

Research Article



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

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ABSTRACT

Type 2 diabetes mellitus is on the increase worldwide. Current treatments possess undesirable side-effects and therefore investigations into alternative remedies, which may be cost-effective and devoid of such side-effects, are on-going. Increased oxidative stress has been suggested as mechanism underlying diabetes and its related complications. The supernatant and mycelial extracts from the static and shake cultures of the fungus (*Emericella unguis* 8429) isolated from the soft coral *Sinularia* sp. were investigated for their α -glucosidase inhibitory activity, cytotoxic, antioxidant and antimicrobial activities. The results showed that the supernatant static (A) had the highest significant α -glucosidase inhibitory activity (65.45%), followed by the mycelial static (B) (51.8%). These results seem to be more potent than positive control, Acarbose (41.8%). A and B extracts were tested against HELA (cervical), MCF-7 (breast), HCT116 (colon) and HEPG2 (liver) carcinoma cell lines. They exhibited cytotoxic effect against all investigated cell lines, in a dose dependent way. Extracts A and B had the highest free radical scavenging activity against DPPH and superoxide anion radicals. Also, they exhibited high activity against representative strains of Gram-positive and Gram-negative bacteria as well as yeast and fungi. The GC/MS analysis of (A) indicated the presence of 14 compounds including p-coumaric acid, m-coumaric acid, hexadecanoic acid, 2-piperidinecarboxylic acid (butenyl derivative) (48%) and its isomer (36%), 6-benzoyl-5-methoxy-10-phenyl-pyranocoumarin, D-friedo-olean-14-en-3-one and other compounds. Based on our results, it could be concluded that, 2ry metabolites of the isolated coral derived fungus *Emericella unguis* 8429 possess significantly high α -glucosidase inhibition capacity, beside concomitant high antioxidant, antimicrobial and mild cytotoxic activities.

Keywords: Antioxidant, Antimicrobial activities, Corals fungi, Cytotoxic, *Emericella unguis* 8429, GC/MS analysis, α -Glucosidase inhibitors.

INTRODUCTION

Diabetes mellitus is a chronic disorder of metabolism caused by an absolute or relative lack of insulin. It is characterized by hyperglycemia (high blood sugar) in postprandial and/or fasting state, and its severe form is accompanied by ketosis and protein wasting.¹ It is also associated with a number of complications like retinopathy, neuropathy and peripheral vascular insufficiencies.² Glucose homeostasis is the key for treating diabetes. The treatment of type II diabetes is complicated by several risk factors inherent to the disease. Elevated postprandial hyperglycemia (PPHG) is one of the risk factors.³ PPHG is elevated by the action of α -glucosidase and α -amylase. Inhibition of these enzymes plays a major role in managing PPHG in diabetic patients. Inhibition of α -glucosidase enzyme activity leads to a reduction in disaccharide hydrolysis, which has beneficial effects on glycemic index control in diabetic patients.^{4,5}

Increased oxidative stress has been suggested as a mechanism underlying diabetes and its related complications.⁶ In diabetes, increased production of free radicals, especially reactive oxygen species (ROS), caused by persistent hyperglycemia, can initiate peroxidation of lipids, which in turn stimulates non-enzymatic glycation of

protein, inactivation of enzymes and alterations in the structure and function of collagen, basement and other membranes which collectively produces the late diabetic complications.⁷ Oxidative stress in diabetes co-exists with an altered cellular redox status and a sharp reduction in the antioxidant defense status, which can subsequently increase the deleterious effects of free radicals.⁸

Nowadays, researchers pay increasing attention towards the potential of marine organisms as an alternative source to isolated novel metabolites.⁹ Natural products such as secondary metabolites from plant, animals and microbes are important source for bioactive molecules, that in many cases, have been developed into medication.¹⁰ Marine organisms, particularly marine-derived fungi, are rich source of structurally biologically active metabolites and studies suggested that some bioactive compounds isolated from marine organisms had shown to exhibit anti-cancer, anti-microbial, anti-fungal or anti-inflammatory and other pharmacological activities.¹¹ This proves that they are rich source of bioactive compounds with therapeutic potential.

