Natural Compounds from Sponge-Derived Fungi

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Abstarct: Fungi associated with the marine sponge *Gellius bubastens* from El-Temsah Lake were isolated. All isolated sponge-derived fungi were screened for the presence of biologically active secondary metabolites in their cultures. Fungi that organic extracts of their cultures showed bioactivities were identified. The lipid content of hexane extracts of saline cultures of three fungal isolates was analyzed with GC/MS and led to identification of compounds with reported diverse bioactivities. Identification of marine fungi was based on DNA sequencing combined with searchable databases. The diversity of the microbial communities associated with *G. bubastens* collected from El-Temsah Lake may attract chemists to explore such area as a source of new, diverse and interesting secondary metabolites due to symbiosis. This work represents the first report of a study of the secondary metabolites diversity of marine-derived fungi isolated from its sponge host collected from El-Temsah Lake.

Keywords: El-Temsah Lake, Marine-derived fungi, antibacterial, antifungal, PCR.

INTRODUCTION

Marine microorganisms are of considerable current interest as a new and promising source of biologically active compounds; many of which can be used for drug development (Mitova *et al.*, 2003). Even though the sea has yielded numerous promising drug candidates, the development of real drugs from this source has been extraordinarily slow mainly due to the pressing supply problem. In addition, the probability of finding useful metabolites from marine organisms is obviously dependent on the number and quantity of samples screened. A further biotechnological approach aiming at the solution of the pressing supply problem of marine-derived drugs addresses the possible involvement of associated microorganisms (symbionts) with marine invertebrates (Proksch *et al.*, 2002). Therefore working on symbionts instead of marine macroorganisms has further expanded the diversity of structures that can be obtained without spending great efforts to collect vast amounts of the marine macro creatures.

Fungi are ubiquitous in nature and have, for centuries, provided us with a variety of useful compounds with a variety of pharmacological activities (Piorko, 2007). Sponga *et al.* (1999) isolated 395 fungi from marine sediments and sponges of the Mediterranean Sea, Red Sea, and Atlantic Ocean and found that marine sources are richer in fungal strains than soil samples. Marine sponges are considered as a rich source of novel microorganisms with potential pharmacological activities. The surfaces and internal spaces of sponges provide a specialized environmental niche for microorganisms (Hentschel *et al.*, 2001).

In our study we report the isolation of fungi associated with the marine sponge *G. bubastens* collected from El-Temsah Lake. The aim of this research is to identify the fungal isolates with molecular characterization. In addition, screening of organic extracts of the saline cultures of the fungal isolates for their biological activities was performed in order to explore El-Temsah Lake as a source of diverse and interesting secondary metabolites due to symbiosis.

MATERIALS AND METHODS

Instrumentation:

Gas Chromatography instrument (Trace GC2000, Thermo) was equipped with a capillary column (DB-5, 5%-Phenyl-methylpolysiloxane, 0.25 mm x 30 m, Agilent) and mass spectrometer (SSQ7000, Finngan Mat). Polymerase Chain Reactions (PCR) were done using PCR machine (Bio-RAD, DNA Engine-Peltier Thermal Cycle). Capillary Electrophoresis instrument (ABI 3730XL) was used for DNA sequencing.

Marine sample collection and identification:

The sponge sample was collected from El-Temsah Lake, Suez Canal., Abou Sultan, Isamilia, Egypt. Sample collection was done in July 2006 at the depth of 1-2 meters. The marine sponge was identified as *Gellius bubastens.*

Isolation of sponge-associated fungi:

In order to ensure fungal isolates to be symbiotic, surface sterilization of the collected sponge had to be made. A piece of sponge tissues was washed with 5% sodium hypochlorite followed by 70 % ethanol and finally with sterile artificial sea water (ASW). The pre-washed sponge was cut into small pieces with a sterile blade. A small piece (~1g) of the sponge tissues (previously surface sterilized) was ground well into a sterile mortar. The ground sample was placed into 15 mL-sterile falcon tube and the total volume was made up to 10 mL with sterile ASW. This was followed by ten-fold dilution with ASW till 5 concentrations. 10 µL of each of the five prepared concentrations and some small pieces of the cut sponge were inoculated onto different agar nutrient media; malt agar (MA, Difco), sabouraud dextrose agar (SDA, Difco), Czapek's dox yeast agar (CZYA), yeast peptone dextrose agar (YPDA (g/L each) yeast (10), peptone (20), dextrose (20)), natural potato dextrose agar (NPDA (g/L each) pealed potato (500 g), dextrose (20 g)) and natural minimal medium (NMM (g/L each) sponge tissue (30), $NaNO_3$ (3)). Further plating led to isolation of essentially pure fungal isolates.

