (2016).[23]

All test microbes were obtained from the Culture Collection Center (Microbial Chemistry Department and National Research Center, NRC), Egypt.

Antibiofilm activity

MTP assays were conducted to test antibiofilm activity of the fungal crude extracts and pure compound. The test was performed using 96 well-flat bottom polystyrene titer plates and four clinical microbes were also used (*P. aeruginosa, S. aureus, E. coli, and B. subtilis*) according to Christensen *et al.* (1985)[24] and Hamed *et al.* (2020).[25] The optical density was determined at 570 nm using a spectrostar nanomicroplate reader (BMG LABTECH GmbH, Allmendgrun, Germany).

Determination of total antioxidant capacity (TAC) and total phenolic content (TPC)

The antioxidant activity of the ethyl acetate crude extracts along with pure compound was determined by phosphor molybdenum assay according to Ghareeb *et al.* (2014 & 2016)[26,27] and Saad *et al.* (2017)[28]. The antioxidant activity was conducted as the number of ascorbic acid equivalent (AAE), in triplicate. The total phenolic content of fungal endophytic ethyl acetate extract was also estimated using phosphomolybdenum assay according to Shoeb et al. (2014).[29]

Extraction, purification and structure elucidation

Purification started by fractionation using liquid-liquid partitioning according to the Kupchan original protocol.[30] The extraction procedures mainly depend on the polarity of interest compounds (which can be determined by thin layer chromatography with varying polarity eluents or by analytical HPLC. The most potent fraction was further purified using Size-exclusion chromatography technique via using Sephadex LH-20. The separation was based on molecular weight. The purified compound was subjected to NMR spectroscopy for structure elucidation as well as Thermo Scientific LTQ Orbitrap XL Mass Spectrometer for molecular formula identification.

Anticancer activity

Four different cell lines were used, human lung fibroblast normal cell line (WI-38) , colorectal carcinoma colon cancer (HCT-116), breast cancer of the mammary gland (MCF- 7), and hepatocellular cancer (Hep-G2). The cell lines were obtained from American Type Culture Collection (ATCC), through Biological Products and Vaccine Holding Company (VACSERA), Cairo, Egypt. In reference, doxorubicin has been used as a traditional anticancer drug.

Using the MTT test, the abovementioned cell lines were employed to assess the inhibitory effects on cell growth of pure compounds extracted from endophytic fungus. The colorimetric assay depends on the conversion of yellow tetrazolium bromide (MTT) in viable cells into a violet formazan derivative by mitochondrial succinate dehydrogenase in viable cells. The cell lines were cultivated using 10 % fetal bovine serum in the RPMI-1640 medium. Antibiotics added in a 5% CO_2 incubator were 100 units/ml of penicillin and 100µg / ml of streptomycin at 37°C. The cell lines were seeded for 48 h under 5 percent CO_2 in a 96-well plate at a density of 1.0×10^4 cells/well at 37°C. The cells were treated with different concentrations of compounds after incubation for 24 h. A 20 µl of MTT solution at 5 mg/ml was used in the drug treatment, added and incubated for 4 h, and 100µl of DMSO was added to dissolve the purple formazan formed in each well. The colorimetric assay was recorded at 570 nm using a plate reader (EXL 800, USA). The relative cell viability in percentage was calculated as (A570 of treated samples/A570 of untreated sample) x 100.[32,33]

RESULT AND DISCUSSION

Sample collection and isolation of endophytes

The plant sample was gathered from Wadi-Elnatrun Valley, Egypt. The plant was identified based on the morphological features as *Hyoscyamus muticus* L. Ten endophytic fungi were isolated from *Hyoscyamus muticus* L, coded and kept at microbial culture collection, Microbial Chemistry Department, Dokki, Giza, Egypt.

