

Research Article



Tyrosinase, Acetylcholinesterase Inhibitory Potential, Antioxidant and Antimicrobial Activities of Sponge Derived Fungi with correlation to their GC/MS Analysis

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ABSTRACT

The increase in production and accumulation of melanins are causes of increase skin acquired Hyper pigmentation. Marine-derived fungi are of great interest as new promising sources of biologically active products. Two fungi (FS1 and FS3) were isolated from the Sponges; *Amphimedon viridis* and *Agelas* sp. The antioxidant (DPPH), tyrosinase, acetyl cholinesterase (AChE) inhibitory activities and antimicrobial activity were evaluated. The fungus FS3 (*Aspergillus sydowii* strain W4-2) had the highest free radical scavenging activity against DPPH (culture static). The mycelia (culture shaking) for the isolated two fungi have mild scavenging activity. These data are mentioned for the first time. Screening the tyrosinase inhibitory activity: only FS3 had a significant activity for its supernatant (culture static). Screening the AChE inhibitory activity: only FS3 had a significant activity for its mycelia (culture static). The supernatant and mycelial extracts from the shake and static cultures; respectively, of the FS3 are highly effective against *Pseudomonas aeruginosa*, *Staph. aureus* and *Candida albicans*. The GC/MS analysis revealed the identification of 38 compounds in supernatant and mycelia static of the fungus *Aspergillus sydowii*. Hydrocinnamic acid, 5-hydroxy-7, 8, 4'-trimethoxy isoflavone, 1,3,8-trihydroxy-2-isopropyl-6-methoxy-9,10-anthraquinone, 6,7-dimethoxy-5,4'-dihydroxy flavone, Benzene acetic acid- 2-hydroxy, Benzene acetic acid- 4-hydroxy, Hydrocoumaric acid were found in its supernatant while, Palmitic acid, Linoleic acid, Oleic acid, cis-13-Eicosenoic acid, cis-13-Docosenoic acid, 9,12-Octadecadienoic acid-3-dihydroxy propyl ester, β -Sitosterol were identified in its mycelia. It could be concluded that, the isolated marine fungus *Aspergillus sydowii* strain W4-2 showed different biological activities as tyrosinase, AChE inhibitor, antioxidant, antimicrobial rather than the unidentified fungus (FS1).

Keywords: Antioxidant, Antimicrobial activities, GC/MS analysis, Marine fungi, Sponges, Tyrosinase and Acetyl cholinesterase inhibitors.

INTRODUCTION

A few recent investigations have established that marine-derived fungi (MDF) associated with sponges are an excellent source of novel bioactive metabolites with the potential to function as drugs or drug leads. Given the high species diversity and wide distribution of sponges, it is reasonable to expect that they harbour marine-derived fungi with the ability to produce diverse secondary metabolites.¹ Among the MDF from various marine sources, those associated with sponges have higher species diversity and are prolific producers of novel secondary metabolites.^{2,3} When compared with bacterial associates of sponges, very little is known about the diversity, nature of association and ecological significance of fungal associates. It is only in recent years that the fungal symbionts of marine sponges have received any attention, mainly related to their capacity to produce novel bioactive metabolites.⁴ MDF associated with sponges produce numerous novel bioactive metabolites, including antitumor, antibiotic, antifungal, antialgal, anti insect, antioxidant and acetyl cholinesterase inhibitors.^{3,5-8} Sponge-associated fungal species produce novel metabolites with unique bioactivities in comparison with their terrestrial conspecifics.⁹ Marine-derived fungi, living in a stressful habitat, are of great interest as new promising sources of

biologically active products. Since marine organisms live in a biologically competitive environment with unique conditions of pH, temperature, pressure, oxygen, light, nutrients and salinity, the chemical diversity of the secondary metabolites from marine fungi is considerably high.¹⁰⁻¹²