To this day, there are no reports that marine compounds isolated from microorganisms of the Red Sea area of Egypt have anti-diabetic and cytotoxic activities. Hence, we tried to study soft coral associated fungi and some of



their biological activities. The present investigation is an outcome of such a study on the fungus *Emericella unguis* 8429 associated with a soft coral *Sinularia* sp. and screen for its anti-diabetic, antioxidant, cytotoxic activities, using *in-vitro* techniques to screen these potentials.

MATERIALS AND METHODS

Soft coral materials

The soft coral, *Sinularia* sp., (from which fungus *Emericella unguis* 8429 was isolated) was collected from Hurghada coast, Red Sea, Egypt. The site is Shaa'b Al areq latitude, N 27° 25' 08.9", E 33° 51' 0.5" the sample was collected at depth of 5m - 8m in January 2013 and kept frozen until the work-up. The morphological taxonomy of the soft corals was identified by Mohamed A. Ghani – environmental researcher -Red Sea Marine parks, Hurghada, Red Sea, Egypt.

Identification of fungal cultures

Fungal culture was identified as *Emericella unguis* 8429 according to a molecular biology protocol by DNA isolation, amplification (PCR) and sequencing of the ITS region.¹²

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.¹²

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.¹³

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.

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,¹⁴ 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:

$$\% \text{ Inhibition} = [(A_{co} - A_t) / A_{co}] \times 100$$

Where, A_{co} is absorbance of the control and A_t is absorbance of the sample.

DPPH radical scavenging activity

DPPH radical scavenging activity of all extracts was analyzed according to a modified procedure of Matsushige and his group.¹⁵ 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. Mean of three measurements for each compound was calculated. Percentage inhibition was calculated using equation given below:

$$\% \text{ Inhibition} = [(A_{co} - A_t) / A_{co}] \times 100$$

Where, A_{co} is absorbance of the control and A_t is absorbance of the sample.

Superoxide anion scavenging activity

Superoxide anion scavenging activity was determined according to a modified method of Matsushige and his group.¹⁵ Reaction mixtures containing 1.4 mL of 50 mM Na₂CO₃ (pH 10.2), 100 µL of 3 mM xanthine, 100 µL of 3 mM EDTA, 100 µL of BSA (1.5 mg/mL), 100 µL of 75 mM Nitro blue tetrazolium, and 50 µL of each compound (100 µg/ml) were preincubated at 30°C for 10 min, and 50 µL of xanthine oxidase (0.3 unit/mL) was added. After incubation at 30 °C for 20 min, 200 µL of 6 mM CuCl₂ was

added to stop the reactions and the absorbance was measured at 560 nm. Percentage inhibition was calculated using equation given below:

$$\% \text{ Inhibition} = [(A_{co} - A_t) / A_{co}] \times 100$$

Where, A_{co} is absorbance of the control and A_t is absorbance of the sample.

Antimicrobial activity Test

Disc agar plate method was done to evaluate the antimicrobial activity of fungal extracts.¹⁶ 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).¹²

Evaluation of cytotoxic activity of A and B

The cytotoxic activity of culture (A) and mycelial (B)(static extracts) were tested against four cancer cell lines including; HELA (cervical), MCF-7 (breast), HCT116 (colon) and HEPG2 (liver). All cell lines were obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.). All cell lines were cultured in RPMI-1640 medium (Sigma Aldrich Chemical Co., St. Louis. Mo. U.S.A) supplemented with 10% FBS (Fetal bovine serum), penicillin (100 U/mL) and streptomycin (2mg/mL) at 5% CO₂ in a 37°C incubator. The cells were plated in 96-well plate at a density of 3.0×10^3 in 150µL of medium per well. Tested extracts dissolved in DMSO were added to the wells in triplicates with concentrations of 0, 5, 12.5, 25 and 50 µg/mL for 48h. The cytotoxic activity was determined using Sulphorhodamine-B (SRB) assay following the method reported by Vichai and Kirtikara.¹⁷ The IC₅₀ values (the concentrations of extract required to produce 50% inhibition of cell growth) were also calculated.