El-Zayat *et al.* (2008) isolated 18 fungi from the surface of *H. muticus* plant.[34] Three species have only been isolated as endophytic fungi from the medicinal plant *Hyoscyamus muticus* L. Abdel-Motaal *et al.* (2010) isolated 44 endophytes and they opserved that, *A. fumigatus* is the most common species obtained from all plant tissues at different habitats.[35] Sharma *et al.* (2016) isolated endophytic fungus *Pestalotiopsis neglecta* from medicinal plant leaves (*Cupressus torulosa*). which could be a potential source for bioactive compound.[36]

Evaluation of the antimicrobial activities of fungal endophytes

The crude extracts from the isolated 10 endophytic fungi were prepared by cultivation of the 10 fungal isolates on rice medium followed by ethyl acetate extraction. The antimicrobial activity of the crude extracts was evaluated (*in vitro*) against several test pathogenic microorganisms comprising 2 Gram -ve, 2 Gram +ve bacteria and one yeast. Results showed that, nearly 70% of the endophytic fungi isolated from *H. muticus* L exhibited antibacterial activities. The most promising endophytic fungi from *H. muticus L* plant were AF3, 15F6, 15F8, 15F13 and 15F14 with inhibition zone 17–21, 15–17, 14.5–19.2, 14.5–17, and 7–14 mm, respectively, as recorded in Table 1.

Similarly, El-Said *et al.* (2016) showed that, *Aspergillus versicolor, A. flavus, Eurotium chevalieri, Cordella clarkia, P. funicolusum and Penicillium aurantiogriseum* were the most active fungi isolated from *H. muticus* plant with inhibition zone ranging from 8–16 mm.[37]

Another study by Basheer *et al.* (2018) showed that, *Aspergillus terreus* isolated from *Avicennia marina* (South Safaga) has antimicrobial efficacy against *Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis, Proteus vulgaris,* as well as *Streptococcus pyogenes* with inhibition zone ranging from 16–21 mm and lacks fungal activity against *C. albicans P. chrysogenum* and *A. niger.*[38] while, endophyte *Aspergillus fumigatus* isolated from the same plant lacks microbial activity. *A. fumigatus* isolates from *H. muticus* were the most active isolates versus *Klebsiella pneumoniae* and *Staphylococcus aureus* according to Gherbawy *et al.* (2016).[39]

Observably, different endophytic fungal extracts from the same plant display various antimicrobial activities Table (1). These sensitivity differences could be attributed to the isolate species, level, nature, and mode of action of antimicrobial agents existing in their extracts and on various trial microorganisms as reported by Barbour *et al.* (2004)[40]. In this study, the most potent endophytic isolates (AF3, 15F6, 15F8, 15F13, and 15F14) were selected for other screening methods and investigations.

Antioxidant capacity of endophytic fungal extract using phosphomolybdenum assay

Phosphomolybdenum assay is based on Mo^{+4} reduction to Mo^{+5} through interaction with the tested sample and sequent formation of a green color [phosphate = Mo^{+5}] complex with maximum absorption of 695 nm in the acidic medium.[41,42]. Results showed that, TAC values for the tested crude endophyte extracts ranged from 111.66 – 880.66 mg AAE/g dry extract. The results are in the order: 15F13 > 15F8 > AF3 > 15F6 > 15F14 as presented in Table 2.

Rudgers *et al.* (2007) stated that plant phenolic compounds influenced the community of endophytes.[43] Therefore, the higher the content of active compounds in host plant, the more richness with endophytic microorganisms. Previous reports revealed that endophyte extracts and their derived pure isolates exhibit noticeable antioxidant potentials.[44,45.]

Antibiofilm activity of endophytic extract of fungal isolates against some pathogenic microorganisms

The evaporated ethyl acetate extract was diluted with methanol for MTP test that was performed according to Christensen *et al.* (1985).[24] From the extracts, a concentration (100μg/mL) was selected for biofilm inhibition assay against tested pathogens and their virulence factor in terms of biofilm formation. The test was performed in a 69-well microtiter plate. The crude extract from fungal endophyte AF3 showed better inhibition percentage in (100 μg/mL) with 50.06% against *Bacillus subtilis*, 37.68% against *E coli*, 28.44% against *Pseudomonas aeruginosa* and 60.8% against *Staphylococcus aureus* Figure 1. The results showed that the crude compound of AF3 was the most active against biofilm colonization and cell adherence

In view of the findings of Martin-Rodriguez *et al.* (2014), marine endophyte extracts (*Khuskia* sp., *Fusarium* sp., *Sarocladium* sp., also *Epicoccum* sp.) possess perfect biofilm inhibition percentage.[46]