The increase in production and accumulation of melanins are cause of increase skin alignments e.g. acquired hyperpigmentation such as melisma, proinflammatory melanoderma, solar lentigo etc. The hyper pigmentation of epidermis and dermis depend on either increased numbers of melanocytes or the activity of enzyme.¹³ Melanin is the major pigment for color of human skin. It is secreted by melanocyte cells in basal layer of the epidermis.¹⁴ Tyrosinase, a copper-containing monooxygenase, is a key enzyme that catalyzes melanin synthesis in melanocytes.¹⁵ It catalyzes two major reactions, including hydroxylation of tyrosine oxidation of the o-diphenyl product, L-Dopa. Dopa oxidation produces a highly reactive intermediate that is further oxidized to form melanin by free radical-coupling pathway. If free radicals are inappropriately processed in melanin synthesis, hydrogen peroxide (H₂O₂) is generated, leading to production of hydroxyl radicals (HO[•]) and other reactive oxygen species (ROS).¹⁶ Melanin biosynthesis can



be inhibited by inhibition of tyrosinase. Tyrosinase inhibitors therefore can be clinically useful for the treatment of some dermatological disorders associated with melanin hyper pigmentation. They also find uses in cosmetics for whitening and depigmentation after sunburn.

Tyrosinase is a multifunctional, glycosylated, which catalyzes the first two steps in mammalian melanogenesis. These phenomena have encouraged researchers to seek new potent tyrosinase inhibitors for use in cosmetics. A number of natural ingredients are currently being used in cosmetics based on their properties as antioxidant, anti-inflammatory or anti tyrosinase activity.

To date the most promising target for the symptomatic treatment and slowing of Alzheimer's disease (AD) progression is cholinesterase inhibitors.¹⁷ In AD the destruction of cholinergic neurones causes a depletion of the neurotransmitter, acetylcholine (ACh).¹⁸ By inhibiting acetyl cholinesterase (AChE), the enzyme which catalyses break down of ACh, levels of this neurotransmitter can be elevated and function improved. This approach has a particular success as these cholinergic neurones are found mainly in regions associated with learning and memory.¹⁹

To this day, there are no reports that marine compounds isolated from microorganisms of the Red Sea area of Egypt have been used for the treatment of melanin hyper pigmentation and Alzheimer's disease. Hence, we tried to study sponge associated fungi and some of their biological activities. The present study was undertaken for screening and to evaluate the tyrosinase and acetyl cholinesterase inhibitory activity on the fungus *Aspergillus sydowii* strain W4-2 associated with the sponge *agelas* sp. with correlation to its GC/MS analysis.

MATERIALS AND METHODS

Sponge materials

Sponge samples; *Amphimedon viridis* (from which fungus FS1 was isolated) and *Agelas* sp. (from which fungus FS3 was isolated) were collected from Hurghada coast, Red Sea, Egypt. The site is located north Hurghada at El-Gouna, latitude N 27° 24' 7.5", E 33° 41' 16.8" the samples were collected at depth of 5m - 8m in January 2013 and kept frozen until the work-up. The morphological taxonomy of the sponges was identified by Mohamed A. Ghani – environmental researcher -Red Sea Marine parks, Hurghada, Red Sea, Egypt.

Preparation of animal material

Small pieces of inner tissue of fresh Sponge materials were rinsed three times with sterile sea water (SW); then aseptically cut into small cubes, approx. (0.5 cm³). A total of 50 - 75 cubes of each sample were placed on different isolation media. During the initial investigations, cubes from Sponge sample were placed in EtOH (70 %) for various times between 5 and 30s and subsequently

squeezed three times in sterile sea water (SW) before inoculation.

Isolation of Fungi from Sponge sample

A measured area of Sponge tissue (about 1cm³) was excised from the middle internal mesohyl area of the Sponge using a sterile scalpel. These Sponge cubes were placed directly on the surface of the agar plates⁷ or the excised tissue was then homogenized with sterile aged sea water, using a sterile mortar and pestle. The resultant homogenization was serially diluted until 10⁻⁶ and preincubated at room temperature for 1hr for the activation of dormant cells. From dilution 10⁻³ to 10⁻⁶ 0.1ml of each dilution was used to inoculate suitable solid medium containing antibacterial antibiotics. The plates were then incubated at 30°C for 7-14 days.²⁰ The appeared single fungal colonies were picked up and inoculated on PDA (sea water) slants. The medium used in isolation exhibited the following composition (g/L): yeast extract (1), glucose (1), Ammonium nitrate (1), peptone (0.25), agar (20) and sea water (1000). The pH was adjusted to 7.4.²¹ The medium²² was supplemented with Streptomycin sulphate (0.1g/L) and Penicillin G (0.1g/L).