Identification of fungal isolates:

The fungal DNA was prepared according to the manual of DNeasy Plant Mini Kit (Qiagen, www.qiagen.com) using the specified amounts of buffers. The prepared aqueous solutions of DNA templates were evaporated to dryness in a speed vac.

Polymerase Chain Reaction (PCR)

Preparation of master mixture:

For each reaction (R), a mixture of; Buffer (1 µL/R), deoxynucleoside triphosphates (dNTPs, 1 µL/R), $MgCl_2$ (0.4 µL/R), Taq DNA polymerase (0.04 µL/R), each of Internal Transcribed Spacer-Forward (ITS-1F, 19 bases) and Internal Transcribed Spacer-Reverse (ITS-4R, 20 bases) (20 µmolar, 0.8 µL/R), prepared DNA template (0.5 µL/R) and sterile water (to 10 µL/R); was prepared. For preparing parallel reactions, a master mixture of all components was prepared in a single tube then it was aliquoted into individual 0.2 mL PCR tubes (9.5 µL each). 0.5 µL of DNA template was then added and well mixed to make a total volume of 10 µL in each tube.

Thermal cycling:

All tubes were heated in a PCR machine (Bio-RAD, DNA Engine-Peltier Thermal Cycle) to 94 °C for 4 min for initial denaturation step, then to 94 °C for 30 sec for denaturation (strand separation) step, 51 °C for 30 sec for primer annealing (hybridization) step and then to 72 °C for 1 min as an extending (elongation) step. The last three steps were repeated for 40 times. The final DNA extension was done at 72 °C for 10 min.

Agarose gel electrophoresis:

Each DNA template amplified with PCR was mixed with 3 µL loading dye (BioLab) and applied to a well of ethidium bromide-treated agarose gel (5 µL EtBr to 1% agarose in TAE (Tris Acetate EDTA)). After electrophoresis, the gel was illuminated with UV lamp and DNA slices were cut out of the gel and weighed.

Retrieve of the purified DNA:

Gel slices were extracted using QIAquick gel extraction kit (Qiagen). The concentration of extracted DNA was measured at 230 nm using NanoDrop (ND-1000 spectrophotometer).

DNA sequencing:

Each purified DNA template (50 ng) was mixed with the primer ITS-1F (10 pmoles) in a total volume of 12 µL. DNA sequencing was done using high throughput Capillary Electrophoresis instrument (ABI 3730XL) at the University of Utah DNA sequencing and genomics core facility.

Saline cultures of three fungi and extraction:

20 L of each of CZYB, YPDB and SDB in ASW were prepared and inoculated with *Aspergillus flavus*, *Aspergillus kambarensis* and *Fusarium oxysporum*, respectively. The prepared cultures were kept at 27 °C with shaking for 14 days. The filtered broths were extracted with ethyl acetate (EtOAc) which was further partitioned between 10 % aqueous methanol (MeOH) and hexane. MeOH and hexane fractions were separated, evaporated and weighed.

Biological activity assays:

The biological activities of fractions were tested against *E. coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces pombe* and *Saccharomyces cervisiae* using agar disc diffusion assay (Anderson, 1978). The test microorganisms were obtained from the Virginia Tech Microbiology Culture Collection. While their cytotoxic properties were examined using brine shrimp lethality assay (Kapadia *et al.*, 2002; Pimenta *et al.*, 2003).