Rajivgandhi *et al.* (2018) reported that, crude compound (100 μg/mL) isolated from endophytic actinomycetes demonstrated antibiofilm

Table 1: ***In vitro*** **antimicrobial activity of the isolated endophytic fungi against some pathogenic microorganisms.**

Fungal isolates Pathogens	15F1	15F2	15F3	15F4	15F6	15F8	15F11	15F13	15F14	AF3	Standard
Yeast					Mean diameter (mm) ± SD						**Amphotorcin**
C. albicans	0 ± 00	0 ± 00	0 ± 00	0 ± 00	16 ± 0.15	19.2 ± 0.03	0.6 ± 002	17 ± 0.02	15 ± 0.01	21 ± 0.01	20 ± 002
Gram +ve bacteria											**Vancomycin**
S.aureus	0 ± 00	0 ± 00	0 ± 00	0 ± 00	16 ± 0.02	15 ± 0.14	0 ± 00	15 ± 0.05	14 ± 0.02	18 ± 0.02	20 ± 031
											Ampicillin
B. subtilis	0 ± 00	0 ± 00	0 ± 00	0 ± 00	15 ± 0.05	17 ± 0.25	6.5 ± 0.02	16 ± 0.12	8 ± 0.12	19 ± 0.12	21 ± 0.05
Gram -ve bacteria											**Gentamicin**
P. aeruginosa	0 ± 00	0 ± 00	0 ± 00	0 ± 00	17 ± 0.03	14.5 ± 0.03	6 ± 0.02	14.5 ± 0.02	11 ± 0.08	17 ± 0.12	20 ± 0.05
E. coli	0 ± 00	0 ± 00	0 ± 00	0 ± 00	16 ± 0.18	15 ± 0.15	0 ± 00	15 ± 0.1	7 ± 0.1	17 ± 0.14	20 ± 0.15

Table 2: **Antioxidant capacity (TAC) of crude endophyte extracts.**

Isolate code	Total antioxidant capacity (mg AAE/g dry extract) means ± S. D
15F6	185.0 ± 6.0
15F8	846.66 ± 5.23
15F13	880.66 ± 8.82
15F14	111.66 ± 7.57
AF3	753.33 ± 6.17

AAE: Ascorbic acid equivalent.

