Research Article

Effect of Media Composition on Potentiality Improving α-Glucosidase Inhibitory Activity for the Soft Coral Associated Fungus *Aspergillus unguis* SPMD-EGY

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ABSTRACT

Marine ecosystems were considered as promising source for the isolation of skeletally and biologically unique compounds. From this point of view, we tried to isolate fungal strains associated with soft coral. In this respect, one promising fungal strain (SPMD) was isolated and molecularly identified as *Aspergillus unguis* SPMD-EGY. Fungi, under various culture conditions, were able to produce variable bioactive secondary metabolites. Three different broth media; Czapek-Dox medium, Potato dextrose medium and PYMG medium in addition to one solid medium (rice medium) were used for the cultivation of the fungus. The fungal extracts from the broth media (culture filtrate and mycelium under static and shake conditions) were tested for their potentiality to improve α-glucosidase inhibitory activity. Fungal extract from solid medium(RS, rice medium), exhibited the highest inhibitory activity against α-glucosidase enzyme activity (86%) followed by the fungal extract from PYMG medium, mycelia static extract(PY), with a percent of 81% compared to the drug (Acarbose, 43%). Fungal extract from mycelia shake culture of (PD) broth medium showed the lowest inhibitory effect of all tested media (49%). GC/MS analysis was performed on the highly active extracts. 35 compounds from PYMG medium and 33 from rice medium were identified. The saturated fatty acids represented 24% in (PY) extract, while in (RS), it was 14.4% only; hexadecanoic acid had the highest concentration in both extracts (18 and 10 %, respectively). The unsaturated fatty acids in both extracts represented (8 & 14 % respectively). Unsaturated fatty acids showed higher α-glucosidase inhibitory activity than the saturated fatty acids.

Keywords: Corals, *Aspergillus unguis* SPMD-EGY, Media compositions, α-Glucosidase inhibitor, GC/MS analysis.

INTRODUCTION

The incidence and prevalence of type-II diabetes, representing over 90% of all case of diabetes, are increasing rapidly as time passes. The International Diabetes Federation predicted that the number of people with diabetes would rise from 266 million in 2011 to 552 million by 2030.[1]

It is a clinical syndrome caused by genetic and environmental factors and characterized by high levels of blood glucose. Type-II diabetes mellitus (DM2) has been becoming a global health problem.[1] DM2 is a chronic disease with clinical manifestation of hyperglycemia, due to the insufficient release and/or inactive form of insulin that controls the level of blood glucose.[2]

Various pharmacological approaches have been used to treat diabetes; one of the most beneficial therapies is to reduce the postprandial hyperglycemia. The absorption of glucose can be retarded by inhibiting the carbohydrate hydrolyzing enzymes; α-Amylase and α-glucosidase.[3] α-Glucosidase catalyzes the final step in the digestive process of carbohydrates and its inhibition can retard the uptake of dietary carbohydrates and suppress postprandial hyperglycemia and could be useful for treating diabetic patients. Enzyme inhibitors are now receiving increased attention, not only for studying the enzyme structure and reaction mechanism but also for pharmacological applications.[4]

The development of new drugs for use against α-glucosidase is therefore urgently needed.

Marine-derived fungi are treasure chest of secondary metabolites, of which a considerable number have promising biological or pharmaceutical properties. The hunt for discovering novel bioactive secondary metabolites from marine fungi is therefore gaining increased attention.[5]

Marine fungi have attracted more attention for their good bioactivities against α-glucosidase.[6] Massive efforts have been made to investigate marine-derived compounds. Unique and stressful marine habitats have intense effects on fungal biological activity.[7]

Recent studies revealed that the biosynthetic pathways of fungi can be activated and the chemical diversity of their metabolites can be maximized by alternating their cultivation parameters systematically, such as the components of the media, co-culture, feeding precursors and the addition of enzyme inhibitors.[8]

Marine ecosystems were considered as promising sources for the isolation of different biological compounds with varying degree of effect including antitumor, anticancer and antibiotic. Until now, not all the marine environments were explored. So, they are considered as potent sources for the isolation of new microbes (bacteria, fungi, actinomycetes, cyanobacteria and

diatoms) with potent bioactive secondary metabolites.[9] Marine fungi were considered as a fruitful and unique source for structurally and biologically active secondary metabolites.[10,11] Marine-derived fungi, under various culture conditions, were able to produce variable bioactive secondary metabolites.[12] Because of the highly chemical and harsh physical conditions in the marine environment, the organisms produce a variety of molecules with unique structural features and exhibit various biological activities. The majority of the marine natural products have been isolated from sponges, coelenterates (sea whips, sea fans, and soft corals).[13]

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 α-glucosidase enzyme.[14] 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. Fungi, under various culture conditions, are able to produce variable bioactive secondary metabolites.

