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, antioxidant, antidiabetics, and anticancer activities.

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

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. 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). Endophytic fungi have a symbiotic relationship between fungi and their host plants. Symbiosis refers to the living together of several species by which symbiont and host are beneficiaries. 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.

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. Khan et al. (2012) declared that, Paraconiothyrium sp. generates ascotoxin which influences seed germination of Echinochloa crus-galli and Lactuca sativa. Fungal endophytes possess defensive mechanisms and greatly affect plant community growth. 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 Biotecnology 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 Kyreemeh et al. (2017). To make sure that, isolated fungi are endophytes, the plant part surfaces were sterilized by rinsing with sterile distilled water (SDW), followed by dipping in 70% ethanol for 1 min. the pieces were again rinsed...
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 cm² 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'-TCCGTAGGTTGAACCTGCG-3')/ITS4 (5'-TCCCTCGCTATTGATATGC-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 (Mediteranean 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 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

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)14 and Hamed et al. (2020).20 The optical density was determined at 570 nm using a spectrorast 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 phosphomolybdenum 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 XI Mass Spectrometer for molecular formula identification.

Anticancer activity

Four different cell lines were used, human lung fibroblast normal cell line (WI-38), colorectal cancer 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% CO₂, incubator was 100 units/ml of penicillin and 100µg / ml of streptomycin at 37°C. The cell lines were seeded for 48 h under 5 percent CO₂ in a 96-well plate at a density of 1.0 × 10⁴ 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 100.31-33

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-Motala et al. (2010) isolated 44 endophytes and they observed that, A. fumigatus is the most common species obtained from all plant tissues at different habitats.35 Sharma et al. (2016) isolated endophytic fungi 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 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 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

Similarly, El-Said et al. (2016) showed that, Aspergillus versicolor, A. flavus, Eurotium chevalieri, Cordelia clarkia, P. funicolusum and Penicillium aurantiogriseum were the most active fungi isolated from H. muticus plant with inhibition zone ranging from 8–16 mm. 40

Antioxidant activity 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.

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           | C. albicans | 0 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 | 16 ± 0.15 | 19.2 ± 0.03 | 0.6 ± 0.02 | 17 ± 0.02 | 15 ± 0.01 | 21 ± 0.01 | Amphotericin |
| Gram +ve bacteria | S. aureus | 0 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 | 16 ± 0.02 | 15 ± 0.14 | 0 ± 0.0 | 15 ± 0.05 | 14 ± 0.02 | 18 ± 0.02 | Vancomycin |
| Gram -ve bacteria | B. subtilis | 0 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 | 15 ± 0.05 | 17 ± 0.25 | 6.5 ± 0.02 | 16 ± 0.12 | 8 ± 0.12 | 19 ± 0.12 | Ampicillin |
|                  | P. aeruginosa | 0 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 | 17 ± 0.03 | 14.5 ± 0.03 | 6 ± 0.02 | 14.5 ± 0.02 | 11 ± 0.08 | 17 ± 0.12 | Gentamicin |
|                  | E. coli | 0 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 | 16 ± 0.18 | 15 ± 0.15 | 0 ± 0.0 | 15 ± 0.1 | 7 ± 0.1 | 17 ± 0.14 | 20 ± 0.05 |

AF3: Ascorbic acid equivalent.

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                                                 |

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. 43 Rajivgandhi et al. (2018) reported that, crude compound (100 μg/mL) isolated from endophytic actinomycetes demonstrated antibiotic
activity with 82% and 77% inhibition against bacterial films of both *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, respectively. 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. 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 GenBank database using BLAST to indicate the score similarity and 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). 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. 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. 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 (C₁₂ H₁₂ O₃) and molecular weight of 204.22 (Supplementary 1). In 'H-NMR spectrum, it showed characteristic signals in the aromatic region between δH 6.95 to δH 7.45 ppm, resonating methyl protons appeared at δH 1.5 ppm. Two olefinic protons were resonated at δH 6.33 and 5.77 ppm. The hydroxyl proton of the carboxylic group was resonated at δH 11.69 ppm.

**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...
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**Figure 2:** Constructed phylogenetic tree of *Aspergillus tubengines*. 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. To overcome such infectious diseases, novel microorganism and plant bioactive compounds may stand by the best substitutional provenance of potential medicines. 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.

**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).

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

| Pure compound | *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.          | WI-35 (μg/ml) | HCT-116 (μg/ml) | Hep-G2 (μg/ml) | MCF-7 (μg/ml) |
|----------------|---------------|-----------------|----------------|---------------|
| 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 nominees as reported by Salvador-Reyes and Luesch (2015) and Hamed et al. (2020). Many endophytes have been described and documented to generate new compounds are successful in anticancer inspection. In another study, endophyte isolate Aspergillus sp. was led to manufacture utmost yield of 100 μg/g of mycelia dry weight. Also, Budhiraja et al. (2013) isolated Aspergillus sp. from Gloriosa superba. A new compound colchatarelane was isolated along with three known compounds namely ergosterol, 4-hydroxy-pthalic acid-dimethyl ester and 5-(hydroxymethyl) furan-2 carboxaldehyde which was tested for cytotoxic effect on seven cell lines. Colchatarelane was found to be influential against THP-1 and MCF-7 with IC50 values of 30 and 50 μg/mL, respectively. Moreover, Lee et al. (2010) outline scarce compounds from Aspergillus versicolor isolated from Petrosia sp had IC50 opposed to HCT-15 colon cancer cell line less than 30 μg/mL.

CONCLUSION

In this study, 10 endophytic fungal strains were isolated from Hyoscyamus muticus plant, biological screening including antibacterial, antibiotic, and antioxidant activities of 10 fungal ethyl acetate extracts, showed that, Aspergillus tubenginesse AH4 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 tubenginesse AH4 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 μg AAE/g. The IC50 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

Aspergillus tubenginses

Hyoscyamus muticus

Endophytic fungal isolation

Bio-guided fractionation & Isolation

Cytotoxicity IC50: 22.61 µg/ml (MCF7)
TAC: 409.92 mg AAE/µl compound
Biofilm inhibition (%): 69.51 (P. aeruginosa)

Antibacterial activity

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