



Enhancement of Acetylcholinesterase Inhibitory Activity for the Soft Coral Associated Fungus *Aspergillus unguis* SPMD-EGY by Media Composition

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ABSTRACT

The soft coral fungus, *Aspergillus unguis* SPMD-EGY, was grown on different media to select the potential medium producing significant high acetyl-cholinesterase (AChE) inhibitory activity. Extract from Rice solid medium exhibited the highest inhibitory activity of AChE followed by PYMG medium in static conditions (culture filtrate and mycelia). Potato dextrose broth (PD) showed no AChE inhibitory activity but moderate activity was found with DOX broth medium under static conditions (culture filtrate and mycelia). Comparative study has been done for PYMG medium and our previous work. The chemical composition of the potent extracts was evaluated using GC/MS analysis. Rice solid medium extract (RS, 89% AChE inhibitory activity) solely contains nitrogenous compounds, fatty acids and their esters. PYMG medium (PY, 85% activity) showed the presence of two major compounds: 1,3-bis[2-hydroxyphenyl]-2-propen-1-one (33.6%) and D-friedoolean-14-en-3-one (39.8%), mild presence for the compounds 1,6,7-trimethyl-3-phenyl-9H-xanthen-9-one (3%) and 2,4,4'-trihydroxy-chalcone (3.2%). Our previous work (PYp, 61% activity) was characterized by the significant presence of 2-Piperidinecarboxylic acid (butenyl derivative)(48%) and its isomer (36%). PD medium extract (-1% activity), only 5 compounds were identified, where hexadecanoic acid had the highest concentration (11%), besides the mild presence for the compounds: glucose oxime, D-gluconic acid, octadecanoic acid.

Keywords: Corals, *Aspergillus unguis* SPMD-EGY, Media compositions, Acetylcholinesterase (AChE) inhibitor, GC/MS analysis.

INTRODUCTION

Marine fungi represent a huge potential for new natural products and an increased number of new metabolites have become known over the past years, while much of the hidden potential still needs to be uncovered. Special focus is given to the production of groups of derivatives of metabolites by the fungi and to significant differences in biological activities due to small structural changes.¹

Fungi are important organisms in the production of bioactive secondary metabolites. Around 38% of the active compounds isolated until 2005 were of fungal origin,² and this context has not changed much in the late years.

The success of fungal metabolites can be attributed to many factors, like the advances in the industrial production of biotechnological metabolites and the possibility of working with techniques such as the "OSMAC"(one strain-many compounds).³ Many drugs currently in the market, possessing a variety of activities such as antitumor, immune-suppressants, antibiotics, hypocholesterolemic agents, antifungals, antiparasites, anti-inflammatory and enzyme inhibitors, were obtained from fungal metabolism.⁴

Acetylcholine (ACh) is the principal neurotransmitter which functions in all autonomic ganglia and is the only neurochemical that triggers motor division of the somatic nervous system. While normal cholinergic activity is

defined by the sequence of release, binding and enzymatic deactivation of ACh by acetyl-cholinesterase (AChE), abnormal cholinergic activity on the other hand is characterized by a deficit or short-fall in cholinergic transmissions at synapses and has been attributed to reduced production of acetylcholine (ACh) or its excess deactivation/hydrolysis by AChE.⁵ Commercially available synthetic acetyl-cholinesterase inhibitors (AChEIs) include donepezil, rivastigmine, galantamine, and tacrine influence the dynamics of ACh by inhibiting the activity of AChE thus increasing the availability and interaction time of ACh with cholinergic receptors of synaptic cells. Considering the drawbacks of synthetic AChEIs which include gastrointestinal disturbances, moderate effectiveness, high cost and short half-life, natural product based compounds have been increasingly explored for better effects. Desirable properties of natural product based compounds include a comparatively better penetration of the blood-brain barrier better than the pharmaceutical options and better specificity for human type AChE.⁶ The symptoms of Alzheimer's disease are connected to the reduction of brain neurotransmitters, such as acetylcholine. Therefore, the treatment is based on the attempt to restore cholinergic function, using inhibitors of acetylcholinesterase (AChE), an enzyme that acts on acetylcholine degradation in the synaptic cleft.⁷

Fungal metabolites have been shown their potential in the production of novel compounds⁸ for treatment of



Alzheimer's disease; a progressive and irreversible neurodegenerative disorder that leads to memory loss and cognitive disorders.⁹

In the course of our continuing search for biologically-active substances from fungi derived from soft corals and sponges. From our previous work, the isolated fungus tentatively identified as "*Emericella unguis* 8429"-showed moderate inhibitory activity against acetyl cholinesterase enzyme.¹⁰ This result encouraged us to extend our work by studying the effect of some different parameters that could be useful in improving the acetyl cholinesterase inhibitory activity of this fungus.