Screening medium (Wickerham Medium for Liquid Culture)

For both shake and static cultures this broth medium with the following ingredients (g/L): Yeast extract (3), Malt extract (3), peptone (5), glucose (10) and sea water to make 1000 mL.

Small and large scale cultivation for screening

One fungal slant (7-10 old) was used to inoculate two Erlenmeyer flasks; (1L) and each containing 300ml of Wickerham medium for liquid cultures; by making spore suspension using 10ml sterile marine water. The cultures were then incubated at room temperature (shaking and static) for 8 days. Large scale cultivation was carried out using twenty 1L Erlenmeyer flasks for liquid cultures.

Extraction of secondary metabolites

The fungi were harvested at the end of incubation period, centrifuged at 8,000 rpm and subjected to extraction. The culture supernatant was extracted with ethyl acetate (3x or till exhaustion) and then evaporated under vacuum. On the other hand the fungal mycelia were first extracted using acetone and evaporated till dryness. The residual part was re-extracted using small volume of ethyl acetate.

Identification of fungal cultures

Fungal culture (FS3) was identified according to a molecular biological protocol by DNA isolation, amplification (PCR) and sequencing of the ITS region. The primers ITS2 (GCTGCGTTCTTCATCGATGC) and ITS3 (GCATCGATGAAGAACGCAGC) were used at PCR while ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used at sequencing. The purification of the PCR products was carried to remove unincorporated PCR primers and dNTPs from PCR



products by using Montage PCR Clean up kit (Millipore). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). *Candida* sp. was used as control.

GC/MS analyses

A Finnigan MAT SSQ 7000 mass spectrometer was coupled with a Varian 3400 gas chromatograph. DB-1 column, 30 m x 0.32 mm (internal diameter), was employed with helium as carrier gas (He pressure, 20 Mpa/cm²), injector temperature, 310°C; GC temperature program, 85 - 310°C at 3°C/min (10 min. initial hold). The mass spectra were recorded in electron ionization (EI) mode at 70 eV. The scan repetition rate was 0.5 s over a mass range of 39 - 650 atomic mass units (amu).

Sample preparation for GC/MS analyses

1mg of the dried extract was prepared for chromatography by derivatization for 30 min at 85°C with 15 µl pyridine + 20 µl N,O, bis-(trimethylsilyl) trifluoroacetamide (BSTFA) and analyzed by GC/MS.²³

Identification of compounds

The identification was accomplished using computer search user-generated reference libraries, incorporating mass spectra. Peaks were examined by single-ion chromatographic reconstruction to confirm their homogeneity. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the bases of its mass spectral fragmentation. Reference compounds were co-chromatographed when possible to confirm GC retention times.

Antimicrobial activity Test

Disc agar plate method was done to evaluate the antimicrobial activity of fungal extracts.²⁴ Investigated samples were solubilized in methanol. The antimicrobial activities of 0.5-cm-diameter filter paper disc saturated with about 1mg sample were tested against four different microbial strains, i.e., *Staphylococcus aureus* (G+ve bacteria), *Pseudomonas aeruginosa* (G-ve bacteria), *Candida albicans* (yeast) and *Aspergillus niger* (fungi). Both bacterial and yeast test microbes were grown on nutrient agar (DSNZ 1) medium (g/L): beef extract (3), peptone (10), and agar (20). Whereas fungal test microbe was grown on Szapek-Dox (DSMZ130) medium (g/L): sucrose (30), NaNO₃ (3), MgSO₄.7H₂O (0.5), KCl (0.5), FeSO₄.7H₂O (0.001), K₂HPO₄ (1) and agar (20). The culture of each microorganism was diluted by sterile distilled water to 10⁷ to 10⁸ CFU/ml to be used as inoculum. 0.1ml of the previous inoculum was used to inoculate 1L of agar medium (just before solidification) then poured in Petri dishes (10cm diameter containing 25ml). Discs (5 mm diameter) were placed on the surface of the agar plates previously inoculated with the test microbe and

incubated for 24 hrs. for bacteria and yeast but for 48 hrs., for fungus at 37 and 30 °C, respectively.

