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ANTIMICROBIAL, CYTOTOXIC AND HEMOLYTIC ACTIVITIES OF MARINE ALGAE-ASSOCIATED FUNGAL ISOLATES IN VIETNAM

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Abstract. In the context of sources for natural products discovery are going scarcer, exploiting biotechnologically potential compounds from marine microbial symbionts is considered a relatively new trend. In our study a total of fifteen fungal strains were isolated from marine algal samples belonging to species *Kappaphycus cottonii*, *K. striatus*, *Gracilaria eucheumatoides* and *Betaphycus gelatinus* collected in Nha Trang in 2017. The *in vitro* biological activities, including antimicrobial, cytotoxic and hemolytic activities of ethyl acetate extracts of the fungal strains were determined. From fifteen fungal extracts, six displayed antimicrobial activity against at least one test strain. At 20 $\mu g.ml^{-1}$, four fungal extracts were found to express cytotoxic activity on two human cancer cell lines hepatocellular carcinoma (Hep-G2) and breast adenocarcinoma (MCF-7), with *G. eucheumatoides* being the source of the highest number of producer strains. Hemolytic activity was observed in rabbit erythrocytes under almost all fungal extracts' effect. No apparent relationship was observed between the biological activities of fungal isolates. The biological assessments uncovered several fungal candidates, such as Bge-1.1, Kco-2.1 and Geu-1.1 with relatively potent antimicrobial and cytotoxic activities while expressing less hemolytic effect at concentrations from 20 $\mu g.ml^{-1}$ to 200 $\mu g.ml^{-1}$. The results evidenced the potential of exploiting natural products from associated marine microorganisms, especially those for the purpose of pharmaceutical applications.

Keywords: Microbial isolates, marine algae, antimicrobial activity, cytotoxicity, hemolytic activity.

INTRODUCTION

Marine organisms are a productive and promising source of natural products. The potent biological activity of many marine natural products is of relevance for their ecological function and also the basis of their biomedical importance [1]. By Kong and coworkers' statistics, approximately 71% of the molecular scaffolds in the Dictionary of Marine Natural Products were exclusively utilized by marine organisms. Besides, in comparison to terrestrial ones, marine organisms are superior in terms of biological activities [2]. While marine macroorganisms have been proved to be a great source of novel natural products, marine microorganisms, especially those that are isolated from macroorganisms, are considered a tremendous but relatively untapped reservoir. Earlier studies mentioned novel metabolites from marine-derived fungi as chemically and biologically diverse compounds [3]. Among them, over 85% were produced by epi- and endophytes. Unlike the vast majority of symbiotic marine microorganisms that can hardly be isolated and cultured in laboratory, numerous epi- and endobiotic marine fungi are

culturable and may produce novel secondary metabolites in laboratory cultures [4]. With the advantage of metabolizing rapidly and controllably, exploiting natural products from marine fungal symbionts is turning out to be a new trend in modern biochemistry.

Marine macroalgae (also seaweed) are macroscopic marine organisms that are known as a vital component in the ocean's food chain, as well as an important oxygen-generator in our planet. Especially, the algae play a rising role in marine agriculture and industry as being a source of various bioactive compounds such as carrageenans and agarans [5], fucoidan [6], proteins [7], fatty acids and dietary fibre [8]. In Vietnam, with a total of 833 species, of which 415 belong to Rhodophyta, 183 are Chlorophyta, 147 are Phaeophyceae and 88 are Cyanobacteria [9], our macroalgal species richness is considerably high, indicating that Vietnam is potentially a diversity hotspot for macroalgae [10].

The red algae *Kappaphycus cottonii*, *K. striatus*, *Gracilaria eucheumatoides* and *Betaphycus gelatinus* are common representatives of macroalgae in Central Vietnam's sea [10]. In our research, we investigated the biological activities of associated fungal strains isolated from the algal species and screened by biologically guided assays in order to find out potentially applicable microbial candidates.

