Research Article

# Antioxidant, Acetylcholinesterase and α-Glucosidase Potentials of Metabolites from the Marine Fungus *Aspergillus unguis* RSPG_204 Associated with the Sponge (*Agelas* sp.)

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**Accepted on:** 30-11-2014; **Finalized on:** 31-12-2014.

**ABSTRACT**

Marine-derived fungi are a rich source of structurally diverse bioactive secondary metabolites with unprecedented skeletons and have shown various health beneficial biological activities. The fungus *Aspergillus unguis RSPG_204* associated to the marine Sponge (*Agelas* sp., Red Sea , Egypt) was investigated. The supernatant and mycelial extracts [culture static (clt.st.)] had the highest free radical scavenging activity against superoxide anion radical. The mycelial extract (clt.st.) revealed significant acetyl cholinesterase inhibitory activity (47.5%). The supernatant (clt.st.) is the only one showed very low (3.6%) activity against tyrosinase while, it had a high α-glucosidase inhibitory activity (42.3%). The supernatant and mycelial extracts (culture shakes) had no biological activities. 55 compounds were identified by GC/MS analysis; 48 compounds in supernatant static (A) and 12 compounds in its mycelial extract (B). 8-Methoxy-5-methyl-2,3-dihydro-1-H-benzazepin-2-one (3.5%), 2-Piperidinone (1.6%), Benzoic acid (1.65%); Pyrimidine-2,4–dihydroxy (0.76%); 2,5-Diketopiperazine (0.25%), 3-Phenyl-3-hydoxypropanoic acid (0.5%), 4-Hydroxy Benzoic acid (1.38%), Benzeneacetic acid-4-hydroxy (1.29%), 9-Amino-3,3-dimethyl-3,4-dihydro-1(2H)acridone (0.4%), Kojic acid (2.3%), Hydrocinnamic acid, p-hydroxy (1.3%), N-Isopropyl-2-phenyl-6-pentanyl-4-pyridinamine (2.36%), Cinnamic acid-p-hydroxy (0.62%) were identified in (A). Hexadecanoic acid (5.91%), Heptadecanoic acid (0.61%), 9,12-Octadecadienoic acid (14.3%), 9-Octadecenoic acid (4.7%), Octadecanoic acid (2.22%), Octadecanoic acid-2,3-hydroxypropylester (0.62%), 2',4'-Diethoxy-6'-methoxy-3,4-methylenedioxychalcone (1.05%) were identified in (B). It could be concluded that, the fungus *Aspergillus unguis RSPG_204* 2ry metabolites showed significant acetyl cholinesterase and high α-glucosidase inhibition, beside its high antioxidant activities. Our study provides (for the first time) primary evidence suggesting that these 2ry metabolites in further in-vivo studies could play an important role as acetyl cholinesterase and α-glucosidase inhibitors, besides their antioxidant activities.

**Keywords:** Sponge, *Aspergillus unguis* RSPG_204, acetyl cholinesterase and α-Glucosidase inhibitors, Antioxidant, GC/MS analysis.

## INTRODUCTION

Over the last few decades, marine organisms are constantly yielding inspiring ideas related to the variety of unusual chemical structures of their metabolites, biosynthesis and the wide spectrum of biological functions and properties. The reasons for a modest interest for marine organisms' research by the Pharmaceutical industry essentially rest on availability and regular, economically acceptable, supply of these "rich sources of new compounds". Among the species within the marine animal kingdom, sponges are the most primitive multicellular organisms that have been studied.[1] Marine sponges for the past decades have been considered as a very fertile field for the discovery of bioactive natural chemical substances with respect to the diversity of their primary and secondary chemical components and metabolites. Sponges (Porifera), as primitive filter-feeders, have a high frequency of bioactive components for their chemical defences against environmental stress factors.[2] In this context, fungi associated with sponges have been found to yield a variety of structurally diverse natural products, e. g. microsphaeropsin and ulocladol.[3] Marine-derived fungi are a rich source of structurally diverse bioactive secondary metabolites with unprecedented skeletons and have shown various health beneficial biological activities.[4,5] A number of metabolites from marine-derived fungi possess antioxidant, antimicrobial, anti-tyrosinase or skin whitening, cytotoxic, quinine reductase induction, and antiplasmodial activities.