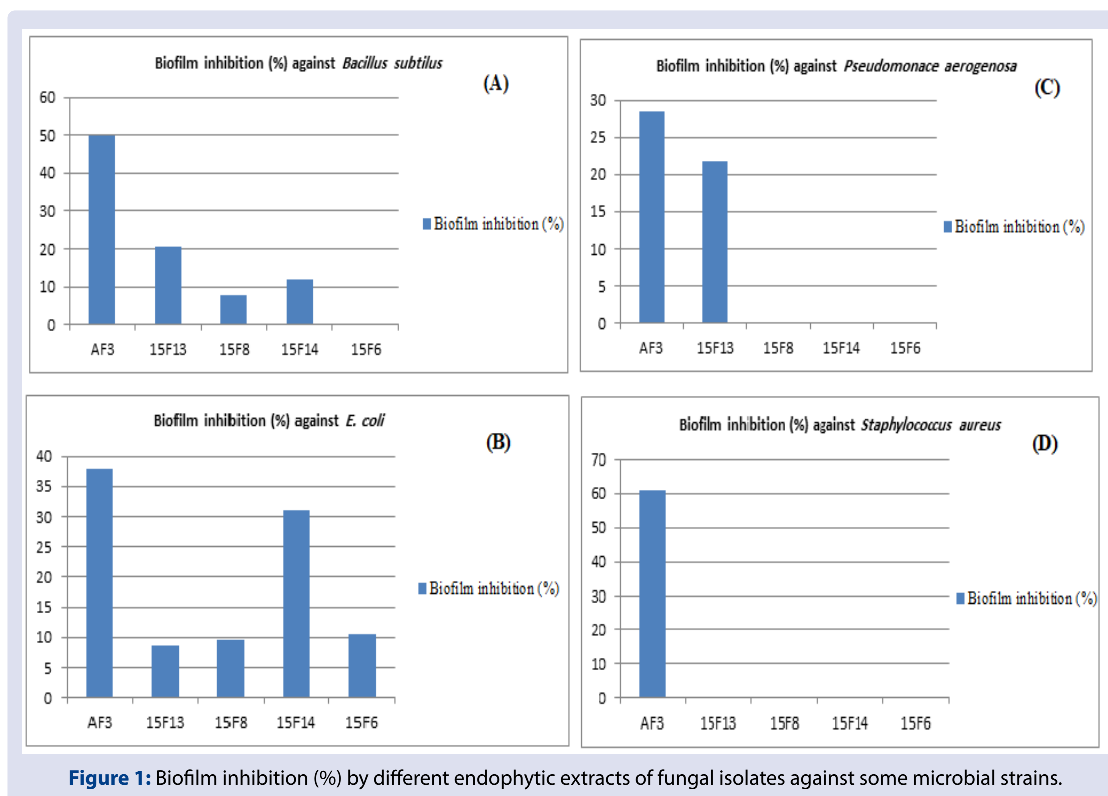

Figure 1: Biofilm inhibition (%) by different endophytic extracts of fungal isolates against some microbial strains.

activity with 82% and 77% inhibition against bacterial films of both *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, respectively.[47] Meenambiga and Rajagopal (2018) reported that, the majority of solvent extracts reported possess strong inhibitory effect on biofilm formed by *C. Albicans* on chloroform extract with elevated activity.[48] The reduction percentage of biofilm inhibition were 74.86% and 72.53% respectively, for chloroform and ethyl acetate, and 60.61% and 52.60 % for hexane and methanol extracts.

Molecular identification the most active endophytic fungal isolate

The 18S rRNA gene sequence was used to identify and oppose other identified sequences available in the GeneBank database using BLAST to indicate the score similaritie sand calculate the statistically significant differences of matches (http://www.blast.ncbi.nlm.nih.gov/Blast). The results established a very close similarity with *Aspergillus tubenginses* using the 18S rRNA gene sequence with 100 % homology of the isolate AF3. The phylogenetic analysis and tree were composed using the neighboring method (Figure 2) by MEGA 7 program according to Kumar *et al.* (2016).[49] Based on the analysis of the DNA sequence and morphological characteristics of the AF3 isolate (Supplementary 1), the isolated strain was identified as *Aspergillus tubenginses* ASH4 and deposited in GenBank with the accession no. MN784618.1.

Extraction and Bio-guided fractionation and purification

Large scale fermentation of the most potent endophytic fungus *Aspergillus tubenginses* ASH4 was carried out on rice medium, incubated for 15 days, extracted by ethyl acetate and evaporated using a rotary evaporator at 40°C. Purification was started by fractionation using liquid-liquid partitioning according to the method of Kupchan original protocol.[30] Five aqueous fractions: methanol, *n*-hexane, *n*-butanol, dichloromethane (DCM) and water were performed as shown in Figure 3. Antimicrobial screening indicated that only dichloromethane (DCM) followed by n-hexane extracts for *Aspergillus tubenginses* ASH4 exhibited antimicrobial activity against the tested pathogenic microorganisms.

Conversely, antioxidant evaluation of the five fractions, showed that butanol and methanol extracts of *Aspergillus tubenginses* ASH4 exhibited high antioxidant activity**.** Also, the results revealed various total phenolic concentrations of ethyl acetate fractions in endophytic fungal extracts as manifest in Table 3. The estimation varied from 48.0 –134.62 mg gallic acid equivalent (GAE)/g of dry weight. The highest concentration of phenols was observed in extract of ASH4 BuOH (134.62 mg GAE/g extract). Considering findings of other studies, the polyphenolic-rich extracts could loss an electron through their redox reaction with a molybdotungstate reagent (Folin Ciocalteu's reagent). This electron transfer reaction induces a blue color, which can be quantified simply by spectrophotometry at 765 nm.[42] Also, based on the antimicrobial screening (Table 4), the fraction dichloromethane (DCM) was active, and therefore was selected for further processing using different chromatographic techniques. Compound (1) was identified as anofinic acid (2,2-dimethyl-2H-1-benzopyran-6-carboxylic acid).