MATERIALS AND METHODS

Fungal isolation

The interior portion of a fresh soft coral; S*inularia* sp. was cut into small cubes of approximately 1 cm^3. These cubes were sterilized by immersing them in sterile sea water three times followed by ethyl alcohol (70%) for about 30sec. The sterilized cubes were used, as they are or after sterilized homogenization and dilution up to 10^{-6}, to inoculate a fungal isolation medium of the following ingredients (g/l): yeast extract (1), glucose (1), $NHNO_3$ (1), peptone (0.25), agar (18-20) and sea water (1000ml). The pH of the medium was subjected to 6.±0.2 and two antibacterial agents were also supplemented (g/l): Penicillin G (0.1) and Streptomycin sulphate (0.1). 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.[14]

Molecular identification

Molecular identification of fungal strain has been established by DNA extraction, PCR and sequencing. Identification of the isolated fungus was employed using the nuclear ribosomal internal transcribed spacer (ITS) region 1, 2, along with the short structural gene (5.8S) as they have been investigated in the supporting information of previous studies.[14,15] The ITS region was selected to identify fungi because it has been recently recognized as a common marker for fungal identification.[16]

Selection of the culture media

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

A. Potato dextrose broth (g/l): 200g of peeled potato and glucose (20).

B. Peptone yeast extract malt extract glucose medium [PYMG]: (g/l)yeast extract (3), malt extract (3), peptone (5) and glucose (5);

C. 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.

D. 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.[14]

While for the rice solid medium, it was directly extracted with ethyl acetate, followed by filtration and evaporation.[17]

α-Glucosidase inhibition assay

α-glucosidase Inhibitors which act as competitive inhibitors of intestinal α-glucosidase can delay the digestion and subsequent absorption of elevated blood glucose levels.

The α-glucosidase inhibitory activity was assessed by the standard method,[18-21] with slight modifications. Briefly, a volume of 60 µl of sample solution and 50 µl of 0.1 M phosphate buffer (pH 6.8) containing α-glucosidase solution (0.2 U/ml) was incubated in 96 well plates at 37 °C for 20 min. After pre-incubation, 50 µl of 5 mM p-nitrophenyl-α-D-glucopyranoside (PNPG) solution in 0.1 M phosphate buffer (pH 6.8) was added to each well and incubated at 37 °C for another 20 min. Then the reaction was stopped by adding 160 µl of 0.2 M NaCO3 into each well, and absorbance readings (A) were recorded at 405 nm by micro-plate reader and compared to a control which had 60 µl of buffer solution in place of the extract. For blank incubation (to allow for absorbance produced by the extract), enzyme solution was replaced by buffer solution and absorbance recorded. Commercially available 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.

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/cm^2), injector temperature, 310°C; 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 analyses

1mg of the dried extract was prepared for chromatography by derivatization for 30 min at 85^{o}C with 20µl pyridine + 30 µl N,O, bis-(trimethylsilyl) trifluoroacetamide (BSTFA) and analyzed by GC/MS.[22-24]

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

By applying the produced sequence from DNA of fungal isolate SPMD into BLAST search, it has been found that it has a similarity of about 99% with the previously identified fungus *Aspergillus unguis*.

The phylogenic tree of the fungal isolate was also constructed (Figure 1). Based on the above identification techniques, our local marine fungal isolate was identified as *Aspergillus unguis* SPMD-EGY with the GeneBank accession number KM203833 (http://www.ncbi.nlm.nih.gov/nuccore/KM203833).

The fungal strain (SPMD) culture was deposited in the Microbial Chemistry Department Collection of Microorganisms. In our previous work, this fungus was tentatively identified as *Emericella unguis* 8429,[14] but now it is completely identified and registered in the Gen Bank with the name "*Aspergillus unguis* SPMD-EGY" with the accession number KM203833 (http://www.ncbi.nlm.nih.gov/nuccore/KM203833).
Conventional fungal identification protocols have been commonly applied and several new species till now are identified according to this method,[25] which was considered as time consuming and not accurate.