RESULTS AND DISCUSSION

Postprandial hyperglycemia is the key problem in diabetes mellitus. Ingestion of carbohydrate rich diet causes elevation in blood glucose level by the rapid absorption of carbohydrates in the intestine aided by the action of glycoside hydrolases which break complex carbohydrates into absorbable monosaccharides.¹⁸ Therefore the use of glycosidase inhibitor such as α-glucosidase inhibitors would be a prospective therapeutic agent for the effective management of the postprandial glucose excursion to enable overall smooth glucose profile.¹⁹ Alpha-glucosidase inhibitor inhibits the disaccharide digestion and impedes inhibitors have been isolated from marine derived fungi to develop as an alternative drug with increased potency and lesser adverse effects than the existing drugs.²⁰

GC/MS analysis

GC/MS analysis of the supernatant extract (A) from the static culture of the identified fungus (*Emericella unguis*

8429) isolated from the soft coral *Sinularia* sp. revealed the identification of 14 compounds; p-coumaric acid (0.8%), m-coumaric acid (0.31%), hexadecanoic acid (1.04%), 2-piperidinecarboxylic acid (butenyl derivative) (48%) and its isomer (36%), 6-benzoyl-5-methoxy-10-phenyl-pyranocoumarin (0.4%), D-friedo-olean-14-en-3-one (0.11%) and other compounds (Table 1, Figure 1).

Previous investigations into the secondary metabolite content of fungi derived from marine animals other than sponges have led to reports on *Emericella unguis* isolated from a mollusc and a medusa and found to produce the antibacterial depsideguinol, unguisins A and B.^{21,22}

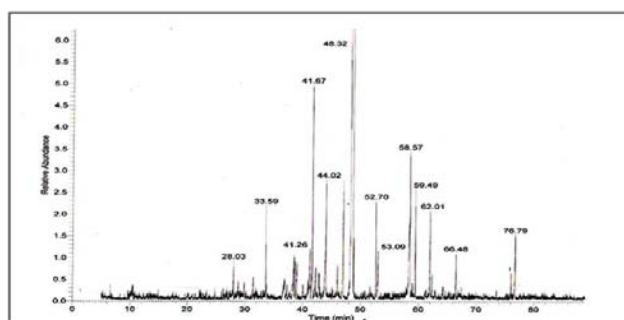


Figure 1: Chromatographic study (GC/MS analysis) for alcoholic extract of Supernatant (A) for the fungus (*Emericella unguis* 8429)

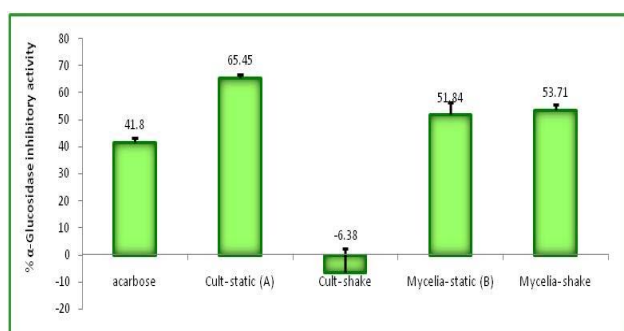


Figure 2: The % α-Glucosidase inhibitory activity of 2ry metabolites extracts from culture and mycelia (static and shake) of the fungus (*Emericella unguis* 8429). Values are expressed as mean ± SD, n = 3 (200 µg/ml for all tested extracts and acarbose).