Anofinic acid (Figure 3) was isolated as a yellow fine crystal from the DCM fraction using Sephadex LH-20 subcolumn eluted with a gradient mix elution system using aqueous methanol. It showed a molecular formula ($C_{12}H_{12}O_3$) and molecular weight of 204.22 (Supplementary 1). In ^{1}H-NMR spectrum, it showed characteristic signals in the aromatic region between δ_H 6.95 to δ_H 7.45 ppm, resonating methyl protons appeared at δ_H 1.5 ppm. Two olefinic protons were resonated at δ_H 6.33 and 5.77 ppm. The hydroxyl proton of the carboxylic group was resonated at δ_H 11.69 ppm.[31]

Evaluation of bioactivity of pure compound antibacterial activity

Data illustrated in Figure 5 represent the antimicrobial activity of the pure compound from *Aspergillus tubenginses* ASH4 against a set of microorganisms comprising Gram-ve bacteria and Gram+ve bacteria (*P. aeruginosa, S. aureus, B. subtilis* and *E. coli*). The results revealed that

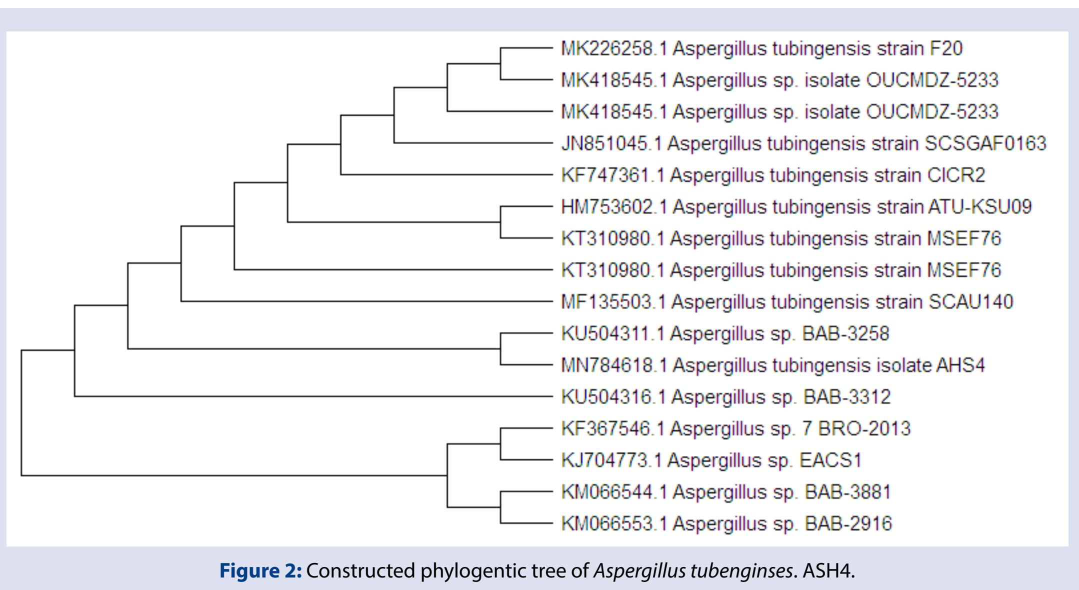

Figure 2: Constructed phylogentic tree of *Aspergillus tubenginses*. ASH4.

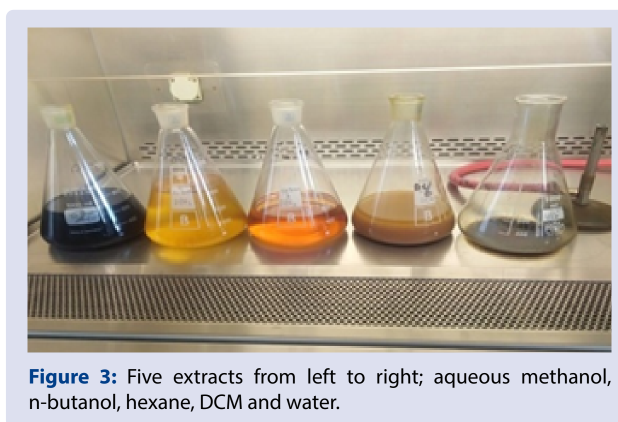

Figure 3: Five extracts from left to right; aqueous methanol, n-butanol, hexane, DCM and water.