MATERIALS AND METHODS

Fungal isolation

The internal part of *Sinularia* sp. (a fresh soft coral) was cut to minute samples of about 1cm³. These samples were immersed in sterile sea water three times followed by ethyl alcohol (70%) for about 30sec for sterilization. The sterilized samples were used, as they are or after homogenization under sterile conditions and dilution up to 10⁻⁶, to inoculate a fungal isolation medium.¹¹

The inoculated plates were incubated at 30°C until the appearance of single pure colonies which were then picked and maintained on potato dextrose agar medium (PDA) and kept at 4°C and further use.¹⁰

Molecular identification

Molecular identification of fungal strain has been established by DNA extraction, PCR and sequencing as previously mentioned.¹¹

Selection of the culture media

The isolate *Aspergillus unguis* SPMD-EGY was grown on different culture media which were:

1. Potato dextrose broth (g/l): 200g of peeled potato and glucose (20).
2. Peptone yeast extract malt extract glucose medium [PYMG] (g/l): yeast extract (3), malt extract (3), peptone (5) and glucose (5);
3. Czapek'sDox broth (g/l): sucrose (30), sodium nitrate (3), dipotassium phosphate (1), potassium chloride (0.5), magnesium sulphate (0.5) and ferrous sulphate (0.1). All media were dissolved in distilled Water.
4. Rice solid medium of the following ingredients: rice (100g) and distilled water 100ml in 1l-Erlenmeyer flasks.

Extraction of secondary metabolites

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,¹⁰ while for the rice solid medium, it was directly extracted with ethyl acetate, followed by filtration and evaporation.¹²

Acetyl cholinesterase (AChE) inhibitory activity:

To investigate the AChE-inhibitory activity, the previously described method was followed^{13, 14} with slight modified spectrophotometric procedure. Electric-eel AChE (Sigma) was utilized as source of cholinesterase. Acetyl thiocholine 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 acetyl thiocholine 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 acetyl thiocholine, at a wavelength of 412 nm (15 min). All the reactions were performed in triplicate in 96-well micro-plate. Commercially available Distigmine bromide was used as a standard and compared with all extracts. The AChE-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.

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 20µl pyridine + 30 µl N₂O, bis-(trimethylsilyl) trifluoroacetamide (BSTFA) and analyzed by GC/MS.¹⁵⁻¹⁷

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.

RESULTS AND DISCUSSION



Fungal identification

The fungus was previously identified¹¹ using the molecular protocol (18SrRNA) as *Aspergillus unguis* SPMD-EGY and was deposited in the Gene Bank with the accession Number KM203833 and appears in the PubMed through the following link (site) (<http://www.ncbi.nlm.nih.gov/nuccore/KM203833>).

Effect of culture composition on Acetyl cholinesterase inhibition activity

One promising fungal strain (SPMD) was isolated and molecularly identified as *Aspergillus unguis* SPMD-EGY. From our previous work, the isolated fungus-tentatively identified as "*Emericella unguis* 8429"-showed moderate inhibitory activity against acetyl cholinesterase enzyme.¹⁰ This result encouraged us to extend our work by studying the effect of different parameters that could be useful in improving the biological activity of this fungus. Three different broth media; Potato dextrose broth (PD), PYMG and Czapek-Dox (DOX) media, in addition to one solid medium (Rice medium) were evaluated for their potentiality to improve acetyl cholinesterase inhibitory activity.