MATERIAL AND METHODS

Isolation of microbial strains from algal samples. Fifteen endo- and epiphytic strains were isolated from algae samples, belonging to species *K. cottonii*, *K. striatus*, *G. eucheumatoides* and *B. gelatinus* collected in Nha Trang (Khanh Hoa province, Vietnam) in June 2017. The fresh algae samples were morphologically identified *in situ* and kept in seawater before being transported to laboratory and isolated. Fungal endophytes and epiphytes were isolated by the method described by Zhang et al., (2009) [11] with minor modification. Briefly, for epiphytes isolation, algae samples were cut into 5 mm long pieces and placed on plates (potato dextrose agar (PDA) with 50 ppm penicillin and 50 ppm streptomycin added) for 1 h and then removed. Endophytic strains were isolated by surface-sterilizing in 70% EtOH (1 min), 2.6% $NaClO_2$ (3 min), and 70% EtOH (1 min), respectively, and followed by placing on PDA plates. Plates were incubated at 25^oC and observed daily for fungal hyphal development [11]. Isolated fungal strains were then re-cultivated, purified and transferred to fresh agar slants and stored at 4^oC for further studies. The properties of isolated fungal strains are listed in table 1.

Table 1. Properties of fungal isolates from algal samples.

No.	Fungal strain name	Host algal species	Hyphal colour*	Fungal taxonomy**
1	Kco-1.1	*K. cottonii*	Brown	*Phaeosphaeriopsis* sp.
2	Kco-1.2	*K. cottonii*	Black	*Cladosporium* sp.
3	Kco-2.1	*K. cottonii*	Black	*Aspergillus* sp.
4	Kco-2.2	*K. cottonii*	Green	*Curvularia* sp.
5	Kco-2.3	*K. cottonii*	Gray	*Penicillium* sp.
6	Kst-1.1	*K. striatus*	Black	*Aspergillus* sp.
7	Kst-2.1	*K. striatus*	Black	*Cladosporium* sp.
8	Kst-2.2	*K. striatus*	Brown	*Chaetomium* sp.
9	Geu-1.1	*G. eucheumatoides*	Purple	*Nodulisporium* sp.
10	Geu-1.2	*G. eucheumatoides*	Black	*Aspergillus* sp.
11	Geu-1.3	*G. eucheumatoides*	Black	*Chaetomium* sp.
12	Geu-1.4	*G. eucheumatoides*	Gray	*Nigrospora* sp.
13	Bge-1.1	*B. gelatinus*	Black	*Aspergillus* sp.
14	Bge-2.1	*B. gelatinus*	Black	*Nigrospora* sp.
15	Bge-2.2	*B. gelatinus*	Blue-green	*Curvularia* sp.

Note: *: Hyphal colour in Czapek's medium with 3.5% NaCl; **: Preliminary taxonomy based on fungal morphological characteristics.

Preparation of microbial extracts. Isolated fungal strains were cultivated at 27°C for 14 days in Erlenmeyer flasks containing potatoes dextrose broth (PDB) in marine water with constant shaking (200 rpm) in an incubator shaker (IKA, Germany). The fungal fermentation broth was then extracted with organic solvents ethyl acetate (XL, China) by vigorous soaking at ambient temperature. The crude extracts obtained were then concentrated at reduced pressure in a rotary vacuum evaporator (EYELA, Japan) (50°C at 40 rpm) and subsequently dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany) for biological assessments.

Assessment of in vitro antimicrobial activity. The *in vitro* antimicrobial activity of the extracts was assessed employing eight microbial strains, namely *Escherichia coli* ATCC 25922 (Gram negative), *Pseudomonas aeruginosa* ATCC 25923 (Gram negative), *Bacillus subtillis* ATCC 11774 (Gram positive), *Staphylococcus aureus* subsp. *aureus* ATCC 11632 (Gram positive), *Aspergillus niger* 439 (filamentous fungi), *Fusarium oxysporum* M42 (filamentous fungi), *Candida albicans* ATCC 7754 (yeast) and *Saccharomyces cerevisiae* SH 20 (yeast). A modification of the broth dilution test method (Vlietinck (1998), Langfied et al., (2004)) in sterile 96-well microtiter plates was used to detect antimicrobial activity of selected fungal strains. Briefly, test samples with their serial diluted concentrations were added into wells that were subsequently filled with test microbial suspensions (10^6–10^8 cells/ml). Medium containing 1% DMSO was used as a negative control, and streptomycin sulfate and nystatin were used as a positive control for anti-bacterial and anti-fungal activities, respectively. The microplates were subsequently incubated for 24 h at 37°C for bacteria and 48 h at 25°C for fungi. Minimum inhibitory concentrations (MICs), the lowest concentrations of samples that inhibit the visible growth of a particular test microorganism, were then observed and recorded.