Free radicals, generated as by-products of normal cellular metabolism, have been implicated in the etiology of several diseases such as liver cirrhosis and diabetes. Increased oxidative stress has been suggested as mechanism underlying diabetes and its related complications.[6]

Diabetes mellitus is a chronic metabolic disease with the highest rates of prevalence and mortality in both developed and developing countries. It has been reported to associate with oxidative damage. Prevention of oxidative damage with natural antioxidants and control of postprandial hyperglycemia, by inhibiting digestive enzymes such as α-glucosidase, a main glycosidase hydrolases found on the luminal surface of enterocytes, are two important diabetic prevention strategies.[7,8]

Reactive oxygen species (ROS) and free radical mediated reactions are the major reasons for the pathological events such as aging, coronary heart ailment and Alzheimer's disease.[9] The oxidative damage of a tissue is

indirectly prevented by increasing cells' natural defenses and/or directly by scavenging the free radical species.[10]

Alzheimer's disease is a progressive degenerative neurologic disorder resulting in impaired memory and behavior. Most treatment strategies have been based on the cholinergic hypothesis. Cholinergic neurotransmission is specially affected in patients with Alzheimer's disease. One of the most promising approaches for treating this disease is to enhance the acetylcholine level in brain using acetylcholinesterase inhibitors.[11]

As far as known till now, there are no reports on the bioactive metabolites identified from the fungus *Aspergillus unguis RSPG_204* associated to the marine Sponge (*Agelas* sp. Red Sea, Egypt) and their antioxidant, anti-diabetic, acetylcholine and tyrosinase inhibition activities. Previously we studied the antimicrobial and cytotoxic activities of this fungus.[12] Hence, we tried to study some of its biological activities (in-vitro), with concomitant GC/MS analysis to the highly biologically active extracts.

## MATERIALS AND METHODS

### Microorganism and culture conditions

*Aspergillus unguis RSPG_204* was isolated from the Sponge *Agelas* sp. collected from Hurgada cosat, red sea, Egypt. The sponge was cut into small pieces and inoculated on agar plates containing appropriate constituents. Produced pure fungal colonies were maintained on potato dextrose agar medium (PDA) for 7-10 days and kept at 4 $^{o}$C until the second subculture and then used for further studies.[13,14] *Aspergillus* sp. was screened for its ability to produce bioactive secondary metabolites by cultivating it on Wickerham broth medium. Bioactive metabolites were extracted by ethyl acetate (3x) for the culture supernatant and acetone followed by ethyl acetate for fungal mycelia. The fungus was previously identified using 18srRNA sequencing techniques and the produced sequence was applied to Blast in Gene Bank.[12]

### GC/MS analysis

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/cm$^2$), injector temperature, 310ºC; GC temperature program, 85 - 310$^{o}$C at 3 $^{o}$ 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 analysis and identification

1mg of the dried extract was prepared for chromatography by derivatization for 30 min at 85$^{\circ}$C with 15 µl pyridine + 20 µl N,O, bis-(trimethylsilyl) trifluoroacetamide (BSTFA) and analyzed by GC/MS.[15,16] The identification was accomplished using computer search user-generated reference libraries, incorporating mass spectra.

### Superoxide anion scavenging activity

Superoxide anion scavenging activity was determined according to a modified method of Matsushige and his group.[17] The absorbance was measured at 560 nm. All the reactions were performed in triplicate in 96-well micro-plate.

### Mushroom Tyrosinase Activity

The inhibitory action of methanol extracts of both culture and mycelia of the isolated fungus on Mushroom tyrosinase activity was evaluated according to the procedure described previously with a minor modification[18]. The reaction mixture was followed spectrophotometrically at 492 nm in a microplate reader. All the reactions were performed in triplicate in 96-well micro-plate.

### Acetylcholinesterase (AChE) inhibitory activity

To investigate the AChE-inhibitory activity we followed the method previously described,[19] with slight modified spectrophotometric procedure as published in our previous study[16]. Electric-eel AChE (Sigma) was utilized; the enzymatic hydrolysis of acetylthiocholine was measured at a wavelength of 412 nm (15 min). All the reactions were performed in triplicate in 96-well micro-plate.