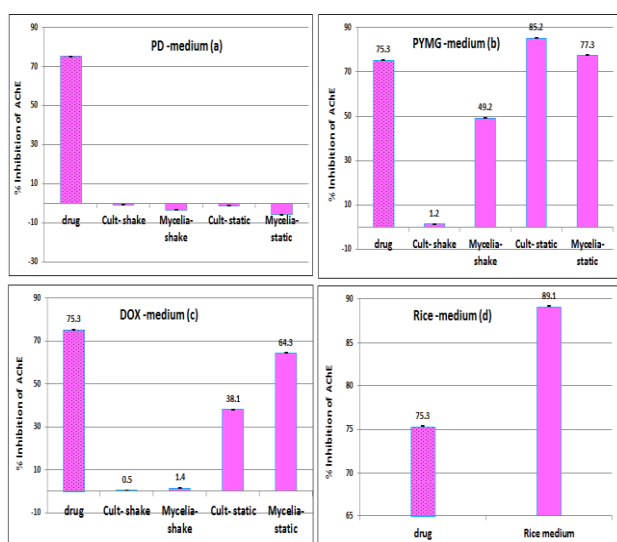


Figure 1: % Acetyl cholinesterase (AChE) inhibitory activity of 2ry metabolites extracts from culture and mycelia (static and shake) of different media. Values are expressed as mean \pm SD, n = 3 (200 μ g/ml for all tested extracts and drug; Distigmine bromide).

The Rice solid medium extract showed the highest significant acetyl cholinesterase inhibitory activity (89%, Figure 1d), followed by culture and mycelia static extracts of PYMG medium (85 and 77%, Figure 1b), if compared to the drug (Distigmine bromide, 75%). In the (DOX) medium (Figure 1c), mycelia static extract showed moderate inhibitory activity (64%), its culture static showed mild inhibitory activity (38 %), while mycelia and culture shake had no inhibitory activity. The PD medium (Figure 1a), showed no inhibitory activity.

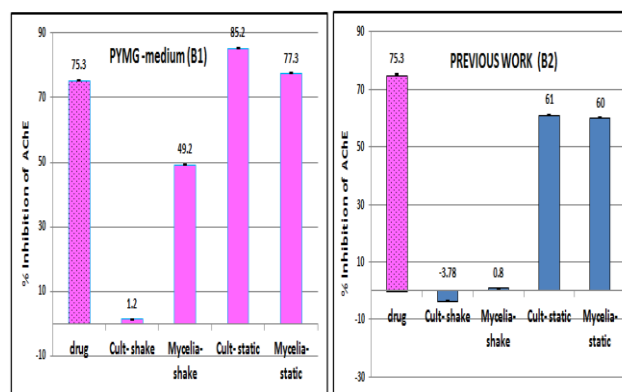


Figure 2: Demonstrative comparison between PYMG medium (5% glucose, distilled water) and our previous work medium (10% glucose, sea water)

From these findings, it was clear that the inhibitory effect of fungal extract depends on the type of culture medium used. It was clear from Figure 2 (B1 & B2), that a significant increase has been shown with culture and mycelia static [from 61-60 to 85-77 %]. The big surprise is for mycelia shake (from 0.8 to 49%), while culture shake still had no effect in both media. It was clearly observed that using distilled water instead of sea water affects the activity of the fungus. This could be attributed to the stress exerted by sea water that affects the endocellular 2ry metabolites formation and consequently the activity. Also, the reduction in glucose concentration (from 10 to 5 %) could be another reason for the significant highly increase in the acetyl cholinesterase inhibitory activity from (61 to 85%). From this suggestion; it is obviously clear that both endo cellular and exo cellular 2ry metabolites in both media have been affected.

Saleem et al. mentioned that "OSMAC is based on the premise that a single fungal species, upon submission to different cultivation conditions, can produce a great diversity of new bioactive molecules. Among the parameters that can be varied using OSMAC strategy, can be pointed the composition of culture medium, aeration, period of cultivation, pH, temperature and addition of agents to induce or inhibit the production of metabolites.¹⁸ Some stressing factors such as high osmotic levels and water restraint have also been used in order to promote metabolic diversification in fungi.^{19,20}

Singh et al.²¹ studied the production of acetyl cholinesterase inhibitors from *Alternaria* sp. grown on different media including malt yeast extract broth (MYEB), Szapek-Dox medium, Sabouroud dextrose broth, potato dextrose broth (PDB), lactose based medium broth (LMB), dextrose peptone broth and glucose broth. They found that the highest acetyl cholinesterase inhibitory activity was found with PDB medium followed by MYEB and LPM. *Microshaeropsis olivacea*, an endophytic fungus isolated from the phloem of the tree *Pilgerodendron uriferum* D.Don, was cultivated on solid rice medium and the isolated compounds (Graphilactone A and Botrallin) exhibited moderate inhibitory activity towards acetyl

cholinesterase with IC_{50} of 8.1 and 6.1 $\mu\text{g/ml}$, respectively.²² Three isolated compound from the marine fungus *Talaromyces* sp. namely; Talaromycesone, Talaxanthenone and AS-186c; exhibited acetylcholine esterase inhibitory activity with IC_{50} of 7.49, 1.61 and 2.60 μM respectively, when grown on modified Wickerham medium (similar to PYMG).²³