Targeting specific regions within the ribosomal RNA gene clusters using global primers through PCR amplification is an alternative strategy for the fungal identification to the species level and also for analyzing fungal diversity.[26]

Effect of culture composition on the production of bioactive metabolites with α-Glucosidase inhibition activity

One promising fungal strain (SPMD) was isolated and molecularly identified as *Aspergillus unguis* SPMD-EGY. Three different broth media; Potato dextrose broth (PD), PYMG and Czapek-Dox (DOX) media, in addition to one solid medium (Rice medium) were tested for their potentiality to improve α-glucosidase inhibitory activity. Rice medium showed the highest significant result. The PD medium (Figure 2a), showed that only mycelia shake extract had moderate α-glucosidase inhibitory activity (49%) compared to the drug (acarbose, 43%). While, the other extracts (culture shake, mycelia static and culture static) had no inhibitory activity. On the other hand, the mycelia static extract of PYMG medium (Figure 2b) showed the highest inhibitory activity (81%) compared to the drug, while mycelia shake and culture static showed moderate inhibitory activity (57 & 59 %) respectively. The culture shake extract showed no inhibitory activity at all. In the (DOX) medium (Figure 2c), mycelia static extract showed moderate inhibitory activity (58%) compared to the drug, while, its mycelial shake and culture static showed mild inhibitory activity (46 & 45 %) respectively. The Rice solid medium extract showed the highest significant α-glucosidase inhibitory activity (86%) compared to the drug. From these findings, it has been found that the inhibitory effect of microbial extract depends on the type of culture medium used. Kang reported that the optimal inhibitory effect of α-glucosidase enzyme of compounds produced from *Aspergillus oryzae* N159-1 was obtained in tryptic soy broth medium and its α-glucosidase inhibitory activity was 65.9%.[27] Intracellular α-glucosidase inhibitory activity was also investigated using other different culture media including yeast extract-malt extract, yeast-peptone-dextrose, and potato-dextrose medium with an inhibitory effect of 52.1%, 49.1%, and 48.1%, respectively. Media with various composition resulted in different effect on growth, fatty acid composition and α-glucosidase inhibitory activity from the fungus *Colletotrichum* sp. ISCI3.[28] It has been found that xylose as carbon source in the medium of *Colletotrichum* sp. had highest growth while fructose gave highest fatty acid content. Therefore, every fungus may have an exceptional preference toward specific sugar type and the effect of different sugar source on growth does not have a correlation with their fatty acid production in the culture.[29] Yeast extract as a nitrogen source exhibited highest effect on fatty acid production. Yeast extract when used as nitrogen source in the culture medium of fungi is more common than beef extract and soy tone. Highest fatty acid content in the

media having yeast extract was found in the culture of *M. ramanniana* and *M. alliaceae* YN-15.[30,31]

From our previous work, the isolated fungus-tentatively identified as "*Emericella unguis* 8429"-showed moderate inhibitory activity against α-glucosidase enzyme.[14] 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. From Figure 3 (B1 & B2), it was obviously found that no significant difference has been shown with culture shake, mycelia shake and culture static in both media. The only difference was detected in case of mycelia static with a difference of about 30%.

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 α-glucosidase inhibitory activity from (51 to 81%). From this suggestion; it is obviously clear that exocellular 2ry metabolites in both media have not been affected.

GC/MS analysis

GC/MS analysis of the mycelia static extract of PYMG medium (PY) and Rice solid (RS) medium extract of the identified fungus (*Aspergillus unguis* SPMD-EGY) isolated from the soft coral *Sinularia* sp. revealed the identification of 43 compounds; 35 in (PY) and 33 in (RS) extracts.

Compounds were only present in (RS) extract

2,6-Ditbutyl-4-hydroxy-4-phenyl-1-imino-2,5-cyclohexadiene(5%),1-[4-hydroxyphenyl]-3-[2,4-di-hydrox-yphenyl]-2-propen-1-one, Octadecanoic acid- 2,3-dihydroxy-propyl ester, Docosanoic acid-1,2,3-propanetriyl ester, 1,4-Benzenediol,2,6-bis(1,1dimethylethyl) and its isomer.