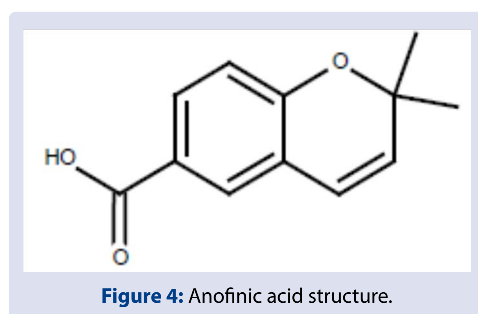

Figure 4: Anofinic acid structure.

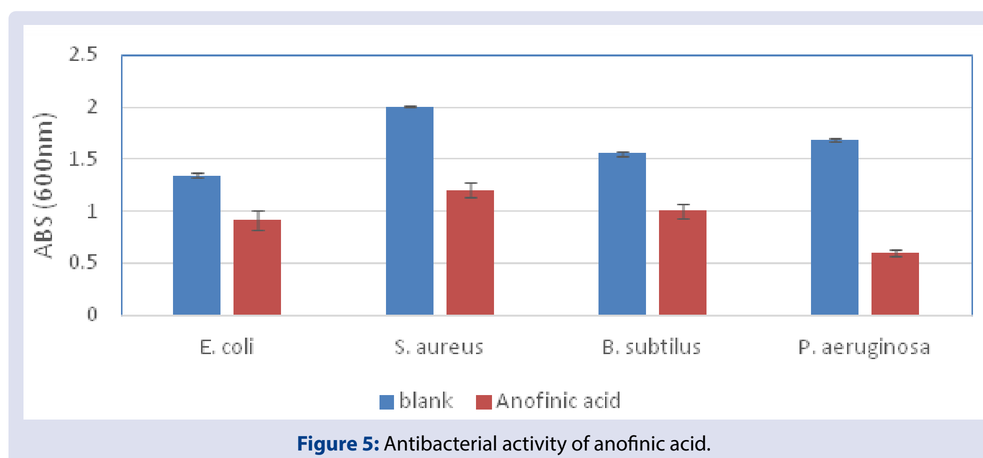

Figure 5: Antibacterial activity of anofinic acid.

anofinic acid exhibited antibacterial activity against all tested bacteria.

Recently, the introduction of new pathogens, such as SARS, H1N1 and different forms of influenza, has become a significant public health threat. Most of these emerged diseases are occasioned by microorganisms and occasional microbes become increasingly drug resistant with time.[50] To overcome such infectious diseases, novel microorganism and plant bioactive compounds may stand by the best substitutional provenance of potential medicines.[51] Since, plant is the prime source of bioactive compounds, endophytes may nevertheless compete a vital role in the quest for novel compounds of biological activity.[52]

Antibiofilm activity of pure compound

Using microtiter biofilm plate assay, the biofilm inhibition activity of the pure compound of *Aspergillus tubenginses* ASH4 was measured against four clinical pathogenic bacteria (*P. aeruginosa, S. aureus, E. coli* and *B. subtilis*), and the results were compared with untreated biofilms (control) for each pathogen (Table 5).

TAC

The results presented in Table 6 show that the TAC of pure anofinic acid is 409.92 mg AAE/g compound. Ghareeb *et al.* (2019b)[45] evaluated the TAC of the pure compounds isolated from terrestrial fungus *Penicillium* sp. SAM16-EGY, in which TAC values ranged from 332.16–608.59 mg AAE/g compound.

Anticancer activity

This study revealed that, for pure compound (anofinic acid), IC_{50} value of 28.14 µg/well was recognized against the colon carcinoma cells, IC_{50} value of 31.65 µg/well for hepatocellular carcinoma, and IC_{50} value of 22.61 µg/well for breast carcinoma. Also, for crude fungal extract IC_{50} value of 9.18 µg/well for colon, IC_{50} 19.37 µg/well against hepatocellular carcinoma and IC_{50} 5.89 µg/well against breast carcinoma (Table 7). The results indicated that the crude extracted from *Aspergillus tubengensis,* ASH4 shows significant strong antiproliferative activity against colon, hepatocellular and breast carcinoma cell line as compared with control (DOX). Also, anofinic acid showed moderate antiproliferative activity

Table 3: Antioxidant capacity and total phenolic content of *Aspergillus tubenginses* ASH4 different fractions.