DPPH radical scavenging activity

DPPH radical scavenging activity of all extracts was analyzed according to a modified procedure of Matsushige and his group.²⁵ 1 ml of methanol solution for each extract (100µg/ml) was added to 1 ml of methanol solution of DPPH (60µM). The prepared solutions were mixed and left for 30 min at room temperature. The optical density was measured at 520 nm using a spectrophotometer (UV-1650PC Shimadzu, Japan). Mean of three measurements for each extract was calculated.

Mushroom Tyrosinase Activity

To evaluate the inhibitory action of methanol extract on tyrosinase, tyrosinase isolated from mushrooms was utilized as described previously with a minor modification.^{26,27} In brief, 140 µl 50 mM phosphate buffer (PH 6.8), 10 µl of extract (1mg/ml, dissolved in MeOH), 40µl of 1.5mM L-tyrosine solution and 20µl of Mushroom tyrosinase (1500 U/ml) were added to a 96 well microplate. The assay mixture was incubated at 25°C for 30 min. Following incubation, the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically at 492 nm in a microplate reader.

Acetyl cholinesterase (AChE) inhibitory activity

To investigate the AChE-inhibitory activity we followed the method previously described,^{28,29} with slight modified Spectrophotometric procedure. Electric-eel AChE (Sigma) was utilized as source of cholinesterase. Acetylthiocholine iodide (Sigma) was used as substrate for AChE, to perform the reaction. 5,5-Dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma) was utilized for the determination of cholinesterase assay. Investigated samples were solubilized in ethanol. Reaction mixture contained 150 µL of (100 mM) sodium phosphate buffer (pH 8.0), 10 µL of DTNB, 10 µL of test-extract solution and 20 µL of acetyl cholinesterase solution were mixed and incubated for 15 min (25°C). 10 mL of acetylthiocholine was added to initiate the reaction. Hydrolysis of acetylthiocholine was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine, at a wavelength of 412 nm (15 min). All the reactions were performed in triplicate in 96-well micro-plate.

RESULTS AND DISCUSSION

Natural antioxidants offer an alternative source to promote healthy life. For example tyrosinase inhibitors are clinically useful for the treatment of some dermatological disorders associated with melanin hyper pigmentation and acetyl cholinesterase inhibitors are used to treat Alzheimer's disease.



Identification of fungal cultures

BLAST search for the fungus isolate FS3 revealed 99% similarity to *Aspergillus sydowii* strain W4-2. The phylogentic tree of this fungal isolate was also constructed (Figure 1).

GC/MS analysis

The GC/MS analysis revealed that 38 compounds were identified; 22 compounds in supernatant static (A) and 23 compounds in its mycelia (B) of the fungus *Aspergillus sydowii* strain W4-2 isolated from the Sponge (*Agelas* sp.).

The GC/MS analysis for supernatant static (A) revealed the presence of hydrocinnamic acid (0.64%), 5-hydroxy-7, 8, 4'-trimethoxy-isoflavone (1.65%); 1,3,8-trihydroxy-2-isopropyl-6-methoxy-9,10-anthraquinone (1.32%); 6,7-dimethoxy-5,4'-dihydroxy flavone (1.4%); Benzene acetic acid-2-hydroxy (0.85%); Benzene acetic acid-4-hydroxy (2.1%); p-Hydro-coumaric acid (0.61%) and other

compounds (Table 1, Figure 2). Hydrocinnamic acid was found not only depressed the activity of the steady state activity but also prolonged the lag time for the monophenolase with dose-dependence anti tyrosinase activity.³⁰ Several isoflavones derivatives with hydroxyl groups in the A ring are considered as tyrosinase inhibitors.³¹ The anthraquinone, physcion (1,8-dihydroxy-2-methoxy-3-methylanthraquinone) was found to show similar tyrosinase inhibitory activity with that of kojic acid.³² Some flavones were also demonstrated to be a slow-binding inhibitors just like kojic acid and tropolone.³¹ 4-Hydroxy-phenylacetic acid and 2-hydroxy-phenylacetic acid were reported as hypopigmenting agents.³³ p-Coumaric acid, pentacosyl dihydro-p-coumarate strongly inhibited L-DOPA oxidation by mushroom tyrosinase. It was found that, the most active compound p-coumaric acid was 22 times more active in tyrosinase inhibitory activity than kojic acid.³⁴