Compounds present with higher concentration in (RS) than in (PY) extract

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%).

Compounds present only in (Py) extract

8-Methoxy-2,3-dihydro-1h-benzazepin-2-one-5-acetic acid(6%), N-Acetyl-N-methyl alanine (3%), Methyl-7-(2,4,6-trimethylphenyl)-5H-furo[2,3c] thiopyran-4-carboxylate, 2,6-Di-t-butyl-4-hydroxy-4-phenyl-1-imino-2,5-cyclohexadiene, 1,1,4,4-Tetramethyl2,3diphenyl-1,4-di-silacyclohex-2-ene (isomer), Tetradecanoic acid, 7,8-Dihydroxy-benzo(5,6g)1H,3H-quinazoline-2,4-dione, Methyl-2{2[3(methoxycarbonyl)phenyl]1-ethynyl}5-hydroxyethynyl)benzoate, Heptadecenoic acid, Heptadecanoic acid and 9,12-Octadecadienoic acid-2-hydroxy-ethyl ester.

Compounds with higher concentration in (PY) extract

Glycerol, Butanedioic acid, 6(4t-Butylphenyl)-1,3,5-hexatriynyl)trimethylsilane, 1,1,4,4-Tetramethyl-2,3-diphenyl-1,4-disilacyclohex-2ene, Hexadecanoic acid (18%), Octadecanoic acid, Diisooctyl phthalate (14%).

The saturated fatty acids represented 24% in (PY) extract, while in (RS), it was 14.4% only; hexadecanoic acid had the highest concentration in both extracts (18 and 10 %, respectively). The **unsaturated fatty acids** in both extracts represented (8.1 & 14.4 % respectively). It was reported that, unsaturated fatty acids were higher α-glucosidase inhibitory activity than the saturated fatty acids and the methyl esters form of unsaturated fatty acids showed slightly less active than the free acids.[32]

In previous studies, oleic acid showed the strongest anti-α-glucosidase activity, followed by linoleic acid, and their activities were more potent than acarbose.[33]

Di-butyl phthalate was found to inhibit alpha-glucosidase. Di-butyl phthalate was shown to be a reversible, slow-binding, non-competitive inhibitor of yeast alpha-glucosidase.[34]

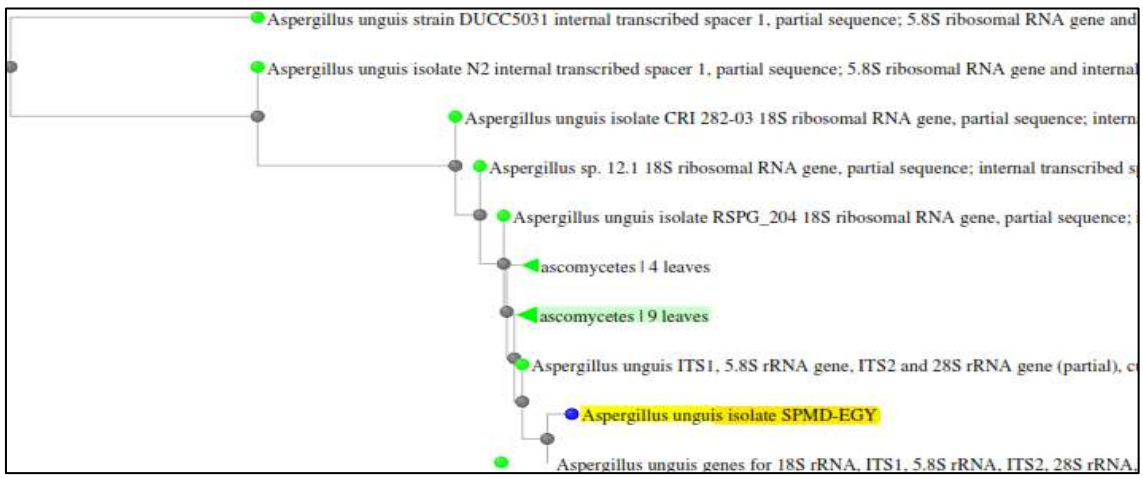

Figure 1: The phylogenetic tree of *Aspergillus unguis* SPMD-EGY isolated from marine soft coral *Sinularia* sp.