### α-Glucosidase inhibition assay

The α-glucosidase inhibitory activity was assessed by the standard method with slight modifications.[20] Absorbance readings (A) were recorded at 405 nm by micro-plate reader. Acarbose was used as a standard and compared with all extracts. The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated as follows:

% Inhibition = [ (Aco – At) / Aco ] X 100

Where, Aco is absorbance of the control and At is absorbance of the sample.

## RESULT AND DISCUSSION

### GC/MS analysis

The GC/MS analysis revealed that 55 compounds were identified; 48 compounds in supernatant static extract (A) and 12 compounds in the mycelial extract (B) of the fungus *Aspergillus unguis RSPG_204* isolated from the Sponge (*Agelas* sp.).

The GC/MS analysis for supernatant static extract (A) revealed the presence of 8-methoxy-5-methyl-2,3-dihydro-1-H-benzazepin-2-one (3.5%), 2-Piperidinone (1.6%), Benzoic acid (1.65%); Pyrimidine, 2,4 –dihydroxy (0.76%); 2,5-Diketopiperazine (0.25%); Benzenepropanoic acid-à- hydroxyl (0.4%), 3-Phenyl-3-hydoxypropanoic acid (0.5%), 4-Hydroxy Benzoic acid

(1.38%), Benzeneacetic acid-4-hydroxy (1.29%), 9-Amino-3,3-dimethyl-3,4-dihydro-1(2H)acridone (0.4%), Kojic acid (2.3%), Hydrocinnamic acid-p-hydroxy (1.3%), N-Isopropyl-2-phenyl-6-pentanyl-4-pyridinamine (2.36%), Cinnamic acid-p-hydroxy (0.62%), and other compounds (Table 1, Figure 1).