Table 1: Chemical composition assessed by GC/MS of alcoholic extracts of Supernatant (A) and mycelia (B) for *Aspergillus sydowii* strain W4-2

No	Compound	RT	A	B	No	Compound	RT	A	B
1	N-Ethyl,N-vinylacetamide	5.04	---	0.89	20	Palmitic acid (C16:0)	47.0	1.06	5.35
2	1,2-Dihydroxy-ethane	5.29	---	0.62	21	a'-D-Galactopyranoside, methyl 2,4,6-tris-O-(trimethylsilyl)-, acetate	47.1	0.56	---
3	Carbamic acid	6.47	---	0.61	22	Linoleic acid (C18:2, $\Delta^{9,12}$)	52.1	---	5.88
4	Glycerol	18.1	---	1.92	23	Oleic acid (C18:1, Δ^9)	52.2	---	4.27
5	Butanedioic acid	19.7	---	0.32	24	2-[5'-(Ethoxycarbonylmethyl)-N-methyl-4'-oxothiazolidin-2'-ylidene]-1-phenylethanethione	52.7	---	0.95
6	Hydrocinnamic acid	20.9	0.64	---	25	2,6-Dinitro-4,4'-di-tert-butylbiphenyl	52.7	0.38	---
7	5-hydroxy-7, 8, 4'-trimethoxy isoflavone	26.8	1.65	---	26	Tetramethoxydibenzofuran-4-carbaldehyde	53.2	0.7	---
8	1,3,8-trihydroxy-2-isopropyl-6-methoxy-9,10-anthraquinone	27.8	1.32	---	27	cis-13-Eicosenoic acid	57.3	---	0.55
9	1,2,3,4-Tetra-methoxybenzene	28.6	2.09	---	28	Docosenoic acid, methyl ester	59.6	---	0.29
10	6,7-dimethoxy-5,4'-dihydroxy flavone	29.1	1.4	---	29	Di-(2-ethylhexyl)phthalate	61.0	0.66	6.34
11	Benzene acetic acid- 2-hydroxy	30.0	0.85	---	30	Hexadecanoic acid, 2,3-dihydroxy-propyl ester	62.1	2.82	0.79
12	Benzeneacetic acid- 4-hydroxy	33.0	2.1	0.59	31	cis-13-Docosenoic acid	62.2	0.46	2.1
13	2,6-dimethoxybenzene-1,4-dihydroxy	34.0	0.48	---	32	Bis[6-(8-methoxy-1,2-dihydro-2,2,4-trimethylquinolyl)malononitrile	63.1	---	2.96
14	2-(p-Methoxyphenyl) oxazolo[5,4-b]pyridine	37.0	1.37	---	33	9,12-Octadecadienoic acid,3-dihydroxy propyl ester	65.9	---	0.5
15	p-Hydro-coumaric acid	37.8	0.61	---	34	Octadecanoic acid, 2,3-dihydroxy propyl ester	66.56	0.98	0.47
16	2,3-Dimethoxy anthracene-9,10-dione	38.8	0.78	---	35	ergosta-5,7,22-trien-3 α -yloxy)trimethyl	76.1	---	1.31
17	2-Ethyl-3-ketohexanoate	42.0	3.61	0.51	36	Stigmasterol	77.0	---	0.3
18	Indole- 3-acetic acid	44.9	0.73	---	37	β -Sitosterol	78.2	---	0.59
19	3-Hydroxy-2'-[2'- Hydroxyethynyl]-2,2'-bipyridine	45.4	0.43	---	38	Hexadecanoic acid, tetradecyl ester	82.5	---	0.5

RT = retention time. TIC = The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation



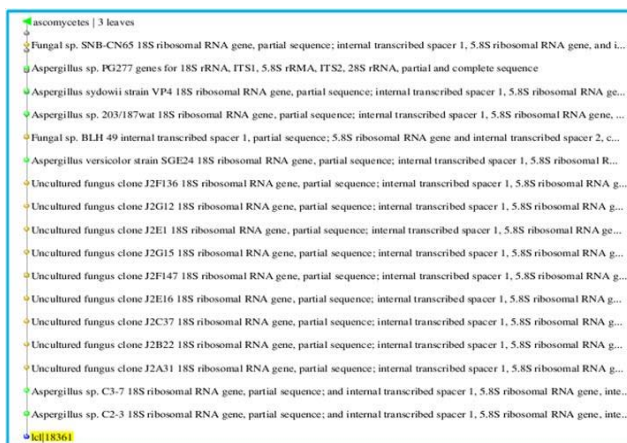


Figure 1: The phylogenetic tree of fungus *Aspergillus sydowii* strain W4-2 isolated from the Sponge (*Agelass* sp.)