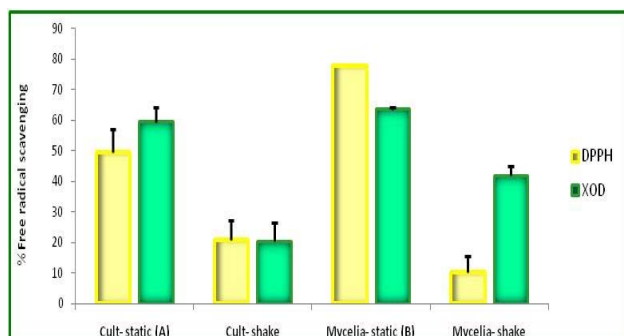
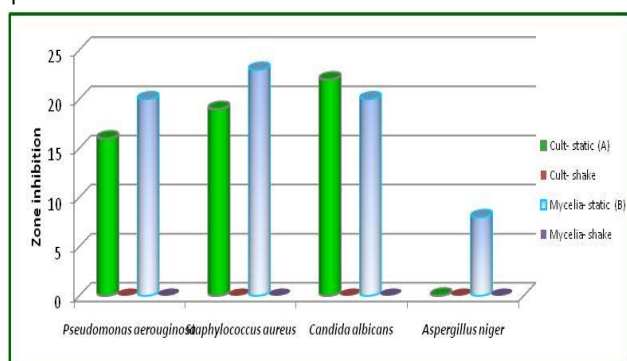
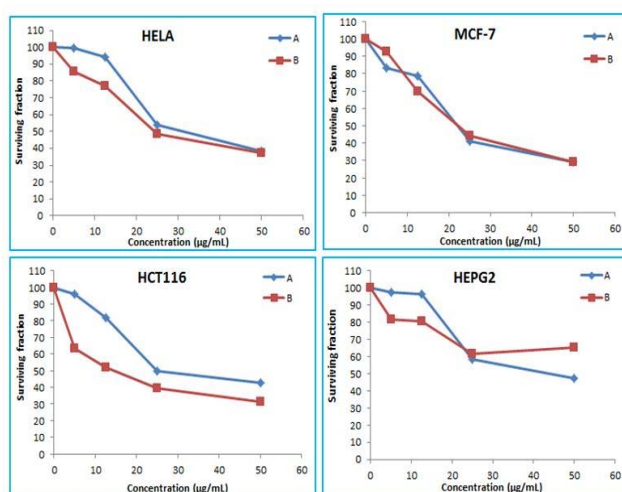


Figure 3: Free radical scavenging activity [DPPH and XOD] of 2ry metabolites extracts from culture and mycelia (static and shake) of the fungus (*Emericella unguis* 8429). Values are expressed as mean ± SD, n = 3 at a concentration of (100 µg/ml for all tested extracts).

Table 1: Chemical composition assessed by GC/MS analysis of alcoholic extract of the supernatant extract (A) from the static culture of the fungus (*Emericella unguis* 8429)

No	Compound	RT	Area % (TIC ^a)
1	3-(3,4-Dichloro-2-methoxyphenyl)-6-methoxyindan-1-one	37.26	0.1
2	4-[1,2-Dimethyl-6-(2-hydroxy-ethoxy-methoxy)cyclohexyl]but-3-en-2-on	38.47	0.3
3	9-(2,6-Bis(1-methylethenyl)phenyl)fluorene	38.69	0.34
4	m-Coumaric acid	39.0	0.31
5	1,3-[2-(hydroxyphenyl)-2-propen-1-one	41.66	1.32
6	3,3',5,5'-Tetra-tert-butyl-2,2'-dihydroxybiphenyl	42.75	0.13
7	6,6'-Bis[2-(hydroxy)-1-ethynyl]-2,2'-bipyridine	42.85	0.12
8	p-Coumaric acid	44.01	0.82
9	Hexadecanoic acid	47.04	1.04
10	D-Friedoolean-14-en-3-one	48.31	0.11
11	6-Benzoyl-5-methoxy-10-phenyl-pyranocoumarin	48.38	0.4
12	2-Piperidinecarboxylic acid (butenyl derivative)	48.6	48.74
13	2-Piperidinecarboxylic acid (butenyl derivative) [isomer]	48.7	36.19
14	N-(p-Chlorophenyl)-[1-(t-butylsulfinyl)-2-naphthyl]methanimine	59.49	0.5

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

**Figure 4:** Antimicrobial activity of 2ry metabolites extracts from culture and mycelia (static and shake) of the fungus (*Emericella unguis* 8429)**Figure 5:** Effects of 2ry metabolites extracts from culture (A) and mycelia (B) (static) of the fungus (*Emericella unguis* 8429) on HELA (Cervical), MCF-7 (Breast), HCT116 (Colon), HEPG2 (Liver) carcinoma cell lines viability.