From the above mentioned, it is obvious that fungal strain also plays an important role; *Alternaria* sp. grown on potato dextrose broth (PDB) showed the highest acetylcholinesterase inhibitory activity, while for our fungus [*Aspergillus unguis* SPMD-EGY], this medium showed no inhibitory activity. Also, the fungus *Microshaeropsis olivacea* which was cultivated on solid rice medium exhibited moderate inhibitory activity towards acetylcholinesterase, although our fungus [*Aspergillus unguis* SPMD-EGY] showed the highest significant acetylcholinesterase inhibitory activity (89%).

GC/MS analysis

GC/MS analysis of the Rice solid medium extract with the highest Acetyl cholinesterase inhibitory activity (89%) was mentioned before.¹¹

A comparative GC/MS analysis for the culture static extracts of PYMG medium (PY, 85% AChE inhibitory activity), our previous work (PYp, 61%) and PD medium extract (-1%) of the identified fungus (*Aspergillus unguis* SPMD-EGY) isolated from the soft coral *Sinularia* sp. revealed the identification of 30 compounds; 13 compounds in (PY), 14 compounds in (PYp) and 5 compounds in (PD) culture static extracts.

The culture static extract of PYMG medium (PY, 85% activity) showed the presence of two major compounds in

very high significant concentration: 1,3-bis[2-hydroxyphenyl]-2-propen-1-one (33.6%) and D-friedoolean-14-en-3-one (39.8%), mild presence for the compounds 1,6,7-trimethyl-3-phenyl-9H-xanthen-9-one (3%) and 2,4,4'-trihydroxy-chalcone (3.2%). 3-(2-Cyano-phenyl-thio)9-chlorobenzo[4.5]thieno[2,3b]quinoxaline (0.6), indolizine]-8-propenoic acid-4'-oxide derivative (1.2), Diisooctyl-phthalate (1.3). Minor presence for the compounds: 7-Methyldinaphtho[2,1b;1',2'd]silole; 6,7-Dimethoxy-1(methyl-thio)3-phenyl-naphthalene; 1,1,4,4-tetramethyl-2,3-diphenyl-1,4-disilacyclohex-2-ene; 1,2,3,4-tetrahydro-6-methoxy-1,1,4,4-tetramethyl-5-(phenylethenyl)-anthracene; benzyl-3-(2-chloroethyl)-5-iodo-4-methylpyrrole-2-carboxylate. All the above mentioned compounds are solely present in (PY) extract.

The two major compound; 1,3-bis[2-hydroxyphenyl]-2-propen-1-one (33.6%) and D-friedoolean-14-en-3-one (39.8%), were present in very minor concentration (1.3 and 0.11, respectively) in (PYp) extract (Table 1, Figure 3).

The culture static extract of our previous work (PYp, 61% activity) was characterized by the significant presence of 2-Piperidinecarboxylic acid (butenyl derivative) (48%) and its isomer (36%), besides minor presence for the compounds: 3-(3,4-Dichloro-2-methoxyphenyl)-6-methoxyindan-1-one; 4-[1,2-Dimethyl-6-(2-hydroxy-ethoxy-methoxy)cyclohexyl]but-3-en-2-on; 9-(2,6-Bis(1-methylethenyl)phenyl)fluorene; m-Coumaric acid; 3,3',5,5'-Tetra-tert-butyl-2,2'-dihydroxybiphenyl; 6,6'-Bis[2-(hydroxy)-1-ethynyl]-2,2'-bipyridine; p-Coumaric acid; 6-Benzoyl-5-methoxy-10-phenyl-pyrano-coumarin; N-(p-Chlorophenyl)-[1-(t-butyl-sulfinyl)-2-naphthyl]methanimine (Table 1, Figure 3).