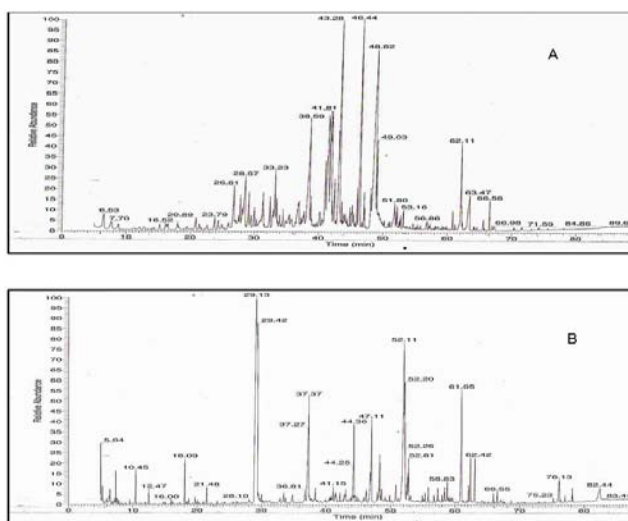


Figure 2: Comparative chromatographic studies (GC/MS analysis) for alcoholic extracts of Supernatant (A) and mycelia (B) for *Aspergillus sydowii* strain W4-2.

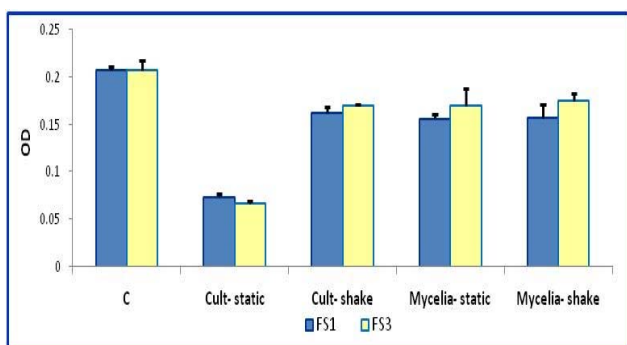


Figure 3: Free radical scavenging activity of 2ry metabolites extracts from culture and mycelia (static and after shaking) in the DPPH radical assay. Values are expressed as mean \pm SD, n = 3 at a concentration of (100 μ g/ml for all tested extracts).

The GC/MS analysis for mycelia static (B) revealed the presence of palmitic acid (5.35%), linoleic acid (5.88%), oleic acid (4.27%), cis-13-eicosenoic acid (0.55%), cis-13-docosenoic acid (2.1%), 9,12-octadecadienoic acid, 3-dihydroxy propyl ester (0.5%), β -sitosterol (0.59%) and other compounds (Table 1, Figure 2).

Öztürk reported that, the best (AChE) inhibitory activity was found for linoleic acid and oleic acid as 0.267 ± 0.05 mg/mL and 0.127 ± 0.03 mg/mL, respectively. Palmitic and stearic acids are more than 4 mg/mL.³⁵ It was found that β -Sitosterol, β -carotene and chlorogenic acid possess great AChE inhibition activity by binding with the active site pocket on target protein.³⁶

DPPH-free radical scavenging activity

The supernatant extracts from the static culture of the identified fungus FS3 (*Aspergillus sydowii* strain W4-2) and the unidentified fungus FS1 isolated from *Agelass* sp. and *Amphimedon viridis*; respectively, showed the highest free radical scavenging activity (FRSA) against DPPH radical, while their mycelial extracts had very low activity (Figure 3). The supernatant and mycelia (culture shaking) for the two fungi isolated showed very low free radical scavenging activity (Figure 3). The DPPH assay is based on the principle that a hydrogen donor is an antioxidant. It measures the activity of an antioxidant to directly scavenge DPPH radical and determining its absorbance spectrophotometrically at 520 nm. The DPPH is stable organic nitrogen centered free radical with a dark purple color that becomes colorless when it reacts with antioxidant to form non-radicals.³⁷ Our data are in agreement with previous studies.^{38,39}