Gas Chromatography/Mass Spectrometry (GC/MS) of hexane extracts:

The separated hexane fractions, for the three studied fungi, showed antimicrobial activities against the studied microorganisms. Therefore, each fraction was saponified (British Pharmacopoeia, 1973; Finar, 1973). Both of the unsaponified matter (**A**) and fatty acid methyl ester (**B**) were analyzed by GC/MS.

Preparation of hexane extracts for GC:

For each fungus, the oily hexane extract was refluxed with 10 % ethanolic potassium hydroxide for 24 h and the mixture was extracted with ether (4 x 50 mL). The combined ethereal extracts were washed with water till free from alkalinity (litmus paper), dried over anhydrous sodium sulphate and evaporated to dryness to give unsaponifiable matter (**A**). The aqueous mother liquor and washings were acidified with concentrated hydrochloric acid. The mixture was extracted with ether and their methyl esters were prepared to give saponifiable matter (**B**).

GC/MS of A and B:

1 µL of chloroformic solutions of (**A**) and (**B**), for the three studied fungi, were applied in a splitless mode to a capillary column. The injector temperature was set to 250 °C and the column was heated using a controlled temperature program (from 50 °C to 300 °C in 3 min). The column head pressure was set to 13 psi. The eluted compounds were detected through a mass range between 50-500 Daltons.

RESULTS AND DISCUSSION

More than sixty fungal isolates were isolated from marine sponge *G. bubastens* collected from El-Temsah Lake. Sequencing of the prepared fungal DNA resulted in the identification of some of these fungal isolates; *Aspergillus kambarensis*, *Aspergillus flavus*, *Aspergillus thomii*, *Aspergillus fasiculatus*, *Aspergillus oryza*, *Fusarium oxysporum*, *Fusarium chlamydosporum*, *Fusarium incarnatum*, *Alternaria tenuissima*, *Nectria haematococca* and *Hypocrea lixii*. All fungal isolates were screened for antimicrobial and cytotoxic activities. Potent antibacterial and antifungal activities were observed for hexane extracts of three marine fungal isolates; *A. flavus*, *A. kambarensis* and *F. oxysporum* cultured in saline media. Their hexane extracts showed antimicrobial activities that were more potent than chloramphenicol and nystatin against studied pathogenic microorganisms at the same tested concentrations. The inhibition zones observed for hexane extracts of *A. flavus*, *A. kambarensis* and *F. oxysporum* were 20, 17 and 19 mm, respectively, from

200 µg extract per disc compared to 16 mm from 200 µg chloramphenicol disc against tested pathogenic bacteria. Therefore, the hexane extracts of these three sponge-derived fungi were analyzed by GC/MS. Analysis of GC/MS data showed a variation of the percentage of secondary metabolites responsible for the biological activities of the corresponding extract. Searching the database, the identified compounds were reported to have an important contribution in many pharmaceutical and pesticide preparations (Tejada *et al.*, 1997; Chapman *et al.*, 1998; Colby *et al.*, 1998; Noorman and Den Otter, 2001; Medina *et al.*, 2004) (Tables 1-3). To the best of our knowledge, this is the first report of the study of the lipid content of hexane extracts of marine-derived fungi.

In conclusion, marine fungi are considered an environmentally friendly source of pharmacologically-active natural products that allow the conservation of marine biological resources. Therefore, chemical and biological investigation of some secondary metabolites of three sponge-associated fungi was studied. The diversity of the microbial communities associated with the sponge *G. bubastens* collected from El-Temsah Lake may attract chemists to explore such area as a source of diverse and interesting secondary metabolites due to symbiosis.