Table 1: Comparative Chemical composition assessed by GC/MS analysis of the static culture Supernatant extracts of the fungus (*Aspergillus unguis* SPMD-EGY) with different media

S.No	Compound	RT	PD (-1%)	PY (85%)	PYp* (61%)
% TIC ^a					
1	morpholine	7.62	2.7	-----	-----
2	3a,7a-Dihydro-3a,4,6-trimethyl-3(trimethylsilyl)1H-pyrazolo[4,3d]pyrimidin-5,7-dione	27.09	-----	0.01	-----
3	7-Methyldinaphtho[2,1b;1',2'd]silole	35.17	-----	0.01	-----
4	3-(3,4-Dichloro-2-methoxyphenyl)-6-methoxyindan-1-one	37.26	-----	-----	0.1
5	4-[1,2-Dimethyl-6-(2-hydroxy-ethoxy-methoxy)cyclohexyl]but-3-en-2-on	38.47	-----	-----	0.3
6	9-(2,6-Bis(1-methylethenyl)phenyl)fluorene	38.69	-----	-----	0.34
7	m-Coumaric acid	39.0	-----	-----	0.31
8	6,7-Dimethoxy-1(methyl-thio)3-phenyl-naphthalene	39.46	-----	0.01	-----
9	1,1,4,4-Tetramethyl-2,3-diphenyl-1,4-disilacyclohex-2-ene	40.16	-----	0.06	-----
10	1,2,3,4-Tetrahydro-6-methoxy-1,1,4,4-tetramethyl-5-(phenylethenyl)-anthracene	41.58	-----	0.03	-----
11	3,3',5,5'-Tetra-tert-butyl-2,2'-dihydroxybiphenyl	42.75	-----	-----	0.13
12	6,6'-Bis[2-(hydroxy)-1-ethynyl]-2,2'-bipyridine	42.85	-----	-----	0.12
13	1,3-Bis[2-hydroxyphenyl]-2-propen-1-one [2,2'-dihydroxy-chalcone]	43.12	-----	33.6	1.32



14	p-Coumaric acid	44.01	----	----	0.82
15	Glucose oxime,	44.49	0.54	----	----
16	D-Gluconic acid,	46.77	2.45	----	----
17	Hexadecanoic acid	47.04	11.64	----	1.04
18	3(2-Cyanophenylthio)9-chlorobenzo[4.5]thieno[2,3b]quinoxaline	47.43	----	0.64	----
19	benzyl-3(2-chloroethyl)5-iodo-4-methylpyrrole-2-carboxylate	47.56	----	0.03	----
20	D-Friedoolean-14-en-3-one	48.31	----	39.8	0.11
21	6-Benzoyl-5-methoxy-10-phenyl-pyranocoumarin	48.38	----	----	0.4
22	2-Piperidine-carboxylic acid (butenyl derivative)	48.6	----	----	48.7
23	2-Piperidinecarboxylic acid (butenyl derivative) [isomer]	48.7	----	----	36.2
24	Methyl ester of [1'R(1'á,4'á,8'á,8a'á)]8-'ethyl-1,2,2',3',6',7',8',8a-'octahydro-1-methyl-2-oxospiro[3H-indole-3,1-(5H)indolizine]8-propenoic acid -4-'oxide	50.31	----	1.23	----
25	1,6,7-Trimethyl-3-phenyl-9H-xanthen-9-one	50.31	----	3.1	----
26	Octadecanoic acid,	51.41	0.7	----	----
27	9(Methoxycarbonyl)-7-(2-bromo-4-methylphenyl) 8-oxo-1,2,7-triazabicyclo[3.4.0]2-nonene	53.54	0.37	----	----
28	2,4,4'-Trihydroxy-chalcone	54.02	----	3.2	----
29	N-(p-Chlorophenyl)-[1-(t-butylsulfinyl)-2-naphthyl]methanimine	59.49	----	----	0.5
30	diisooctylphthalate	63.60	----	1.3	----

RT=retention time. a , TIC = The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation.* Data previously mentioned in reference: Faten K. Abd El-Hady, Mohamed S. Abdel-Aziz, Amr M. Abdou, Kamel H. Shaker, Laila S. Ibrahim, Zeinab A. El-Shahid (2014) "In vitro Anti-diabetic and Cytotoxic Effect of the Coral Derived Fungus (*Emericella unguis* 8429) on Human Colon, Liver, Breast and Cervical carcinoma cell lines" Int. J. Pharm. Sci. Rev. Res., 27(2), 297-301.

