International Journal of Advanced Biotechnology and Research (IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol-7, Issue-2, 2016, pp598-604
http://www.bipublication.com

Research Article

Antimicrobial and anticancer activity and DNA fingerprinting of extracts from Red Sea marine fungal symbiotes.

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[Received-01/05/2016, Accepted-15/05/2016, Published-10/06/2016]

ABSTRACT

Marine microorganisms have become an important source of pharmacologically active metabolites. More specifically, fungi from the marine environment have shown great potential as suggested by the diversity of secondary metabolites. The aim of this study was to look for bioactive natural products from Red Sea derived fungi. Numerous natural products with novel structures and distinct biological activities have been discovered as the secondary metabolites of marine- derived microbes.Due to their pharmaceutical potential secondary metabolites of fungi have been studied for more than 70 years. In an effort to search for new antimicrobial compounds, this study was achieved by screening of 12 fungi samples. These fungal strains isolated from the water, sediment, swab, and tissue of these sponges and soft coral samples collected from the Red Sea, Egypt. The isolated fungi were *invitro* cultured and their total ethanolic extracts were screened for their antimicrobial activities using ATCC strains of bacteria and fungi. The anticancer activity of these marine fungal extracts was also tested. Following selection of the bioactive strains, a bioassay guided fractionation protocol was adapted to detect the biologically active fractions. Finally, molecular identification of the active metabolites producing fungi was done using PCR analysis.In this thesis we report the cultivation, fermentation, extraction, fractionation and assessing the antimicrobial and anticancer activity of marine isolated fungi.

Key words: Red Sea-derived fungi; antimicrobial activity; anticancer activity.

1. INTRODUCTION

1.1 Marine organisms as a source of new and biologically active metabolites

The marine environment is home to an immensely vast and complex array of species and ecosystems, most of which remain undiscovered. This does not come as a surprise, considering that the ocean covers approximately 70% of the planet's surface. Due to their pharmaceutical potential secondary metabolites of fungi have been studied for more than 70 years **(9-15).**

The search for new drugs from fungi started with the discovery of penicillin (Fleming, 1929), a potent antibiotic against Gram-positive bacteria, which was produced by *penicillium natatum*, **(15)**. A further milestone in the history of fungal products for medicinal use was the discovery of the immunosuppressant cyclosporine which is produced, e.g., by *Tolypocladium inflatum* and *Cylindrocarpon lucidum*. **(3)**. It was first discovered as an antifungal metabolite and later found to be

immunosuppressive which made cyclosporine useful for the treatment following organ transplantation. **(5)**. Cancer has posed a great challenge to the fields of medicine and immunology. Finding novel and efficient compounds of natural origin has been a major point of concern for research in the pharmaceutical sciences**. (12).**

This importance of marine natural products in the field of therapeutics may be attributed to their high affinity to the target, little loss of entropy when they bind to a protein and their bioavailability. Moreover, natural compounds are quite flexible in conformational acquisition in aqueous and lipophilic environments. **(10).**

The aim of this study was to assess the antimicrobial and the anticancer potential of Red Sea marine organisms associated fungal symbiotes.

2.MATERIALS AND METHODS

2. Cultivation, fermentation and extraction of fungi sample

2.1 Cultivation on solid media for growing purpose:

Media which used for cultivation of fungi from sponges must have specific characters such as the salinity of the media must be like the sea salinity to support the cultivation of marine fungi. Also the pH of the media must be acidic (pH 5.5-5.6) to support the growth of fungi and suppress bacterial growth. **(2)**.

For these reasons we used Modified Sabouraud Dextrose Agar (MSDA) media for the cultivation of marine derived fungi. Modified Sabouraud Dextrose Agar (MSDA) media used for cultivation of fungi, 65 g/L SDA (Difco) in sterile sea water (SSW).

2.2 Fermentation (cultivation on liquid media for screening purpose:

All isolated fungi after purification were cultivated on specific liquid media for screening purpose. Every fungus was grown on Sabouraud Dextrose Agar (SDA) plate for 5 days, and then a small piece of the fresh prepared colons was transferred to 100 ml conical flask (labeled with permanent ink as fungus key) containing the liquid medium (25 ml/flask) composed of the following contents **(8)**.