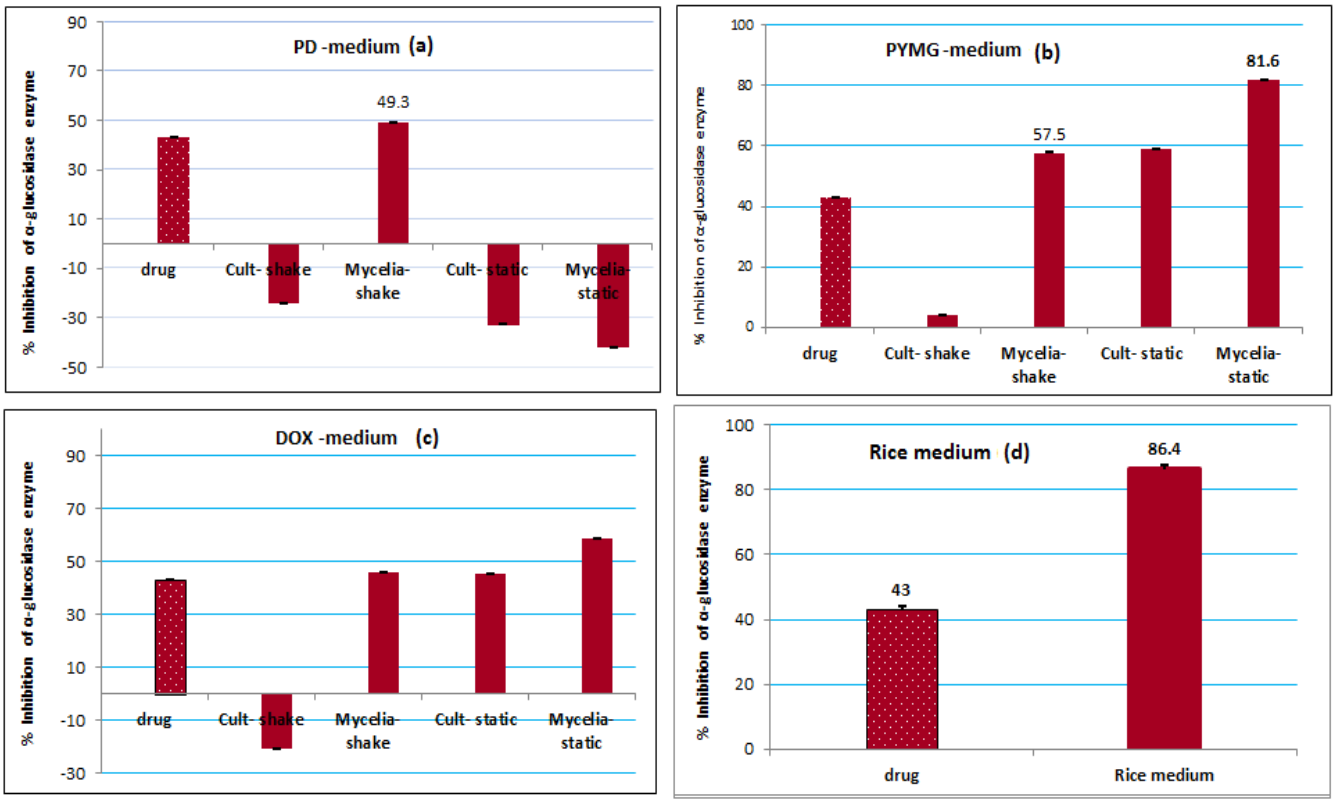

Figure 2: % **α-Glucosidase** inhibitory activity of 2ry metabolites extracts from culture and mycelia (static and shake) of different media. Values are expressed as mean ±SD, n = 3 (200 µg/ml for all tested extracts and drug; acarbose).