In culture static extract of PD medium extract (-1% activity) only 5 compounds were identified, where hexadecanoic acid had the highest concentration (11.6%), besides the presence of mild concentration for the compounds: glucose oxime; D-gluconic acid; octadecanoic acid; 9-(methoxy carbonyl)-7-(2-bromo-4-methylphenyl) 8-oxo-1, 2, 7-triazabicyclo [3.4.0]2-nonene. For PD medium extract; all the above mentioned compounds are solely present in it, with the exception for hexadecanoic acid, which was found in (PYp) in a minor concentration (1%) (Table 1, Figure 3). Compounds only present in Rice solid medium extract (RS, 89% activity) were mentioned before,¹¹ and the major compounds present are 2,6-Ditbutyl-4-hydroxy-4-phenyl-1-imino-2,5-cyclohexadiene (5%); 1-[4-hydroxyphenyl]-3-[2,4-di-hydroxy-phenyl]-2-propen-1-one (2,4,4'-trihydroxy-chalcone); octadecanoic acid-2,3-dihydroxy-propylester; docosanoic acid-1,2,3-propanetriylester; 1,4-benzenediol,2,6-bis(1,1dimethyl ethyl) and its isomer. Tridecanol (1.4%), 5-keto-2,2-dimethyl-heptanoic acid-propyl ester (1.4%), 4(4-methoxyphenyl)-4-(1H-pyrrolo[2,3b]pyridin-3-yl)butan-2-one (4.5%), 9,12-octadecadienoic acid (6.3%) and 9-octadecenoic acid (7.3%).

The solid Rice medium is solely characterized by the presence of saturated, unsaturated fatty acids and their esters (Table 2, Figure 3), which they have a big share in its acetyl cholinesterase inhibitory activity. The culture

static extract of PYMG medium (PY), showed very high and mild concentrations for the two compounds 1,3-bis[2-hydroxyphenyl]-2-propen-1-one[2,2'-dihydroxy-chalcone] (33.6%) and 2,4,4'-trihydroxy-chalcone (3.2%), while in Rice solid medium they present in minor concentrations (1 and 0.6 %, respectively) (Table 2, Figure 3).

A series of 2'-hydroxy- and 2'-hydroxy-4', 6'-dimethoxychalcones were evaluated as inhibitors of human acetyl cholinesterase (AChE). The majority of the compounds were found to show some activity, the most active compounds having IC₅₀ values of 40-85 µM. Kinetic studies on the most active compounds showed that they act as mixed-type inhibitors, this was in agreement with the results of molecular modeling studies, which suggested that they interact with residues in the peripheral anionic site and the gorge region of AChE.²⁴

Hydroxyl (OH) group present in ring A of chalcone has vital role in the inhibition of acetyl cholinesterase activity. Compounds having hydroxyl group at 2 position such as (iso liquiritigenin, 2', 4', 4'-trihydroxy chalcone), (2', 4', 3, 4-tetrahydroxychalcone), (2', 2-dihydroxychalcone) showed inhibitory activity against AChE.