- **Dextrose**_____20 g
- **Peptone**_____5 g
- **Malt extract**_____3 g
- **Yeast extract**_____3 g
- **Sea water after adjusting its pH to 7.0** _1000 ml.

The media was prepared by filtered sterile sea water after adjusting its pH to 7.0 by Cole Parmer pH-meter: It is digital microprocessor controlled pH-meter**(8).**The fungus was grown on incubator shaker (Innova™ 4000 incubator shaker, new Brun swick scientific) at 26 °C and 100 rpm for 14 days.

2.3 Extraction of fungi secondary metabolites:

Extraction method:

The fermented broth was extracted three times with equal volumes (3 x 25 per 25 ml medium) of ethyl acetate **(4-11-13)** solution, the mycelia and supernatant together, and then the broth was filtrated through cheesecloth to separate into mycelia and unmixed solution of liquid broth with the ethyl acetate solution.

The immiscible liquids were separated by a glass separating funnel (avoid any plastic tools). The ethyl acetate extract was collected and concentrated under reduced pressure by a rotary evaporator (Bűchi Rotavapor R-114) to yield a crude extract. The obtained extracts were labeled to be ready for screening tests.

2.4 Biological testing

2.4.1 Screening for antimicrobial production by fungi

All fungi extracts were tested for their antimicrobial activity

Organisms and culture conditions.

2.4.1.1 Antibacterial activity testing

All media and solutions used for culture were sterilized by autoclaving (121 °C, 60 min).

Standard isolates of *staphylococcus aureus,* and *Escherichia coli* were used in this study; these were obtained from American type culture collection (ATCC): -

1) *Escherichia coli* ATCC 25922

2) *Staphylococcus aureus* ATCC 25923

These organisms were stored on Mueller Hinton agar (MH) slants and were propagated

in nutrient broth media. The grown bacteria stored at -80°C by using Glycerol media preservation method as described above in fungi preservation with using Luria-Bertani media (LB media) instate of SDB media.

2.4.1.2 Antifungal activity testing

Candida albicans ATCC was used throughout this study. It was stored on SDA plates and was propagated in yeast extract-peptone-glucose medium (1 % [w/v] yeast extract, 1 % [w/v] peptone, 2 % [w/v] glucose).Batches of medium (20 ml) was inoculated material from Sabouraud Dextrose Agar (SDA) plates containing freshly grown *Candida albicans* cells and incubated overnight in shaker incubator at 28 °C.

Crude extracts of the isolated fungi were tested against the isolates of *Candida albicans*.

2.5 Activity confirmation assays (TLC Bioautographic overlay assay)

Bioautography technique was chosen as a simple and accurate method for biological activity screening .The crude extract of each fungi was applied on small pieces of TLC plate , by using capillary tubes .

The excised pieces were placed on the top of agar plates containing either MHA or SDA and overlaid with thin layer of the same medium containing the test organisms was poured over the plate and incubated at 37° C for 24 hr. The inhibition zones were visualized as clear spots against a background of fungal or bacterial colonies. This technique was applied for the evaluation of antimicrobial activity of fungi extracts.**(7)**.

2.4.2 Screening for anticancer production by fungi

All fungi extracts were tested for their anticancer activity.

Potential cytotoxicity of the compound (s) was tested using the method of SKehan **(14)**(SRB assay):

1. Cells were plated in 96- multiwell plate (10^4 cell/well) for 24 hours before treatment with the compounds(s) to allow attachment of cell to the wall of the plate. to attach to the plates.
2. Different concentrations of compound under test (0, 1, 2.5, 5, and 10µg/ml) were added to the cell monolayer triplicate wells were prepared for each individual dose.
3. Monolayer cells were incubated with the compound(s) for 48hrs at 37^0C and inatomosphere of 5% co2.
4. After 48hrs, cells were fixed, washed and stained with Sulfo_Rhodamine_B stain.
5. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. The plates were washed with 1 % acetic acid and air-dried.
6. Color intensity was measured in an ELISA reader (Enzyme Linked Immune Sorbent Assay).
7. The dye was solubilized with 100 µl/well of 10M tris base (pH 10.5) and optical density (O.D.) of each well was measured spectrophotometric ally at 570 nm with an The mean background absorbance was automatically subtracted and means values of each drug concentration was calculated. The experiment was repeated 3 times.