Fraction	Total antioxidant capacity (mg AAE/g extract) means ± S.D	Total phenolic content (mg GAE/g extract) means ± S.D.
ASH4Methanol	270.66 ± 5.77	102.56 ± 4.16
ASH4 Hexane	53.34 ± 5.03	48.0 ± 4.32
ASH4 DCM	189.56 ± 6.11	89.47 ± 3.29
ASH4 But.	324.39 ± 4.61	134.62 ± 3.89
ASH4 H2O	124.78 ± 3.05	68.57 ± 4.24

AAE: Ascorbic acid equivalent, GAE: Gallic acid *equivalent*

Table 4: Antimicrobial activity of *Aspergillus tubenginses* ASH4 different fractions against some pathogenic microorganisms.

Fractions	Zone of inhibition (mm)				
	P. aeruginosa	*S. aureus*	*E. coli*	*B. subtilis*	*C. albicans*
ASH4 MeOH	10	11	-	-	13
ASH4 H_2O	-	-	-	-	07
ASH4 DCM	14	15	16	13	15
ASH4 BuOH	-	-	-	-	-
ASH4 Hexane	-	-	13	-	14
Control	20	20	20		20

Table 5: Biofilm inhibition (%) of pure compound against pathogenic bacteria.

Pure compound	Anti-biofilm formation (%)			
	E. coli	*S. aureus*	*B. subtilis*	*P. aeruginosa*
Anofinic acid	34.45 ± 3.02	61.39 ± 6.02	54.93 ± 8.02	69.51 ± 2.02

Table 6: Antioxidant capacity (TAC) of the pure anofinic acid.

Compound	Total antioxidant capacity (mg AAE/g compound) means ± S.D.
Anofinic acid	409.92 ± 3.14

AAE: Ascorbic acid equivalent.

Table 7: *In vitro* cytotoxicity IC_{50} (µg/ml) of anofinic acid and crude fungal endophyte compound.

Comp.	Cytotoxicity IC_{50} (µg/ml)			
	WI-35	HCT-116	Hep-G2	MCF-7
DOX (control)	6.72 ± 0.5	5.23 ± 0.3	4.50 ± 0.2	4.17 ± 0.2
AF3 crude	29.01 ± 0.1	9.18 ± 0.8	10.41 ± 0.9	5.89 ± 0.4
Anofinic acid	66.24 ± 3.5	28.18 ± 2.3	31.65 ± 2.6	22.61 ± 1.8

IC_{50} (µg/ml): 1–10 (very strong). 11–20 (strong). 21–50 (moderate). 51–100 (week), and above 100 (no cytotoxic), DOX: Doxorubicin

against examined cell lines cancer of the colorectal colon (HCT-116), breast cancer of the mammary gland (MCF-7), and (Hep-G2).

Extraction of natural bioactive compounds and screening them for pharmacological advantages provide a path for identifying of drug nominee as reported by Salvador-Reyes and Luesch (2015)[53] and Hamed *et al.* (2020)[25]. Many endophytes have been described and documented to generate new compounds are successful in anticancer inspection.[54,55]

In another study, endophyte isolate *Aspergillus* sp. was led to manufacture utmost yield of 100 µg/g of mycelia dry weight.[56] Also, Budhiraja *et al.* (2013) isolated *Aspergillus* sp. from *Gloriosa superba.*[57] A new compound colchatetralene was isolated along with three known compounds namely ergosterol, 4-hydroxy-phthalic acid-dimethyl ester and 5-(hydroxymethyl) furan-2 carbaldehyde which was tested for cytotoxic effect on seven cell lines. Colchatetralene was found to be influential against THP-1 and MCF-7 with IC_{50} values of 30 and 50 µg/mL, respectively. Moreover, Lee *et al.* (2010) outline scarce compounds from *Aspergillus versicolor* isolated from *Petrosia sp* had IC_{50} opposed to HCT-15 colon cancer cell line less than 30 µg/mL.[58]