Assessment of in vitro cytotoxicity. The cytotoxicity of the fungal isolates was determined by Sulforhodamine B (SRB) colorimetric assay [12] employing two human cancer cell lines: Hepatocellular carcinoma (Hep-G2) and breast adenocarcinoma (MCF-7). The assay was adapted for a quantitative measurement of cell growth and viability. Briefly, cancer cells were seeded at $5*10^3$ cells per well in 96-well microtiter plates, and incubated at 37°C (48 h, 5% CO_2). Cultures fixed with trichloroacetic acid were stained for 30 minutes with 0.4% (w/v) SRB. The protein-bound dye was extracted with 10 mM Tris base for determination of optical density (OD) at a single wavelength of 564 nm in 96-well plate reader (Tecan, USA). The percentages of cell survival of test samples were calculated using following formula:

$$\% \text{ cell survival} = 100\% * [OD_{(sample)} - OD_{(day\ 0)}] / [OD_{(DMSO)} - OD_{(day\ 0)}]$$

IC50 values of test samples were calculated by interpolation from linear regression analysis.

Assessment of in vitro hemolytic activity. The hemolytic activity of the fungal isolates was tested for hemolytic activity using method that was described by Sunyer and Tort (1995) with minor modification. Accordingly, rabbit erythrocytes were freshly collected in Alsever's solution, washed thrice in phosphate-buffered saline (PBS, 0.15 M NaCl, pH 6.9) and standardized to $5*10^8$ cells.ml^{-1} prior to assay. The rabbit erythrocyte suspension was added to each tube containing 1 ml of serial dilutions of samples in PBS. After being shaken, the mixture was incubated at 37°C for 60 min, and then centrifuged at 1,000x g for 10 min at 4°C. A 1 ml amount of supernatant fluid from tubes was added to 6.0 ml of 1% sodium carbonate (Sigma Aldrich, Germany) and allowed to stand for 15 min at room temperature before determination of optical density at 541 nm in 96-well plate reader. The hemolytic activity was calculated from the formula:

$$\% \text{ Hemolytic} = 100\% * [OD_{(sample)} - OD_{(blank)}] / OD_{(positive\ control)}$$

The positive control was obtained using Tritron X-100 (0.1%) and 0% hemolysis was obtained with PBS buffer. IC50 values of test samples were calculated by interpolation from linear regression analysis.

RESULTS

Antimicrobial activity. The antimicrobial activity of fifteen fungal extracts is described in table 2. The result shows that 6 out of 15 fungal extracts inhibited the growth of at least one test microorganism at concentration of 200 μg.ml^{-1}. Among them, the crude extract of fungal strain Bge-1.1 showed inhibition effects against two test bacteria, including Gram negative bacterium *E. coli* and Gram positive bacterium *S. aureus*, and two test fungi, including filamentous fungi *A. niger* and yeast *C. albicans*. The crude extract of strain Kco-1.2 was also observed to have relatively potent antibacterial effects against *E. coli*, *P. aeruginosa* and *B. subtilis*. Ethyl acetate extracts of Kco-2.1, Kst-2.1 and Geu-1.1 exhibited to have less inhibitory activity with a MIC concentration of 200 μg.ml^{-1} against *S. aureus*.