**Table 1:** Chemical composition assessed by GC/MS of Supernatant (A) and mycelia (B) for *Aspergillus unguis RSPG_204*

| No | Compound | RT | A% | B%[a] |
|---|---|---|---|---|
| **1** | Butane,2,3 dihydroxy | 5.26 | 6.3 | |
| **2** | Lactic acid | 5.98 | 4.4 | |
| **3** | Hydroxy- Acetic acid, | 6.36 | 0.26 | |
| **4** | Alanine | 7.29 | 0.28 | |
| **5** | Butanoic acid,4-amino | 7.95 | 0.26 | |
| **6** | 8-methoxy-5-methyl-2,3-dihydro-1-H-benzazepin-2-one | 8.51 | 3.5 | |
| **7** | 2-Piperidinone | 8.96 | 1.6 | |
| **8** | Butanoic acid-3methyl-2-hydroxy | 10.31 | 0.18 | |
| **9** | Benzoic acid | 14.35 | 1.65 | |
| **10** | Butanoic acid, 2,2dimethyl-3- hydroxy | 15.63 | 0.36 | |
| **11** | Benzeneacetic acid | 17.02 | 0.42 | |
| **12** | 2,6-Di-t-butyl-4-hydroxy-4-phenyl-1-imino-2,5-cyclohexadiene | 17.31 | 1.84 | |
| **13** | Butanedioic acid | 18.22 | 0.67 | |
| **14** | **Pyrimidine, 2,4 –dihydroxy** | 18.43 | 0.76 | |
| **15** | 4(4-Methoxyphenyl)-4-(1H-pyrrolo[2,3b]pyridine-3-yl)-butan-2-one | 20.16 | 2.43 | |
| **16** | 2,5-Diketopiperazine | 20.94 | 0.25 | |
| **17** | Benzene-propanoic acid | 22.65 | 0.41 | |
| **18** | 2(tert-Butylaminomethylidene)3,3-dimethyl-3,4-dihydro-1(2H)naphthalenone-4-carboxylic acid methyl ester | 25.02 | 1.03 | |
| **19** | 2(tert-Butylaminomethylidene)3,3-dimethyl-3,4-dihydro 1(2H)naphthalenone-4-carboxylic acid | 26.16 | 0.43 | |
| **20** | 1-Methoxy-3-pentyl-6,6a,7,8-tetrahydro-6,6-dimethyl-9H-dibenzo[b,d]pyran-9-one | 26.8 | 2.57 | |
| **21** | 1-Methoxy-3-pentyl-6,6a,7,8-tetrahydro-6,6-dimethyl-9H-dibenzo[b,d]pyran-9-one (isomer) | 27.61 | 1.76 | |
| **22** | 2(tert-Butylaminomethylidene)3,3dimethyl-3,4-dihydro-1-(2H)naphthalenone-4-carboxylic acid methyl ester (isomer) | 28.64 | 1.75 | |
| **23** | Acetic acid, [o-hydroxyphenyl], | 29.32 | 0.79 | |
| **24** | Benzenepropanoic acid-à- hydroxyl | 30.02 | 0.4 | |
| **25** | Benzeneacetic acid,4-hydroxy | 32.11 | 0.5 | |
| **26** | 4-Hydroxy Benzoic acid, | 31.55 | 1.38 | |
| **27** | | | 1.29 | |
| **28** | 9-Amino-3,3-dimethyl-3,4-dihydro-1(2H)acridone | 32.26 | 0.4 | |
| **29** | 4H-Pyran-4-one,5-hydroxy-2hydroxymethyl (Kojic acid) | 33.51 | 2.3 | |
| **30** | Octanedioic acid | 34.35 | 0.24 | |
| **31** | Ethyl 6-methyl-5-oxo-1,2,3,5-tetrahydro-pyrrolo[1,2-a]quinolin-4-carboxylate | 34.59 | | 0.87 |
| **32** | Cyclohexanecarboxylic acid, 3-tridecyl ester | 36.46 | | 0.67 |
| **33** | Hydrocinnamic acid, p-hydroxy | 36.50 | 1.3 | |
| **34** | 1H-Indole-6-methoxy-5(phenylmethoxy) | 37.51 | 0.52 | |
| **35** | Azelaic acid | 37.83 | 0.58 | |
| **36** | N-Isopropyl-2-phenyl-6-pentanyl-4-pyridinamine | 39.76 | 2.36 | |
| **37** | 19-Norandrosterone | 41.45 | 1.14 | |
| **38** | n-Pentadecanoic acid | 41.63 | 0.34 | 0.84 |
| **39** | Cinnamic acid, p-hydroxy | 42.42 | 0.62 | |
| **40** | Ribitol (5-sugar) | 44.2 | | 1.63 |
| **41** | Hexadecanoic acid, | 45.61 | 1.11 | 5.91 |
| **42** | 5H,10H-Dipyrrolo[1,2a:1',2'd]pyrazine-5,10-dione, octahydro-2,7-dihydroxy | 47.24 | 0.59 | |
| **43** | Heptadecanoic acid | 47.71 | 0.44 | |
| **44** | 2',4'- Diethoxy-6'-methoxy-3,4-methylenedioxychalcone | 48.1 | | 1.05 |
| **45** | Heptadecanoic acid | 49.75 | | 0.61 |
| **46** | 9,12-Octadecadienoic acid | 50.37 | 0.30 | **14.3** |
| **47** | Anodendroside A | 50.46 | | 0.4 |
| **48** | 9-Octadecenoic acid, | 50.55 | 0.45 | 4.7 |
| **49** | Octadecanoic acid, | 51.33 | 1.3 | 2.22 |
| **50** | 6,11a-Dihydro-8-hydroxy-3-methoxy-9-benzyloxy-6,6-dimethyl-6H-benzofuro[3,2c]benzopyran | 56.91 | 1.89 | |
| **51** | Pentadecanoic acid, glycerine(1)monoester, | 57.60 | 0.16 | |
| **52** | N(p-Chlorophenyl)1(tbutylsulfinyl)2-naphthyl] methanimine | 57.75 | 0.36 | |
| **53** | N(p-Chlorophenyl)1(t-butylsulfinyl)2-naphthyl] methanimine (isomer) | 58.23 | 0.99 | |
| **54** | Hexadecanoic acid, 2,3-dihydroxy | 60.66 | 0.25 | |
| **55** | Octadecanoic acid, 2,3-hydroxypropyl ester | 66.43 | | 0.62 |