CONCLUSION

In this study, 10 endophytic fungal strains were isolated from *Hyoscyamus muticus* plant, biological screening including antibacterial, antibiofilm, and antioxidant activities of 10 fungal ethyl acetate extracts, showed that, *Aspergillus tubenginses* ASH4 exhibited maximum antibacterial activity against tested pathogenic strains with inhibition zone of 21 mm, and the TAC values for the tested crude extract of *Aspergillus tubenginses* ASH4 was 753.33 mg AAE/g dry extract. Moreover, the isolated pure compound anofinic acid inhibited biofilm formation up to 69.51%. Moreover, the antioxidant activity of anofinic acid was 409.92 mg AAE/g. The IC_{50} values of anofinic acid against some carcinoma cells such as HCT-116, Hep-G2 and MCF-7 were 728.14, 31.65, and 22.61 µg/well, respectively, described as very strong activities.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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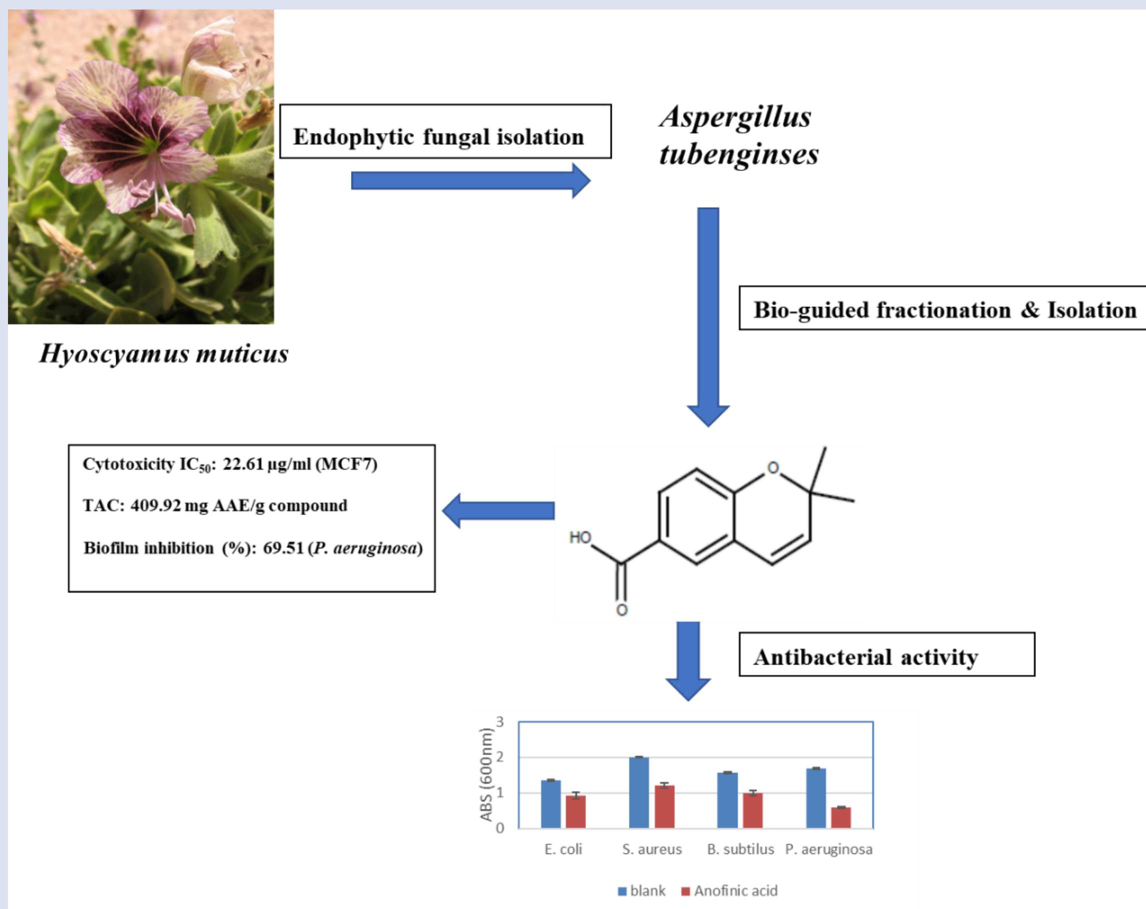
GRAPHICAL ABSTRACT



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