Table 2. Antimicrobial activities of fungal extracts

No.	Sample name	MICs of fungal extracts against test microorganisms (μg.ml^{-1})							
		E. coli	*P. aeruginosa*	*B. subtillis*	*S. aureus*	*A. niger*	*F. oxysporum*	*C. albicans*	*S. cerevisiae*
1	Kco-1.1	-	-	-	-	-	-	-	-
2	Kco-1.2	200	200	100	-	-	-	-	-
3	Kco-2.1	-	-	-	200	-	-	-	-
4	Kco-2.2	-	-	-	-	-	-	-	-
5	Kco-2.3	-	-	-	-	-	-	-	-
6	Kst-1.1	-	-	-	-	-	-	-	-
7	Kst-2.1	-	-	-	200	-	-	-	-
8	Kst-2.2	-	-	-	-	-	-	-	-
9	Geu-1.1	-	-	-	200	-	-	-	-
10	Geu-1.2	-	-	-	-	-	-	-	-
11	Geu-1.3	-	-	-	-	-	-	-	-
12	Geu-1.4	-	-	-	-	-	-	-	-
13	Bge-1.1	200	-	-	200	200	-	200	-
14	Bge-2.1	-	200	-	200	-	-	-	-
15	Bge-2.2	-	-	-	-	-	-	-	-

Note: -: Indicates no inhibition.

Cytotoxic activity. The cytotoxic activity of 15 fungal extracts is shown in table 3. As described, of 15 extracts tested, four isolates, namely Kco-2.1, Geu-1.1, Geu-1.3 and Bge-1.1, were found toxic to both cancer cell lines Hep-G2 and MCF-7.

The result suggests that cytotoxicity and antimicrobial activities are not always relevant. The crude extract of Geu-1.3 for instance, expressed no antimicrobial activity but remarkable cytotoxic activity against two experimental cancer cell lines. Another example is the bacterial-inhibitory ethyl acetate extract of Kco-1.2 (table 2) that exhibited merely faint toxic activity in cell lines Hep-G2 and MCF-7. However, several fungal extracts (Kco-2.1, Geu-1.1, Bge-1.1) showed inhibitory effects in both biological assessments, suggesting the potential of applying these fungal strains with pharmaceutical aspects.

Table 3. Cytotoxicity of fungal extracts

No.	Sample name	Conc. (µg.ml^{-1})	Cell survival percentage (%)		IC50 value (µg.mL^{-1})	
			Hep-G2	MCF-7	Hep-G2	MCF-7
	DMSO		100	100	100	100
	Ellipticine	5				
1	Kco-1.1	20	93.8 ± 1.01	98.5 ± 1.4	-	-
2	Kco-1.2	20	82.8 ± 1.7	91.3 ± 1.5	-	-
3	Kco-2.1	20	37.4 ± 0.9	42.3 ± 0.7	16.25	18.47
4	Kco-2.2	20	82.8 ± 1.7	91.3 ± 1.5	-	-
5	Kco-2.3	20	74.3 ± 0.9	72.5 ± 0.6	-	-
6	Kst-1.1	20	71.7 ± 0.7	80.6 ± 0.5	-	-
7	Kst-2.1	20	74.5 ± 0.0	81.2 ± 0.7	-	-
8	Kst-2.2	20	86.4 ± 0.5	90.7 ± 1.2	-	-
9	Geu-1.1	20	8.46 ± 0.3	5.8 ± 0.4	8.28	10.36
10	Geu-1.2	20	61.3 ± 0.4	76.3 ± 0.2	-	-
11	Geu-1.3	20	17.2 ± 0.9	31.5 ± 1.1	11.07	15.38
12	Geu-1.4	20	91.5 ± 0.6	93.5 ± 0.7	-	-
13	Bge-1.1	20	0.7 ± 0.3	10.5 ± 0.6	5.24	7.32
14	Bge-2.1	20	64.1 ± 1.1	62.3 ± 1.5	-	-
15	Bge-2.2	20	77.21 ± 1.4	68.5 ± 0.4	-	-

***In vitro* hemolytic activity.** *In vitro* hemolytic activity of 15 marine fungal crude extracts is shown in fig. 1 and table 4. The result indicated that the hemolytic activity of almost all the strains were dependent on concentration: The effect of most ethyl acetate extracts (12 out of 15 extracts) on rabbit erythrocytes was obviously higher when the concentration varied from 25 µg.ml^{-1} to 500 µg.ml^{-1}. The result suggested that the fungal strains, especially strains Geu-1.2, Bge-2.2 and Bge-2.1, exhibited hemolytic activity on rabbit erythrocytes at high concentrations (250–500 µg.ml^{-1}). Fig. 1 and table 4 also showed lower hemolytic effect on rabbit red blood cells at all tested concentrations of crude extracts Kco-2.2, Geu-1.3 and Geu-1.4, indicating non-hemolytic effect of the fungal strains.