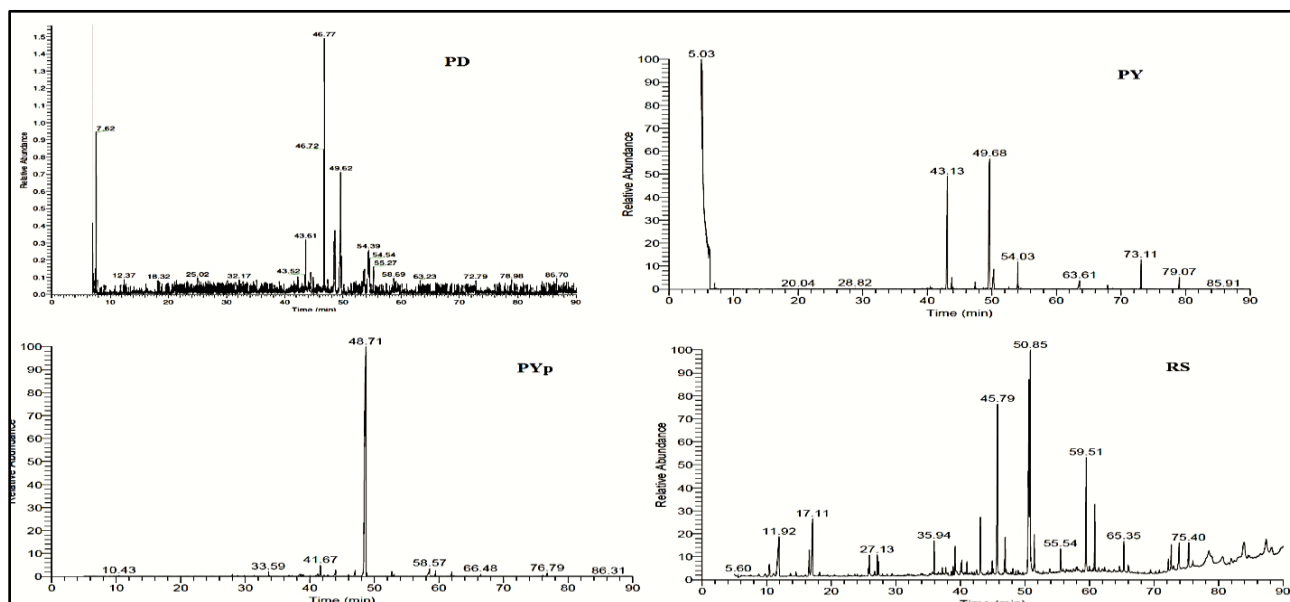


Figure 3: Comparative chromatographic study (GC/MS analysis) of the supernatant extracts of the static cultures with different media and the Rice Solid medium extract of the fungus (*Aspergillus unguis* SPMD-EGY)

Table 2: Demonstrative Table to show the variability in Chemical composition assessed by GC/MS of PYMG culture static (PY) and Rice solid medium (RS) extracts or *Aspergillus unguis* SPMD-EGY

Compounds	RT	PY (85%)	RS % ^{a*} (89%)
Saturated fatty acids			
Butanedioic acid	17.19	-----	0.5
Azelaic acid	37.76	-----	0.4
Pentadecanoic acid	42.49	-----	0.2
Hexadecanoic acid	45.78	-----	10.1
Octadecanoic acid	51.41	-----	1.6
Unsaturated fatty acids			
<i>cis</i> -9-Hexadecenoic acid	44.90	-----	0.6
9,12-Octadecadienoic acid	50.52	-----	6.3
9-Octadecenoic acid	50.89	-----	7.3
11-Octadecenoic acid	50.95	-----	0.2
Fatty acid esters			
5-Keto-2,2-dimethyl-heptanoic acid, propyl ester	19.14	-----	1.4
Hexadecanoic acid-2,3-dihydroxy propyl ester	60.65	-----	2.4
Octadecanoic acid- 2,3-dihydroxy- propyl ester	65.35	-----	0.9
Docosanoic acid,1,2,3-propanetriyl ester	87.39	-----	0.5
Nitrogenous & Thio compounds			
2,6-Di-t-butyl-4-hydroxy-4-phenyl-1-imino-2,5-cyclohexadiene	11.92	-----	5.0
4-(4-Methoxyphenyl)-4-(1-Hpyrrolo[2,3b]pyridine-3-yl)-butan-2-one(isomer)	14.53	-----	0.3
4(4-Methoxyphenyl)-4-(1H-pyrrolo[2,3b]pyridin-3-yl)butan-2-one	19.30	-----	4.5
3a,7a-Dihydro-3a,4,6-trimethyl-3(trimethylsilyl)1H-pyrazolo[4,3d]pyrimidin-5,7-dione	27.09	0.01	-----
7-Methylindinaphtho[2,1b;1',2'd]silole	35.17	0.01	-----
6,7-Dimethoxy1(methyl-thio)3-phenylnaphthalene	39.46	0.01	-----
4,6-Dimethoxy-7(5-methyl-1-pyrrolin-2-yl)2,3-diphenylindole	41.45	-----	0.25

2,2-Dichloro-1,1-bis(4-diethylaminophenyl)ethylene	44.21	-----	0.3
1,1,3-Trifluoro-3,3-bis[ethylamino]1-phenyldisiloxane	46.92	-----	3.3
3(2-Cyanophenylthio)9-chlorobenzo[4.5]thieno[2,3b]quinoxaline	47.43	0.64	-----
benzyl-3(2-chloroethyl)5-iodo-4-methylpyrrole-2-carboxylate	47.56	0.03	-----
others			
Glycerol	10.43	-----	1.37
Tridecanol	19.12	-----	1.4
L-Threitol	27.6	-----	1.7
6(4t-Butylphenyl)1,3,5-hexatriynyl)trimethylsilane	27.89	-----	0.7
1,4-Benzenediol,2,6-bis(1,1dimethylethyl)	35.22	-----	0.2
Arabitol	35.99	-----	2.8
1,4-Benzenediol,2,6-bis(1,1dimethylethyl) (isomer)	36.92	-----	0.17
1,1,4,4-Tetramethyl-2,3-diphenyl-1,4-disilacyclohex-2ene	37.23	0.06	0.6
1,3-Bis[2-hydroxyphenyl]-2-propen-1-one	40.23	33.6	1.0
3-Deoxyglucitol	41.03	-----	0.9
1,2,3,4-Tetrahydro-6-methoxy-1,1,4,4-tetramethyl-5-(phenylethenyl)-anthracene	41.58	0.03	-----
Sorbitol	43.07	-----	4.5
D-Friedoolean-14-en-3-one	48.31	39.8	-----
1,6,7-Trimethyl-3-phenyl-9H-xanthen-9-one	50.31	3.1	-----
Dotriacontane	52.84	-----	0.2
2,4,4'-Trihydroxy-chalcone	54.02	3.2	0.6
Diisooctyl phthalate	59.51	1.3	11.9