   The percentage of cell survival was calculated as follows:

Surviving fraction = O.D. (treated cells)/ O.D. (control cells).

The IC_{50} values (the concentrations of resveratrol required to produce 50% inhibition of cell growth) . **(14)**.

2.6 Bioassay-Guided Fractionation

We used three different mobile phases with different concentrations to fractionate the crude extract of fungi into five fractions. (5% hexane /EtOAc),(50% hexane /EtOAc), (100%EtOAc),(50% EtOAc /methanol),(100% methanol).

Each fraction was concentrated under reduced pressure prior screening for anticancer activity.

2.7 Molecular identification of the fungi (PCR analysis)

The fungus coded with (A1- A2- A4- A6 –A8) was identified by PCR technique which was done according to the following method:

2.7.1 DNA Extraction:

DNA extraction was done using Genomic DNA preparation kit (Jena Bioscience)

2.7.2 PCR thermal profile:

PCR reaction: for total volume of 25 µl:

12.5 µl Master Mix (Qiagen)

2 µl DNA template (20 ng/ µl)
2 µl Primer (20 pmole)
8.5 µl deionized H_2O

Oligonucleotide:

Oligonucleotides used in this study was obtained from (Bio Basic Inc. Canada)
The following Oligonucleotide primer was used for specific detection of the fungi.
18S F:(5'-TTAAGCCATGCATGTCTAAG-3')
18S R: (5'-GACTACGACGGTATCTAATC-3') reversed-primer
Thermal profile for 18S primer 2 min at 94ºC
35 Cycles (30s at 94ºC, 30s at 52 at ºC, 3 min at 72 ºC) 10min at 72 ºC

2.7.3 Gel preparation:

1.5% Agarose gel in 1X TAE buffer
Gel key: Marker (1500bp), Sample A1, Sample A2, Sample A4, Sample A6, Sample A8.

2.7.4 DNA Sequencing:

- PCR products were purified using QIAquick PCR Purification Kit, QIAGEN.
- 2nd PCR was performed using BigDye Terminator v3.1 Cycle Sequencing Kit

For a total volume of 20 µL each reaction contained 8 µL Terminator ready reaction mix, 3.2 pmol Primer, DNA template (template quantity was calculated according to the PCR product size), and deionized water.
Thermal profile for Cycle Sequencing PCR: 1 min at 96ºC , 25 Cycles (10s at 96ºC, 5s at 50 at ºC, 4 min at 60 ºC).

- After additional step of purification with CENTRI-SEP Columns (PRINCETON SEPARATIONS), DNA sequencing was applied by 3500 Genetic Analyzer, Applied Biosystems.

3.RESULTS

3.1 Red sea Marine samples and each isolated fungi samples

A total of 12 fungal strains numbered from A1to A12 were separated from different red sea marine sponge.

Nr	Name of the sponge	Name of the isolated fungi
1	*Callysongia spp*	**A1**
2	*Echinoclathria sp*	**A2**
3	*Hxrtios sp*	**A3**
4	*Lathrunculia sp*	**A4**
5	*Lithphyto sp*	**A5**
6	*Rumphlla torta*	**A6**
7	*Stylissa sp*	**A7**
8	*Sinularia compressa*	**A8**
9	*Sarcophyton sp*	A9
10	*Sinularia sp*	**A10**
11	*Sarcophyton sp*	**A11**
12	*Thallasodendron cilliatium*	**A12**

Table (1). The isolated fungi from each sponge sample.