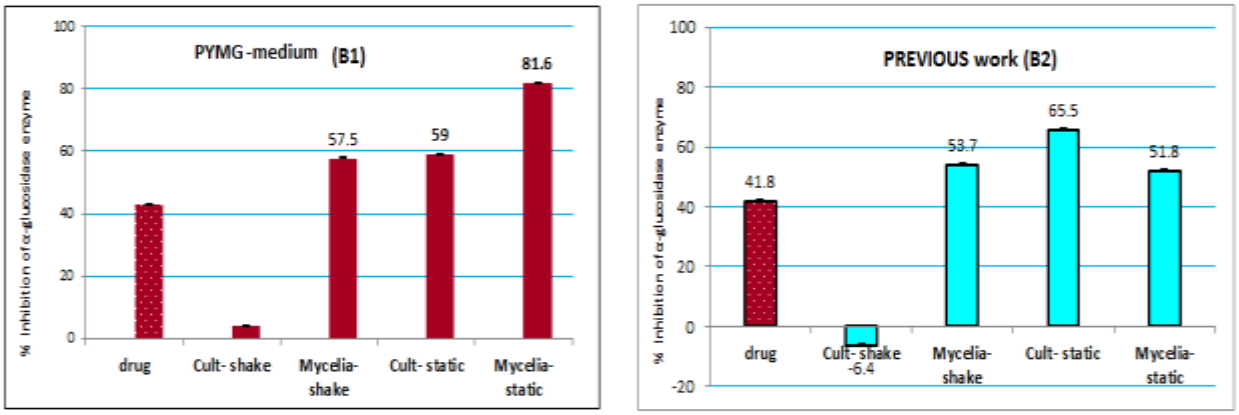

Figure 3: Demonstrative comparison between PYMG medium (5% glucose, distilled water) and previous work medium (10% glucose, sea water)

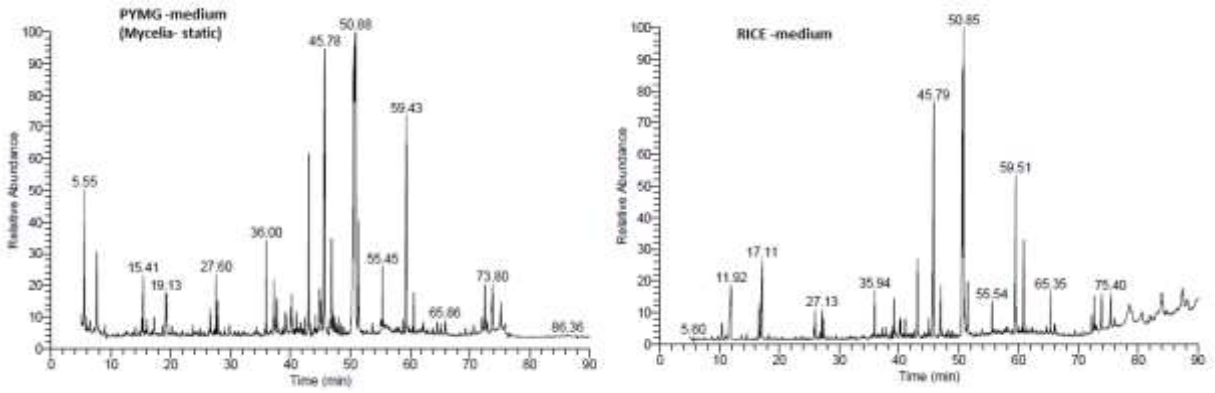

Figure 4: Comparative chromatographic study (GC/MS analysis) of PYMG mycelia static and Rice solid medium extracts for *Aspergillus unguis* SPMD-EGY

Table 1: Chemical composition assessed by GC/MS of PYMG mycelia static (PY) and Rice solid medium(RS) extracts for *Aspergillus unguis* SPMD-EGY