RT=retention time. a; TIC = The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation.* Data previously mentioned in reference: Faten K. Abd El-Hady, Mohamed S. Abdel-Aziz, Kamel H. Shaker, Zeinab A El-Shahid." Effect of Media Composition on Potentiality Improving α -Glucosidase Inhibitory Activity for the Soft Coral Associated Fungus *Aspergillus unguis* SPMD-EGY." Int. J. Pharm. Sci. Rev. Res., 38(2), 2016, 173-180.

Thus, it may be concluded that a hydroxyl group ortho to the side chain is very much responsible for acetyl cholinesterase inhibitory activity. ISL (iso liquiritigenin, 2', 4', 4- trihydroxy chalcone), and BUT (2', 4', 3, 4-tetrahydroxychalcone) having three and four hydroxy groups increased the spontaneous alternations and decreased the transfer latency respectively, indicating improvement in memory. Thus, for the ameliorating effects of chalcones on memory deficits, presence of more than two hydroxyl groups on both the rings is necessary. The above behavioral and biochemical results suggest that chalcones (ISL and BUT) have the ability to improve or ameliorate memory dysfunction, in part, by facilitating the cholinergic transmission in brain.²⁵

It was mentioned before that, D-Friedoolean-14-en-3-one (taraxerone) showed acetyl cholinesterase inhibitory activities (% Inhibition= 88.0 \pm 1.8, and LC₅₀= 42.0 \pm 1.4).²⁶

1,3-dihydroxyxanthone derivatives were evaluated for anti-cholinesterase activity on both the enzymes, most of the compounds exhibited moderate to good

inhibitory activities with the IC₅₀ values at micromolar level concentrations against both acetyl cholinesterase (AChE) and butyryl cholinesterase (BuChE).²⁷ Öztürk reported that, the best (AChE) inhibitory activity was found for 9,12-octadecadienoic acid and 9-octadecenoic acid as 0.267 \pm 0.05 mg/mL and 0.127 \pm 0.03 mg/mL, respectively while, hexadecanoic and octadecanoic acids are more than 4 mg/mL.²⁸ Bis(7-acetoxy-2-ethyl-5-methylheptyl) phthalate (**3**) and diisooctyl phthalate (**2**), were tested against acetyl cholinesterase, the IC₅₀ values of compounds **3** against AChE was 1.65, while compounds **2** showed weak inhibition profile.²⁹ The inhibitory activity of benzene-1,4-diol on AChE activity was evaluated, it effectively inhibited AChE with Ki of 1.22 nM.³⁰

CONCLUSION

The production of acetylcholinesterase inhibitory secondary metabolite from *Aspergillus unguis* SPMD-EGY (formerly *Emercilla unguis*) was previously studied using Wecherham medium to produce 61% inhibitory activity. In the present study different media (three liquid and one solid) were studied for their ability to



enhance the potentiality of the inhibitory activity against acetyl cholinesterase enzyme. Both Rice and PYMG media showed the highest inhibitory effect (89 and 85%) compared to the drug (Distigmine bromide, 75%). In further in-vivo studies, this significant high percent activity could play an important role as acetyl cholinesterase inhibitor. Especially referring to the distinguished variability in the chemical composition of the two highly active extracts, where Rice solid medium extract solely contains nitrogenous compounds, fatty acids and their esters. PYMG medium had two major compounds: 1,3-bis[2-hydroxyphenyl]-2-propen-1-one (33.6%) and D-friedoolean-14-en-3-one (39.8%), mild presence for the compounds 1,6,7-trimethyl-3-phenyl-9H-xanthen-9-one (3%) and 2,4,4'-trihydroxy-chalcone (3.2%).

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