3.2 Antibacterial and antifungal activity testing using TLC bio- autography

The antimicrobial activity of each isolated fungus extract on the tested microorganisms. *E.coli ,Staph.aureus* were used as test strains for the antibacterial testing , *Candida albicans* was used as test strain for antifungal testing. TLC bioautography was used to monitor the activity of the extracts after development on the TLC plates, which aims to monitor the activity of each extract. Of the 12 extracts, 9 extracts showed good antifungal activity and 6 extracts showed good antibacterial activity.Table(2)

3.3 Anticancer activity of the fungal extracts

The ethyl acetate extract (0.5 gm) of each fungus was tested for anticancer activity by SRB (Sulfo-Rhodamine-B) assay .Three fungal strains (A2 A6 _A8) were chosen for further investigation based on their promising IC_{50}s. The IC_{50} values (the concentrations of the drug required to produce 50% inhibition of cell growth). Table (2)

3.4 Anticancer activity for fractions

The chosen three fungal extracts with promising anticancer activity were subjected for further chromatographic fractionation. Different fractions eluted with different solvent combinations were tested for their anticancer activity to determine the exact fraction holding the activity. The IC_{50} values are shown in Tables (3a,b,c)

3.5Molecular identification of the fungi (PCR analysis)

The isolated fungi were identified according to their molecular structure using PCR examination compared with the documented ones fig (1)

4.DISCUSSION

Using sea water as a culture base for media preparation (SDA) during this study was preferred in order to introduce the closest conditions to these fungi natural habitat. The medium pH was adjusted to be 5.5 by adding lactic acid solution to aid the fungal growth (2). The optimal time for fungal growth and secondary metabolites accumulation was monitored throughout the whole cultur period to obtain the best resuits (6). A period of 14 days was found to be the best regarding the anticancer and antimicrobial active metabolited production as well as best extract yeild. Ethyl acetate was chosen to extract the culture media as according to the allotropic theory it is the best to extract both polar and non polar compounds (1). The bioautography is a documented method to assess the antimicrobial activity of natural extract and adapting this technique in this work lead to identification of some marine fungi extracts as antimicrobial agents. E Assessing the anticancer activity of the fungal crude extracts as well as their derived fraction has lead to that (50%ethyl acetate/methanol) fraction shows the highest anticancer activity.

In this study the molecular identification of the fungal DNA was analyzed by PCR technique. From the results most of the isolated fungi are related to the *Aspergillus* and *Paecilomyes* species based on their DNA sequencing results.

5.ACKNOWLEDGMENTS

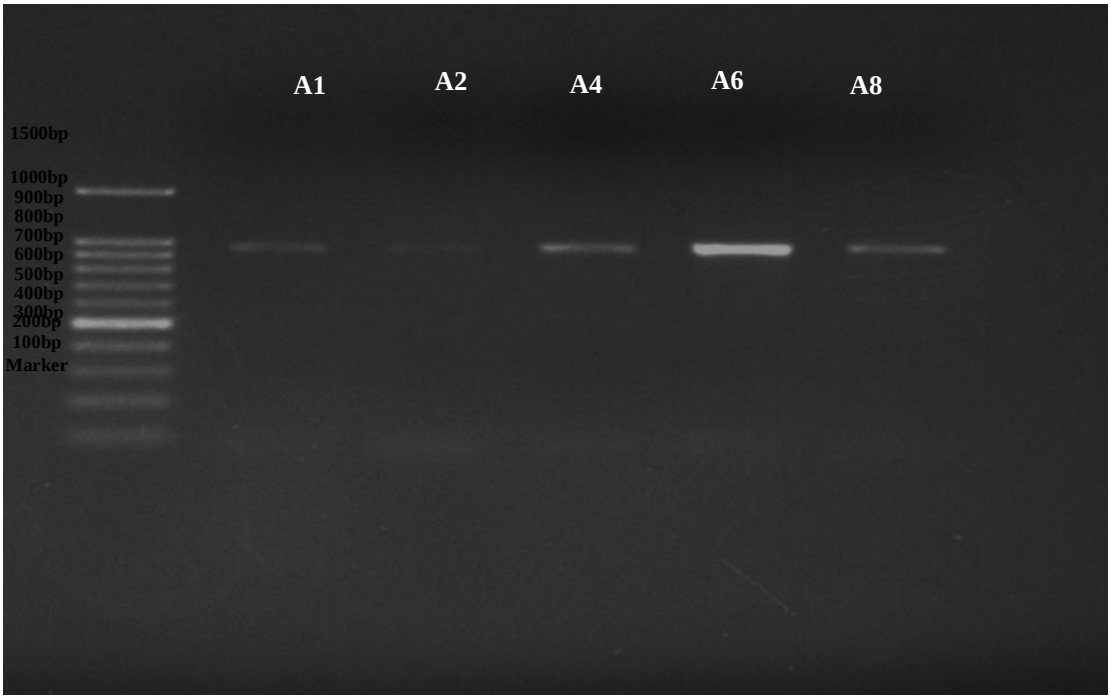
Thanks are due to the National Cancer Institute, Cairo, Egypt for their help during running the anticancer assays.