No	Compound	RT	PY % [a]	RS % [a]
	Saturated fatty acids			
1	Butanedioic acid	17.19	1.0	0.5
2	Azelaic acid	37.76	0.8	0.4
3	Tetradecanoic acid	39.28	0.4	----
4	Pentadecanoic acid	42.49	0.4	0.2
5	**Hexadecanoic acid**	45.78	**18.0**	**10.1**
6	Heptadecanoic acid	48.50	0.3	----
7	Octadecanoic acid	51.41	3.1	1.6
	Unsaturated fatty acids			
8	*cis*-9-Hexadecenoic acid	44.90	0.6	0.6
9	Heptadecenoic acid	48.05	0.3	----
10	9,12-Octadecadienoic acid	50.52	3.4	**6.3**
11	9-Octadecenoic acid	50.89	3.5	**7.3**
12	11-Octadecenoic acid	50.95	0.3	0.2
	Fatty acid esters			
13	5-Keto-2,2-dimethyl-heptanoic acid, propyl ester	19.14	0.6	1.4
14	Hexadecanoic acid-2,3-dihydroxy propyl ester	60.65	0.6	2.4
15	9,12-Octadecadienoic acid-2-hydroxy-ethyl ester	64.53	0.3	----
16	Octadecanoic acid- 2,3-dihydroxy- propyl ester	65.35	----	0.9
17	Docosanoic acid,1,2,3-propanetriyl ester	87.39	----	0.5
	Nitrogenous compounds			
18	8-Methoxy-2,3-dihydro-1h-benzazepin-2-one-5-acetic acid	5.55	**6.2**	----
19	N-Acetyl-N-methyl alanine	7.66	3.1	----
20	2,6-Ditbutyl-4-hydroxy-4-phenyl-1-imino-2,5-cyclohexadiene	11.92	0.4	**5.0**
21	4-(4-Methoxyphenyl)-4-(1-Hpyrrolo[2,3b]pyridine-3-yl)-butan-2-one(isomer)	14.53	----	0.3
22	4(4-Methoxyphenyl)-4-(1H-pyrrolo[2,3b]pyridin-3-yl)butan-2-one	19.30	0.8	**4.5**
23	7,8-Dihydroxy-benzo(5,6g)1H,3H-quinazoline-2,4-dione	40.07	0.7	----
24	4,6-Dimethoxy-7(5-methyl-1-pyrrolin-2-yl)2,3-diphenylindole	41.45	0.22	0.25
25	2,2-Dichloro-1,1-bis(4-diethylaminophenyl)ethylene	44.21	0.8	0.3
26	1,1,3-Trifluoro-3,3-bis[ethylamino]1-phenyldisiloxane	46.92	3.5	3.3
	others			
27	Glycerol	10.43	2.3	1.37
28	Methyl -7(2,4,6-trimethylphenyl)-5H-furo[2,3c] thiopyran-4-carboxylate	15.15	0.5	----
29	Tridecanol	19.12	0.62	1.4
30	L-Threitol	27.6	2.0	1.7
31	6(4t-Butylphenyl)1,3,5-hexatriynyl)trimethylsilane	27.89	1.5	0.7
32	1,4-Benzenediol,2,6-bis(1,1dimethylethyl)	35.22	----	0.2
33	Arabitol	35.99	3.4	2.8
34	1,4-Benzenediol,2,6-bis(1,1dimethylethyl) (isomer)	36.92	----	0.17
35	1,1,4,4-Tetramethyl-2,3-diphenyl-1,4-disilacyclohex-2ene	37.23	0.93	0.6
36	1,1,4,4-Tetramethyl-2,3-diphenyl-1,4-di-silacyclohex-2-ene (isomer)	37.28	0.89	----
37	1,3-Bis[2-hydroxyphenyl]-2-propen-1-one	40.23	1.3	1.0
38	3-Deoxyglucitol	41.03	0.45	0.9
39	Sorbitol	43.07	6.1	4.5
40	Methyl 2{2[3(methoxycarbonyl)phenyl]-1-ethynyl}5-hydroxy-ethynyl)benzoate	44.21	0.8	----
41	1-[4-hydroxyphenyl]-3-[2,4-di-hydroxyphenyl]-2-propen-1-one	51.34	----	0.6
42	Dotriacontane	52.84	----	0.2
43	Diisooctyl phthalate	59.51	**13.8**	**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.

CONCLUSION

The production of alpha-glucosidase inhibitory secondary metabolite from *Aspergilus unguis* (formely *Emercilla ung*uis) was studied using Wecherham medium.

In this work different media (three liquid and one solid) were studied for their ability to enhance the potentiality of the inhibitory behavior of alpha-glucosidase enzyme. Both rice and PYMG media showed the highest effect (86 and 81%) compared to the drug (acarbose, 43%). Fatty acid productions from the extracts of *Aspergillus unguis* from these media were identified using GC/MS analysis. The unsaturated fatty acids in both extracts represented (8 & 14 % respectively).

Unsaturated fatty acids showed higher α-glucosidase inhibitory activity than the saturated fatty acids. The high significant alpha-glucosidase inhibitory activity of fungal extracts prompted us to continue work for further *in vivo* studies.

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