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

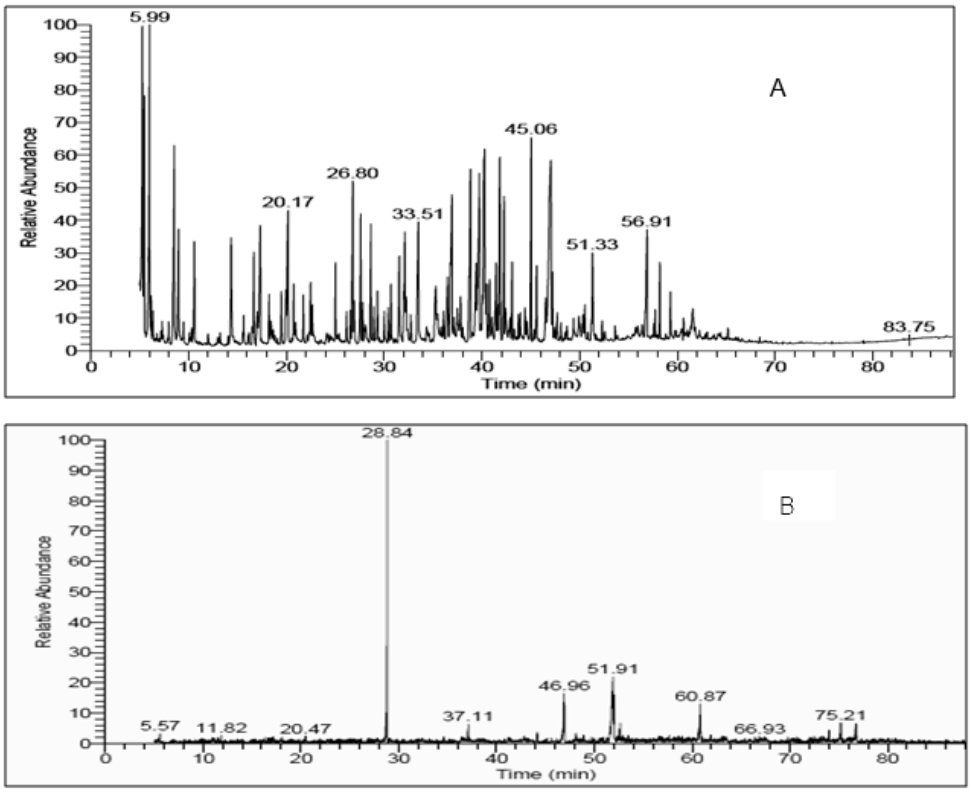


**Figure 1:** Comparative chromatographic studies (GC/MS analysis) for alcoholic extracts of Supernatant (A) and mycelia (B) for *Aspergillus unguis RSPG_204*

Zhang and his group proved the presence of α-glucosidase inhibitory activity of 4-aryl-2-benzazepine-1,5-diones.[21] Du *et al.,* showed remarkably α-glucosidase inhibition of 4-piperidone derivative.[22] It was proven that pyrimidine-fused derivatives show selective inhibitory properties for α- glucosidase over porcine pancreatic α-amylase which is important in terms of their reduced susceptibility for the possible development of intestinal disturbance side effects.[23] The potential inhibition of α-glucosidases of a series of chiral piperazine-2,5-dione derivatives was investigated, where some of them are α-glucosidase inhibitor selectivity.[24] The acridones alkaloids isolated by Wansi and his group showed potent activity against α-glucosidase.[25] Lelono and Tachibana showed that, 4-hydroxy-3-methoxy benzoic acid and 4-hydroxy-3,5-dimethoxy benzoic acid, with 27 and 35% inhibition of α-glucosidase activity, respectively.[26] It was reported that, 4-methoxy-trans-cinnamic acid and its ethyl ester showed the highest potent inhibitory activity among those of trans-cinnamic acid derivatives. 4-methoxy-trans-cinnamic acid was a noncompetitive inhibitor for α-glucosidase.[27] Some cinnamic acid derivatives were found to be inactive in pancreatic α-amylase inhibition. Kinetic analysis revealed that intestinal maltase was inhibited by caffeic acid, ferulic acid, and isoferulic acid in a mixed-inhibition manner. In addition, ferulic acid and isoferulic acid inhibited intestinal sucrase in a mixed type manner, whereas caffeic acid was a non-competitive inhibitor.[28]