6.REFERENCES

1. Burns, E.; Ifrach, I.; Carmeli, S.; Pawlik, J. R.; Ilan, M. (2003): Comparison of anti-predatory defenses of Red Sea and Caribbean sponges. *I. chemical defense. Mar. Ecoloprog. Ser.* 252, pp. 105-114.
2. Collee, J. G.; Fraser, A. G.; Marmion, B. P.; Simmons, A. (1996): Practical medical microbiology. 4th ed. New York: Churchill Livingstone
3. Churchill, A. (2001): Fungi uses as a resource for therapeutic agents. Thesis (PhD). AMA Briefing on food Biotochnology, Ithaca, N.Y.
4. Gesner, S.; Cohen, N.; Ilan, M.; Yarden, O.; Carmeli, S. (2005): Pandangolide 1a, a metabolite of the sponge-associated fungus Cladosporium sp., and the absolute stereochemistry of pandangalide 1 and iso-cladospolide B. *J. Nat. Prod*. 68, pp.1350-1353.
5. Goodman Gilman, A.; Goodman, L. S.; Rall, T. W.; Murade, F. (1985). (eds.) 7th ed., Macmillan, New York, pp.1298-1299.
6. Jang, J. H.; Kanoh, K.; Shizuri, Y. (2006): New dihydrobenzofuran derivative, Awajanoran, from marine-derived Acremonium sp. AWA16-1. Japan Antibiotics Research Association. *J. Antibiot*. 59(7), pp.428-431.
7. Khattab, R. M. A. (2005): Isolation and bioactivities of natural products from Red Sea Holothurians (sea Cucumbers). Thesis (Ph. D.), UNIVERSITY OF Hull, England. Pp.9-10.
8. Lui, R.; Gu, Q.; Zhu, W.; Cui, C; Fan, G; Fang, Y; Zhu, T; Liu, H. (2006): 10-phenyl-[12]-cytochalasins Z_7, Z_8, and Z_9 from the marine-Derived Fungus Spicaria elegans. *J. Nat. Prod*. 69, pp.871-875.
9. Mayer, AM; Rodríguez, AD; Berlinck, RG; Hamann, MT. Marine pharmacology in 2005–6: Marine compounds with anthelmintic, antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the cardiovascular, immune and nervous systems, and other miscellaneous mechanisms of action. Biochim. Biophys. Acta 2009, 1790, 283–308.
10. McGranaghar, P.; Davies, J. C.; Grioth, G.W.; Davies, D. R.; Theodorou, M. K. (1999): The survival of anaerobic fungi in cattle faeces. *FEMS Microbiology Ecology*. 29, pp.293-300.
11. Proksch, P.; Ebel, R. A.; Schupp, P.; Lin, W. H.; Sudarsono; Wray, V.; Steube, K. (2003): Detection of pharmacologically active

natural products using ecology. Selected examplea from indopacific marine invertebrates and sponge-derived fungi. *Pure and applied chemistry*. 75, pp. 343-352.