α -Glucosidase Inhibitory activity

The supernatant and mycelial extracts from the static and shake cultures of the identified fungus (*Emericella unguis* 8429) isolated from the soft coral *Sinularia* sp. were investigated for their in-vitro α -glucosidase inhibitory activity. The results showed that the supernatant static (A) had the highest significant inhibitory activity (65.45%), followed by the mycelial static (B) (51.8%) and mycelial shake (53.7%) extracts. All these extracts seem to be more potent in α -glucosidase inhibitory potential compared to Acarbose (41.8%). It was observed that α -glucosidase is more sensitive towards fungal extracts than did the positive control (acarbose) (Fig. 2). 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 gastro-intestinal 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.²³

Previous studies showed that: p-coumaric acid was examined for its α -glucosidase inhibitory activity, its IC₅₀ value was found as 0.004 mM.²⁴ 3,4-Dihydroxy-5-(hydroxymethyl) piperidine and analogues are found to be strong inhibitors of glycosidases, and are therefore of potential interest in treatment of various disorders.²⁵ 21 α -

Hydroxy-D:A-friedo-olean-3-one and 29-hydroxy-D:A-friedo-olean-3-one showed much stronger inhibitory activity against α -glucosidase than did the positive control (acarbose, IC_{50} 1.02 μ M).²⁶ Pyrano-coumarin derivatives were evaluated for their *in vitro* anti-hyperglycemic activity; they exhibited potential PTP-1B inhibition, thereby revealing their possible mechanism of anti-diabetic action.²⁷

DPPH and superoxide anion free radical scavenging activities

The supernatant and mycelial extracts from the static culture of the identified fungus (*Emricella unguis* 8429) had the highest free radical scavenging activity (FRSA) against DPPH and superoxide anion radicals (Figure 3). While its supernatant and mycelial extracts (culture shaking) showed very low free radical scavenging activity (Figure 3). Comparable investigation revealed that the highly effective α -glucosidase inhibitor extracts (A and B) showed also the highly antioxidant activity (Figure 2,3).

Studies have shown that antioxidants play a key role in reducing diabetic complications.²⁸ Furthermore, positive correlation between α -glucosidase inhibition and antioxidant activity has been reported in numerous studies.²⁹ Increasing evidence has shown that prolonged exposure to elevated glucose induces the production of free radicals, particularly reactive oxygen species (ROS), through glucose auto-oxidation and protein glycosylation.³⁰ Oxidative injury by ROS has been suggested to explain the excess prevalence of vascular complications in diabetes mellitus, which may be mediated by oxidative stress.^{31,32} An impairment in the equilibrium between ROS and antioxidants results in oxidative stress, a deleterious process that can be an important mediator of damage to cell structures, including lipids and membranes, proteins and DNA. Indeed, a variety of defects in antioxidative status have been reported in experimental and in diabetic patients when compared with normal population.^{33,34}

The antimicrobial activity

The antimicrobial activity of *Emricella unguis* 8429 was mentioned.¹² It has been found that both culture static supernatant (A) and mycelial (B) extracts exhibited high activity against Gram positive and Gram negative bacteria as well as yeast test microbes (Figure 4).

Cytotoxic activity

Extracts A and B exhibited cytotoxic effect *in-vitro* against all investigated cell lines, in a dose dependent way (Figure 5). Extraction solvent had no effects on number of viable cells, proving that the cytotoxic effects were exclusively due to extracts components. Extract B showed the highest cytotoxic activity against HCT116 with IC_{50} of 14.5 μ g/ml. Meanwhile, the cytotoxic activities of extracts A and B against MCF-7 were almost the same with IC_{50} values of 22 and 22.1 μ g/mL respectively. On the other

hand both extracts showed non-significant cytotoxic activities against HELA and HEPG2 (Figure 5).

Previous studies showed that: p-coumaric acid at 1500 μ M decreased the number of colon cancer cell line to 43-75% of control after 2-3 days of treatment by inhibiting cell proliferation by interfering different cell cycle phases.^{35,36} Also, the anti proliferative and cytotoxic activity of pyrano-coumarin derivatives were documented earlier.³⁷

CONCLUSION

It could be concluded that, 2ry metabolites of the isolated marine fungus *Emricella unguis* 8429 showed significantly high α -glucosidase inhibition, beside concomitant high antioxidant, antimicrobial and mild cytotoxic activities. Our study provides (for the first time) primary evidence suggesting that the fungus *Emricella unguis* 8429 2ry metabolites in further in-vivo studies could play an important role as α -glucosidase inhibitor, besides their antioxidant, antibacterial, antifungal and cytotoxic activities.

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