Table(1): Results of GC/MS analysis of hexane extract of *A. flavus*

No	Identified Compounds	Uses	MF	R_t	%
1 (A)	1-hexanol, 2-ethyl [octyl alcohol]	In cosmetics	$C_8H_{18}O$	8.96	6.64
2 (A)	2-(1',1'-dideuteroallyl)-4-methylanisole	Fragrance, flavor concentrates	$C_{11}H_{12}D_2O$	19.31	0.55
3 (A)	1-Octadecene	Intraocular preparations	$C_{18}H_{36}$	28.16	0.57
4 (A)	Hexadecanoic acid [Palmitic acid]	In IM* injections (long acting release carrier)	$C_{16}H_{32}O_2$	31.62	0.17
5 (A)	(Z)-9-tricosene [Muscalure]	Pesticide	$C_{23}H_{46}$	32.20	0.66
6 (A)	4,4,7,7-Tetramethyldeca-1,9-diene	In fumigants	$C_{14}H_{26}$	32.66	0.15
7 (A)	Germacrene-C	Antimicrobial	$C_{15}H_{30}$	35.90	0.3
8 (A)	1,2-Benzenedicarboxylic acid, diisooctyl ester	Plasticizer in softeners	$C_{24}H_{38}O_4$	40.43	0.17
9 (A)	butyl 2,4-dimethyl-2-nitro-4-pentenoate	Pesticide	$C_{11}H_{19}NO_4$	44.06	0.28
10 (B)	Benzoic acid, methyl ester [Methyl benzoate]	Fragrance	$C_8H_8O_2$	10.62	0.24
11(B)	Hexanoic acid, methyl ester [methyl caproate]	Flavor concentrates	$C_7H_{14}O_2$	14.22	0.09
12 (B)	Methyl-3-(tetrahydro-2H-pyran-2-yl) oxybenzoate	Herbicide	$C_{13}H_{16}O_4$	19.88	0.66
13 (B)	1,2-Benzenedicarboxylic acid, dimethyl ester [Methyl phthalate]	Plasticizer	$C_{10}H_{10}O_4$	20.37	7.49
14 (B)	Dodecanoic acid, methyl ester [Methyl laurate]	Flavors	$C_{13}H_{26}O_2$	22.03	0.36
15 (B)	Nonanedioic acid, dimethyl ester [Methyl azelate]	Veterinary products	$C_{11}H_{20}O_4$	22.62	3.12
16 (B)	Tetradecanoic acid, methyl ester [Methyl myristate]	Flavors	$C_{15}H_{30}O_2$	26.74	8.11
17 (B)	Hexadecanoic acid, methyl ester [Methyl palmitate]	Veterinary products	$C_{17}H_{34}O_2$	31.14	24.85
18 (B)	Octadecanoic acid, methyl ester [Methyl stearate]	Emulsifier	$C_{19}H_{38}O_2$	34.83	13.87
19 (B)	Methyl 6,8-dioxo-2,7-diphenyl-c-4-(3-pyridyl)-3,7-diazabicyclo[3.3.0]octane-r-2-carboxylate	Weak antimicrobial	$C_{25}H_{21}N_3O_4$	42.91	0.11
20 (B)	2,3-Didecyl-1,4-naphthoquinone	Antimicrobial activities	$C_{30}H_{46}O_2$	49.80	0.27

IM*: Intra-Muscular

Table (2): Results of GC/MS analysis of hexane extract of *A. kambarensis*

No	Identified Compounds	Uses	MF	R_t	%
1 (A)	1-Hexanol, 2-ethyl (Isooctanol)	Manufacture of plasticizers and sunscreens	$C_8H_{18}O$	9.28	75.02
2 (A)	Glycerol carbonate	Solvents in colours, varnishes, glues, cosmetics and pharmaceuticals	$C_4H_6O_4$	31.34	0.06
3 (A)	Hexadecanoic acid (Palmitic acid)	In IM injections (long acting release carrier)	$C_{16}H_{32}O_2$	31.69	1.18
4 (A)	Di-(2-ethylhexyl) phthalate	Plasticizer	$C_{24}H_{38}O_4$	41.48	20.55
5 (B)	1,3-Isobenzofurandione (Phthalic anhydride)	Plasticizer	$C_8H_4O_3$	16.74	1.03
6 (B)	Tetradecanoic acid, methyl ester (Methyl myristate)	Flavor	$C_{15}H_{30}O_2$	26.62	0.26
7 (B)	Methyl pentadecanoate	Insecticide	$C_{16}H_{32}O_2$	28.17	1.26
8 (B)	Methyl elaidate	Liposomes preparations for membrane permeability tests	$C_{19}H_{36}O_2$	34.15	0.67
9 (B)	Octadecanoic acid methyl ester (Methyl stearate)	Emulsifier	$C_{19}H_{38}O_2$	34.65	1.57
10 (B)	Cis-linoleic acid methyl ester	Cosmetics	$C_{19}H_{34}O_2$	35.11	0.26
11 (B)	Docosanol silylated	Wax component	$C_{25}H_{54}OSi$	44.37	0.27