Tyrosinase inhibitory activity

Tyrosinase is a polyphenol oxidase with a dinuclear copper active site and is involved in the formation of mammalian melanin pigments. Over-activity of this enzyme leads to hyper pigmentation of the skin. Chemical agents that demonstrated tyrosinase inhibitory activity has been used to suppress melanogenesis and can be clinically useful for the treatment of some dermatological disorders associated with melanin hyper pigmentation associated with the high production of melanocytes in human cells. Kojic acid is one of the popular chemicals used in Whitening cosmetics but chronic, cytotoxic and mutagenic effects have been proved.⁴⁰ To avoid this problem, more attention has been paid to the use of natural products instead of chemical agents as anti-tyrosinase substances.

The tyrosinase inhibitory activity of fungal extracts is shown in (Figure 4). The supernatant extract from the static culture of the identified fungus FS3 (*Aspergillus sydowii* strain W4-2) revealed moderate tyrosinase inhibitory activity (36%), while its mycelial extract revealed no inhibitory activity. FS3 shake culture extract, static and shake culture extracts of FS1 showed no activity.

Usually, "true inhibitors" are classified into four types, including competitive inhibitors, uncompetitive inhibitors, mixed type (competitive/uncompetitive) inhibitors, and non-competitive inhibitors. A competitive inhibitor is a substance that combines with a free enzyme in a manner that prevents substrate binding. That is, the inhibitor and the substrate are mutually exclusive, often because of

true competition for the same site. A competitive inhibitor might be a copper chelator, non-metabolizable analogs, or derivatives of the true substrate. In contrast, an uncompetitive inhibitor can bind only to the enzyme-substrate complex. A mixed (competitive and uncompetitive mixed) type inhibitor can bind not only with a free enzyme but also with the enzyme-substrate complex. For most mixed-type inhibitors, their equilibrium binding constants for the free enzyme and the enzyme-substrate complex, respectively, are different. However, a special case among the mixed inhibitors is the non-competitive inhibitors, which bind to a free enzyme and an enzyme-substrate complex with the same equilibrium constant.³¹

Data obtained suggest that enzyme inhibition by extracts was a non-competitive inhibition and not competitive one as reported by Chang et al,³¹ (data not shown). This finding support the idea that this fungal extract may be defined as true enzyme inhibitor in two ways: one way through its free radical scavenging activity, and the second way through its non-competitive inhibition of enzyme-substrate complex.³¹ lawi and his group reported that, the anti tyrosinase activity of natural antioxidant is due to the presence of double bond or free hydroxyl group (-OH) that can form a stable Schiff base with a primary amino group in the enzyme.⁴¹

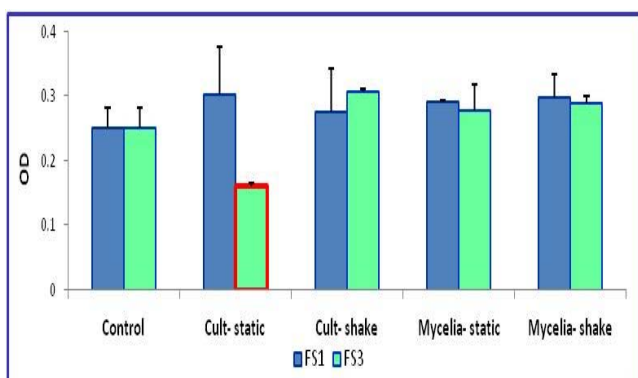


Figure 4: Inhibition of the **Tyrosinase** activity of 2ry metabolites extracts from culture and mycelia (static and after shaking). Values are expressed as mean \pm SD, n = 3 (200 μ g/ml for all tested extracts).