12. da Rocha, AB; Lopes, RM; Schwartsmann, G. Natural products in anticancer therapy. Curr. Opin. Pharmacol 2001, 1, 364–369.
13. Sperry, S.; Samuels, G.J.; Crews, P. (1998): Vertinoid Polyketides from the Saltwater Culture of the Fungus *Trichoderma longibrachiatum* Separated from a *Haliclona* Marine Sponge. *J. Org. Chem.*, 63(26), pp.10011-10014.
14. SKehan P., Storeng R , (1990). Newcoloremtric cytotoxicity assay for anti-cancer drug screening. J Natl Cancer Inst.82:1107-1112.
15. Sykes, R. (2001): Penicillin: from discovery to product. Bulletin of the World Health Organization. 79(8), pp. 778-779. The regional organization for the conservation of the environment of the Red Sea and Gulf of Aden.



Figure(1). PCR produced by 18Sr DNA, size (1500bp) ,the gel electrophoresis of the samples (A1-A2-A4-A6-A8). Used 1.5% Agarose gel in 1X TAE buffer.

Table (2) Antimicrobial and anticancer activity of each fungal extracts.

Fungi sample code	Test microorganism for antimicrobial activity			Anticancer activity (IC50)
	Ec	Sa	Ca	
A1	+	+	_	**44.5 µg/ml**
A2	+	+	+	**17.5 µg/ml**
A3	_	_	+	**44.9 µg/ml**
A4	+	+	+	**40.3 µg/ml**
A5	_	_	+	**50 µg/ml**
A6	_	_	+	**23.9 µg/ml**
A7	_	_	+	**39.5 µg/ml**
A8	+	_	+	**19.4 µg/ml**
A9	_	+	_	**40.3 µg/ml**
A10	+	_	_	**42.8 µg/ml**
A11	_	+	+	**44 µg/ml**
A12	+	+	+	**42.8 µg/ml**

Ca, *Candida albicans* Sa, *Staphylococcus aureus*; Ec, *Escherichia coli*. '-' no inhibition zone ; '+' inhibition zone.

Table (3a). Anticancer activity of the fractions obtained from the crude extract of (sample A2)

Code of sample fraction	Concentration of used solvent mobile phase	Weight of sample	Anticancer activity(IC50)
1-1	5% ethyl acetate /hexane	0.06 gm	38.5 µl/ml
1-2	50% ethyl acetate /hexane	0.04 gm	*
1-3	100% ethyl acetate	0.02 gm	*
1-4	50% ethyl acetate/ methanol	0.01 gm	17.7µl/ml
1-5	100% methanol	0.1gm	16.6µl/ml

Table (3b). Anticancer activity of the fractions obtained from the crude extract of (sample A6)

Code of sample fraction	Concentration of used solvent mobile phase	Weight of sample	Anticancer activity(IC50)
2-1	5% ethyl acetate /hexane	0.07 gm	24.5 µl/ml
2-2	50% ethyl acetate /hexane	0.04 gm	*
2-3	100% ethyl acetate	0.05 gm	*
2-4	50% ethyl acetate/ methanol	0.02 gm	20.4µl/ml
2-5	100% methanol	0.1 gm	35 µl/ml

Table (3c). Anticancer activity of the fractions obtained from the crude extract of (sample A8)

Code of sample fraction	Concentration of used solvent mobile phase	Weight of sample	Anticancer activity(IC50)
3-1	5% ethyl acetate /hexane	0.1 gm	*
3-2	50% ethyl acetate /hexane	0.1gm	42 µl/ml
3-3	100% ethyl acetate	0.03gm	19.9 µl/ml
3-4	50% ethyl acetate/ methanol	0.01gm	15.3 µl/ml
3-5	100% methanol	0.07gm	25.5 µl/ml

(*)No significant activity

From the anticancer results shown for the above fractions, the fractions which were collected with (ethyl acetate/ methanol) as a mobile phase seems to hold anticancer activity.

Table (4). The molecular identification of the isolated fungi according to its code.

Fungus code	**Identified as**
A1	***Aspergillus sp***
A2	***Aspergillus tamari***
A4 – A8	***Aspergillus niger***
A6	***Paecilomyes varioti***