Table (3): Results of GC/MS analysis of hexane extract of *F. oxysporum*

No	Identified Compounds	Uses	MF	R_t	%
1 (A)	1,4,10-Trioxa-7,13-diazacyclopentadecane (Kryptofix 21)	Phase transfere catalyst, synthesis of alkalides and electrides	$C_{10}H_{22}N_2O_3$	17.15	0.72
2 (A)	3,4-Dihydro-2H-1,5-(3"-t-butyl) benzodioxepine	Bronchial dialator activity	$C_{13}H_{18}O_2$	21.61	5.21
3 (A)	1-Dodecanol	Emollient in cosmetics, surfactant	$C_{12}H_{26}O$	25.62	1.79
4 (A)	4-(1-Adamantyl-5-tert-butyl-3,6-dimethylphthalic anhydride	Plasticizer	$C_{24}H_{30}O_3$	27.11	0.31
5 (A)	1-Octadecanol (Stenol)	Emulsifier in cosmetics, creams and ointments	$C_{18}H_{38}O$	29.92	0.74
6 (A)	Glycerol carbonate	Solvents in colours, varnishes, glues, cosmetics and pharmaceuticals	$C_4H_6O_4$	31.34	0.04
7 (A)	Hexadecanoic acid (Palmitic acid)	In IM injections (long acting release carrier)	$C_{16}H_{32}O_2$	31.56	0.10
8 (B)	Methyl 2-amino -1,6-dihydro-6-oxo-4-pyrimidine carbamate	Insecticide	$C_6H_8N_4O_3$	22.51	0.33
9 (B)	Tetradecanoic acid, methyl ester (Methyl myristate)	Flavor	$C_{15}H_{30}O_2$	26.67	12.50
10 (B)	Glycerol carbonate	Solvents in colours, varnishes, glues, cosmetics and pharmaceuticals	$C_4H_6O_4$	29.43	0.13
11 (B)	Hexadecanoic acid, methyl ester (Methyl palmitate)	Veterinary products	$C_{17}H_{34}O_2$	30.94	38.78
12 (B)	Butanoic acid, 3-methyl (Isovaleric acid)	Anticonvulsant agent	$C_5H_{10}O_2$	38.10	0.05
13 (B)	Methyl tetracosanoate	Antidiabetic activity	$C_{25}H_{50}O_2$	44.35	0.05

REFERENCES

Anderson, B. (1978). Improved susceptibility disk assay method employing an agar overlay technique. Antimicrobial Agents and Chemotherapy, 14 (5): 761-764.

British Pharmacopoeia, (1973). The Pharmaceutics Press, London.

Chapman, J. W., J. Knapp, P. Howse and D. Goulson (1998). An evaluation of (Z)-9-tricosene and food odours for attracting houseflies, *Musca domestica*, to baited targets in deep-pit poultry units. Entomologia Experimentalis et Applicata, 89 (2): 183-192.

Colby, S., J. Crock, B. Dowdle-Rizzo, P. Lemaux and R. Croteau (1998). Germacrene C synthase from *Lycopersicon esculentum* cv VFNT cherry tomato: cDNA isolation, characterization, and bacterial expression of the multiple product sesquiterpene cyclase. *Proc Natl Acad Sci USA*, 95: 2216-2221.

Finar, I. L. (1973). In: Organic Chemistry, Longman Groups Limited Press, England 6th Edn., 45-69.

Hentschel, U., M. Schmidt, M. Wagner, L. Fieseler, C. Gernert and J. Hacker (2001). Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. FEMS Microbiological Ecology, 35: 305-312.