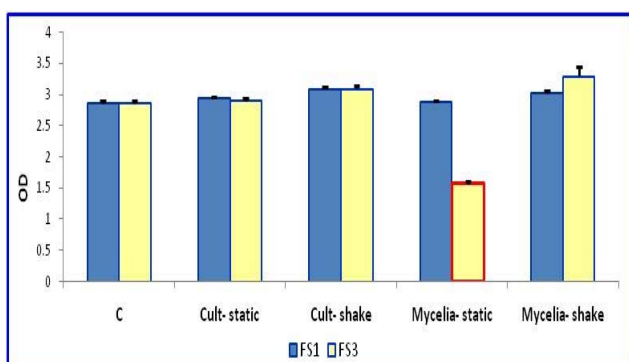


Figure 5: Inhibition of the **acetyl cholinesterase** activity of 2ry metabolites extracts from culture and mycelia (static

and after shaking). Values are expressed as mean \pm SD, n = 3 (200 μ g/ml for all tested extracts).

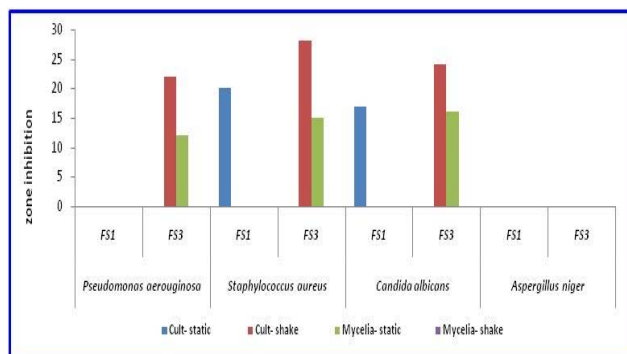


Figure 6: Antimicrobial activity of 2ry metabolites extracts from culture and mycelia (static and after shaking)

Acetyl cholinesterase inhibitory activity

Acetylcholine breakdown in the brain can be prevented by the inhibition of acetyl cholinesterase activity, which subsequently increases the concentration of acetylcholine. This further result in increased communication between nerve cells that use acetylcholine as a chemical messenger producing a therapeutic effect in patients with Alzheimer's disease.⁴²

The effect of fungal extracts on acetyl cholinesterase is shown in (Figure 5). The mycelial extract from the static culture of the identified fungus FS3 (*Aspergillus sydowii* strain W4-2) revealed significant acetyl cholinesterase inhibitory activity (45%), while its supernatant extract revealed no inhibitory activity. FS3 shake culture extract, static and shake culture extracts of FS1 showed no activity.

Antimicrobial activity

The supernatant and mycelial extracts from the shake and static cultures; respectively, of the identified fungus FS3 (*Aspergillus sydowii* strain W4-2) isolated from the sponge *Agelas* sp. is highly effective against *Pseudomonas aeruginosa*, *Staph. aureus* and *Candida albicans*. The supernatant from the static culture of the fungus FS1 isolated from the sponge *Amphimedon viridis* is highly effective against *Staph. aureus* and *Candida albicans* but its shake culture had no activity against any tested microbe. Also, the FS1 mycelial extract from the static culture had no activity against any test microbe.

Marine invertebrates-associated microorganisms gained a great attention as important sources of new bioactive secondary metabolites. Genus *Aspergillus* was investigated previously as a producer of considerable numbers of cytotoxic and biologically active compounds.⁴³ Two new hetero-spirocyclic-lactams, azaspirofurans A and B were isolated from the marine sediment-derived fungus *Aspergillus sydowii* D2-6.⁴⁴ Azaspirofurans A exhibited cytotoxicity against A549 cell line. *Aspergillus sydowii* PSU-F154 was also isolated from sea fan. From this isolate three new sesquiterpenes named; asparagillusenes A, B and (7S)-7-O-methylsydowic

acid and two new hydrogenated xanthone derivatives named; Aspergillusones A and B were separated and identified.⁴⁵

CONCLUSION

It could be concluded that, the isolated marine fungus *Aspergillus sydowii* strain W4-2 (FS3) showed different biological activities as tyrosinase, acetyl cholinesterase inhibitors, antioxidant, antibacterial and antifungal rather than the other unidentified fungus (FS1). Our study provides (for the first time) primary evidence suggesting that the isolated marine fungus *Aspergillus sydowii* strain W4-2 in further in-vivo studies could play an important role as tyrosinase and acetyl cholinesterase inhibitor, besides its antioxidant, antibacterial and antifungal activities.

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