The GC/MS analysis for mycelia static extract (B) revealed the presence of Pentadecanoic acid (0.84%), hexadecanoic acid (5.91%), Heptadecanoic acid (0.61%), 9,12-Octadecadienoic acid **(14.3%),** 9-Octadecenoic acid (4.7%), Octadecanoic acid (2.22%), Octadecanoic acid-2,3-hydroxypropylester (0.62%), 2',4'- Diethoxy-6'-methoxy-3,4-methylenedioxychalcone (1.05%) and other compounds (Table 1, Figure 1). It was mentioned that, the substituent at the C3 and C9 positions of quinoline alkaloids played a critical role in AChE or BChE inhibition.[29] Kang stated that number of hydroxyl groups on the chalcone B-ring could alleviate AChE inhibition; where 3,4-dihydroxy > 4-hydroxy > 2,4-dihydroxy > 2,5-dihydroxy.[30] Öztürk reported that, the best (AChE) inhibitory activity was found for 9,12-Octadecadienoic acid and 9-Octadecadienoic acid as 0.267±0.05 mg/mL and 0.127±0.03 mg/mL, respectively while, hexadecanoic and Octadecanoic acids are more than 4 mg/mL.[31]

**Scavenging ability for superoxide anion radical**

The free radical scavenging activity of different extracts on superoxide anion generated by Xanthine-Xanthine oxidase enzymatic method was evaluated. It could be observed that: the supernatant and mycelial extracts (culture static) for the identified fungus *Aspergillus unguis* RSPG_204 isolated from the Sponge *Agelas* sp. had the highest free radical scavenging activity against superoxide anion radical (Figure 2), while, the supernatant and mycelial extracts (culture shaking) for the isolated fungus had mild free radical scavenging activity (Figure 2). These data are mentioned for the first time.

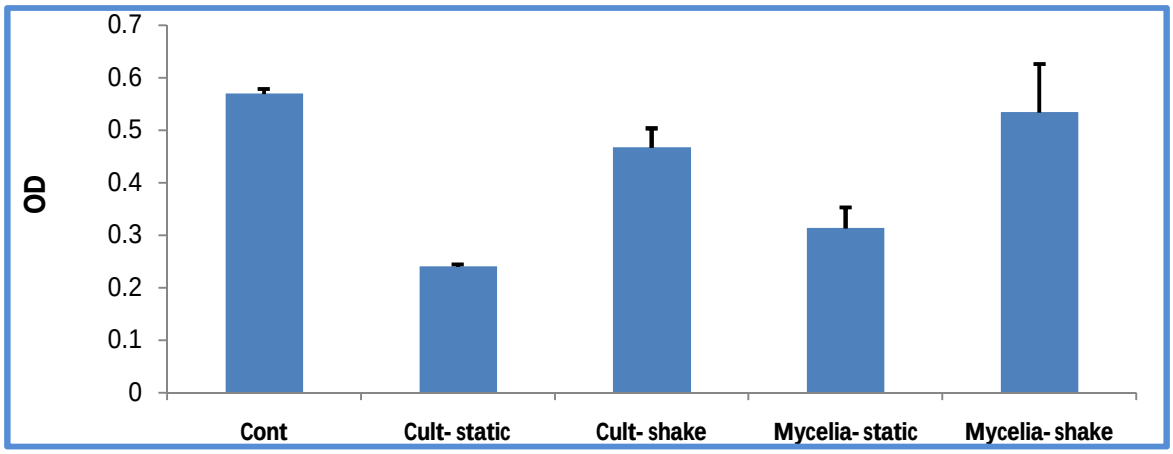


**Figure 2:** Free radical scavenging activity of 2ry metabolites extracts from culture and mycelia (static and after shaking) in the X-XOD radical assay. Values are expressed as mean ± SD, n = 3 at a concentration of (100 µg/ml for all tested extracts).