Kapadia, G. J., M. A. Azuine, H. Tokuda, M. Takasaki, T. Mukainaka, T. Konoshima and H. Nishino (2002). Chemopreventive effect of resveratrol, sesamol, sesame oil and sunflower oil in the Epstein-Barr virus early antigen activation assay and the mouse skin two-stage carcinogenesis. Pharmacol. Res., 45 (6): 499-505.

Medina, L. F., V. Stefani and A. Brandelli (2004). Use of 1,4-naphthoquinones for control of *Erwinia carotovora*. Canadian Journal of Microbiology, 50 (11): 951-956.

Mitova, M., G. Tommonaro and S. De Rosa (2003). A novel cyclopeptide from a bacterium associated with the marine sponge *Ircinia muscarum*. Z. Naturforsch., 58c: 740-745.

Noorman, N. and C. Den Otter (2001). The effects of laboratory culturing on (Z)-9-tricosene (muscalure) quantities on female houseflies. Entomologia Experimentalis et Applicata, 101, 69-80.

Pimenta, L. P. S., G. B. Pinto, J. A. Takahashi, L. G. F. Silva, and M. A. D. Boaventura (2003). Biological Screening of Annonaceous Brazilian Medicinal Plants using *Artemia salina* (Brine Shrimp Test) Phytomedicine, 10: 209-212.

Piorko A. (2007). Fungi: Multifaceted Microbes, in Natural Products from Marine Fungi, Ch. 3, Ganguli B. N., Deshmukh S. K., eds, CRC-Taylor and Francis, Anamaya Publishers, New Delhi, India, pp. 208-235.

Proksch, P. R. A. Edrada and R. Ebel (2002). Drugs from the seas-current status and microbiological implications. Applied Microbiology and Biotechnology, 59: 125-134.

Sponga, F., L. Cavaletti, A. Lazzarini, A. Borghi, I. Ciciliato, D. Losi and F. Marinelli (1999). Biodiversity and potentials of marine-derived microorganisms. Journal of Biotechnology, 70: 65-69.

Tejada, P., M. J. Mendez, S. Madero, C. Cava, A. Barcelo and V. Castellano (1997). 1-Octadecene as a solvent for ferrofluids for intraocular use. European Journal of Ophthalmology, 7 (3): 288-293.

مركبات طبيعية من الفطريات المعزولة من الأسفنجيات البحرية

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أصبحت الكائنات الدقيقة مصدرا غنيا لكثير من المركبات ليس فقط المتنوعة فى تركيبها الكيميائى، بل أيضا فى خواصها وفاعليتها البيولوجية مما جعلها مصدر اهتمام لكثير من العاملين بصناعة الدواء. تناول هذا البحث دراسة بعض الفطريات من أسفنج الجيلليس بوباستنس الموجود ببحيرة التمساح بالاسماعيلية. تم عزل الفطريات التى تعيش فى صورة تكافلية مع الأسفنج وزراعتها فى أوساط غذائية مختلفة واستخلاص نواتج الأيض فى الأوساط السائلة باستخدام المذيبات العضوية وذلك لاختبار هذه المستخلصات من حيث الفاعلية البيولوجية ضد البكتريا والفطريات الضارة. تم التعرف على بعض الفطريات المفصولة بواسطة الحامض النووى الديوكسى ريبوزى. وقد اختير كل من: فطر الاسبرجلس فلافس، و الاسبرجلس كامبارنسس، و الفيوزاريم اوكسيسبورم للدراسة وذلك لوجود فاعلية بيولوجية لمستخلص الهكسان لكل من هذه الفطريات ضد بعض البكتريا والفطريات. تم استخدام الفصل الكروماتوجرافى باستخدام الغاز وطيف الكتلة لتعيين المركبات الموجودة فى مستخلصات الهكسان وقد أثبتت النتائج وجود تنوع فى نواتج الأيض لكل فطر على حدة وهذه المركبات لها دورا مهما فى كثير من الصناعات مثل صناعة الدواء والمبيدات الحشرية وغيرها. وهذا يعد أول بحث يتم نشره عن الفطريات المتطفلة على أسفنج الجيلليس بوباستنس الموجود فى بحيرة التمساح بقناة السويس.