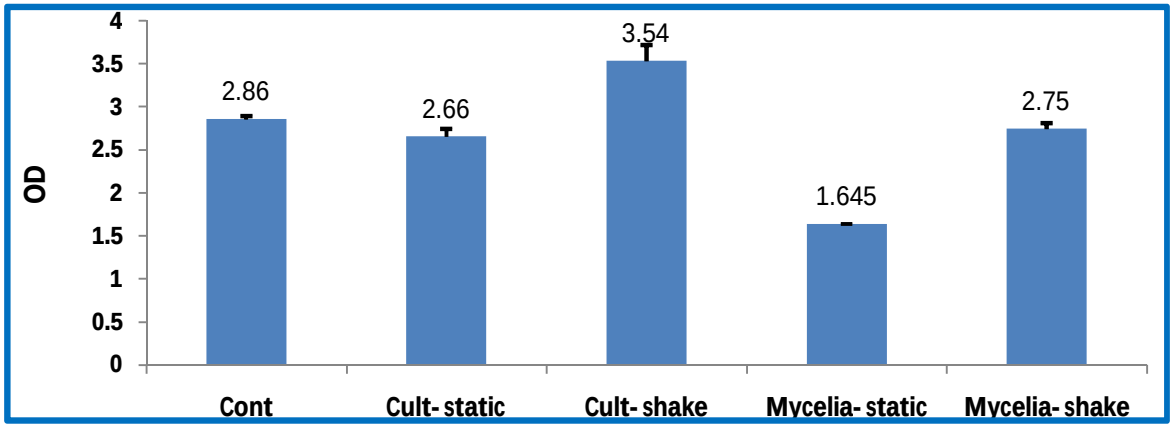


**Figure 3:** Inhibition of the **acetylcholinesterase** activity of 2ry metabolites extracts from culture and mycelia (static and after shaking). Values are expressed as mean ±SD, n = 3 (200 µg/ml for all tested extracts).

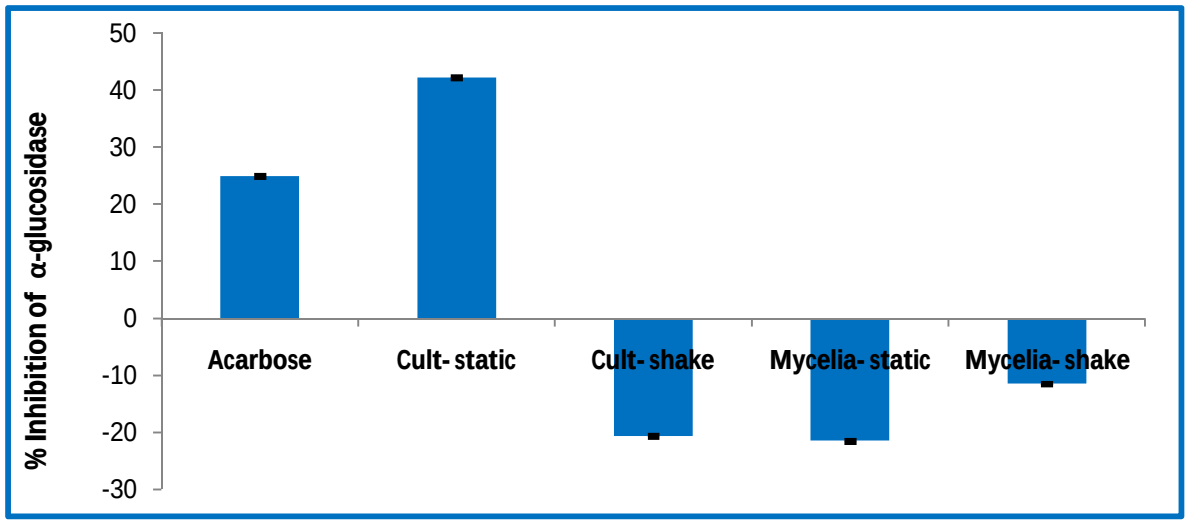


**Figure 4:** % **α-Glucosidase** inhibitory activity of 2ry metabolites extracts from culture and mycelia (static and shake). Values are expressed as mean ±SD, n = 3 (200 µg/ml for all tested extracts and acarbose).

Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. Superoxide anion is an initial free radical formed from mitochondrial electron transport systems. Endogenously, superoxide anions could be produced in large amounts by various metabolic and physiological processes.[32,33] The formation of the superoxide radical leads to a cascade formation of other reactive oxygen species in the cell, such as hydrogen peroxide, hydroxyl radical, peroxy nitrite, or singlet oxygen in living systems.[34] Superoxide radical decreases the activity of other antioxidant defense enzymes such as catalase and glutathione peroxidase.[16]

### Acetylcholinesterase inhibitory activity

The enzyme acetylcholinesterase (AChE) catalyses the hydrolysis of the ester bound of acetylcholine (ACh) to terminate the impulse transmitted action of ACh through cholinergic synapses.[35] Although the basic reason of Alzheimer's disease (AD) is not clear so far, AD is firmly associated with impairment in cholinergic transmission. A number of AChE inhibitors have been considered as candidates for the symptomatic treatment of AD as the most useful relieving strategy.[36] Reversible inhibitors of cholinesterase are currently used in clinical trials examining the treatment of Alzheimer's disease. Anticholinesterase may interact with the central cholinergic system to improve memory and cognitive deficits of the patients by diminishing the breakdown of acetylcholine at the synaptic site in the brain.[11] The effect of fungus extracts on acetylcholinesterase is shown in (Figure 3). It was observed that , the mycelial extract from the static culture of the identified fungus (*Aspergillus unguis* RSPG_204) revealed significant acetyl cholinesterase inhibitory activity (47.5%; 400µg/ml) while its supernatant extract revealed no inhibitory activity. The supernatant and mycelial extracts (culture shake) showed no inhibitory activity against acetyl cholinesterase. This data is in agreement with the data obtained from our previous studies.[16]

### Tyrosinase inhibitory activity

The supernatant extract of the static culture is the only one revealed very low (3.6%) activity against mushroom tyrosinase (data not shown) compared to vitamin C (a potent mushroom tyrosinase inhibitor) and the other extracts showed no inhibitory activity ,indicating no possible role of the isolated fungus in the prevention of skin hyper pigmentation and melanogensis .

### α-Glucosidase Inhibitory activity

The supernatant and mycelial extracts from the static and shake cultures of the identified fungus *Aspergillus unguis* RSPG_204 isolated from the Sponge *Agelas* sp. were investigated for their in-vitro α-glucosidase inhibitory activity (Figure 4). The results showed that the supernatant extract of the static culture had a higher inhibitory activity (42.3%), compared to Acarbose (25%). Acarbose was used as a reference standard for the evaluation of α-glucosidase inhibitory action. α-Glucosidase is one of a number of glucosidases located in the brush-border surface membrane of intestinal cells, and is a key enzyme of carbohydrate digestion. α-Glucosidase inhibitors block the actions of α-glucosidase enzymes in the small intestine, which limits the conversion of oligosaccharides and disaccharides to monosaccharides, necessary for gastrointestinal absorption. Postprandial glucose peaks may be attenuated by delayed glucose absorption. The main benefits attributable to α-glucosidase inhibitors are reductions in both postprandial glycemic levels and in the total range of postprandial glucose levels.[37] All other extracts( mycelial static, culture and mycelial shake) showed no activity.

## CONCLUSION

It could be concluded that, 2ry metabolites of the isolated Sponge fungus, *Aspergillus unguis* strain RSPG_204, showed significant acetyl cholinesterase and high α-glucosidase inhibition, beside concomitant high antioxidant activities. Our study provides (for the first time) primary evidence suggesting that the 2ry metabolites of fungus *Aspergillus unguis* strain RSPG_204 in further in-vivo studies could play an important role as acetylcholinesterase and α-glucosidase inhibitors, besides their antioxidant activities.

**Acknowledgements:** This work is financially supported by the bilateral projects within the Executive Programmed of Scientific and Technological Cooperation between Arab Republic of Egypt and Italian Republic for the years 2013 – 2016. Project no. [A2-12-15].

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**Source of Support:** Nil, **Conflict of Interest:** None.