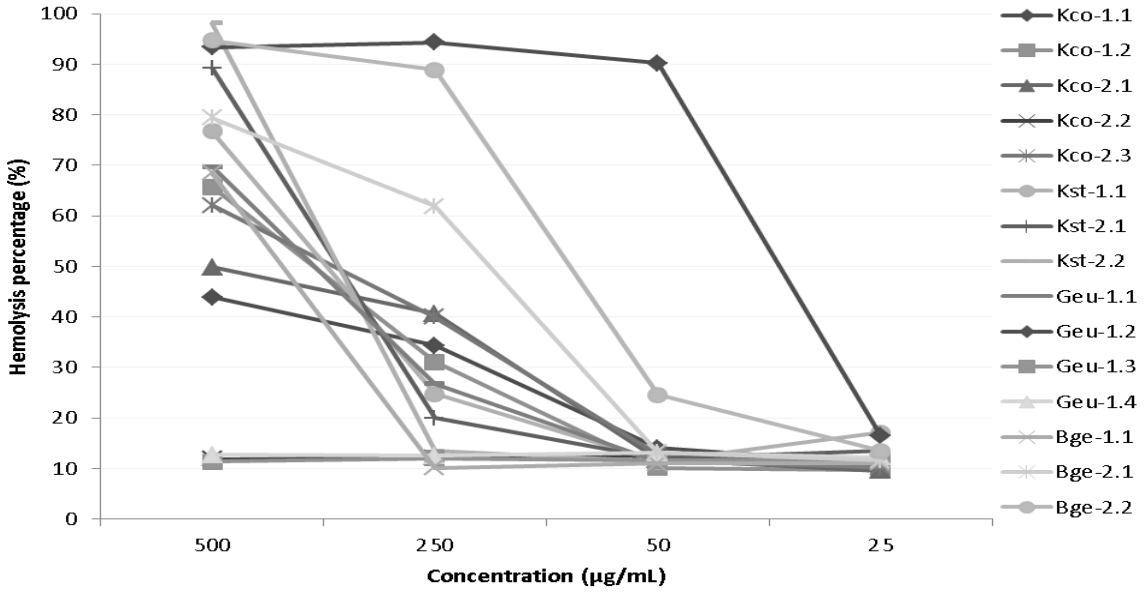

Fig. 1. Hemolysis percentages of fungal extracts in relation to concentratio***n***

Table 4. Hemolytic activity of fungal extracts

No.	Sample name	Conc. (μg.ml^{-1})	Hemolysis percentage (HL, %)	IC50 value (μg/ml)
	PBS buffer	-	8.77±0.12	ND*
1	Kco-1.1	500	43.93±0.09	826.66
2	Kco-1.2	500	65.74±0.12	393.43
3	Kco-2.1	500	49.84±0.14	508.55
4	Kco-2.2	500	11.83±0.15	ND
5	Kco-2.3	500	62.08±0.45	355.63
6	Kst-1.1	500	76.76±0.57	406.93
7	Kst-2.1	500	89.27±0.82	412.20
8	Kst-2.2	500	98.22±1.36	472.20
9	Geu-1.1	500	69.79±0.54	396.92
10	Geu-1.2	500	93.44±1.13	70.53
11	Geu-1.3	500	11.38±0.05	ND
12	Geu-1.4	500	12.72±0.14	ND
13	Bge-1.1	500	68.55±0.41	489.62
14	Bge-2.1	500	79.38±0.05	195.41
15	Bge-2.2	500	94.72±0.65	140.10

Note: *Symbols: ND indicates "not detected".

Since all fungal extracts were not hemolytic to animal erythrocytes at lower concentration of 25 μg.ml^{-1}, it may thus be possible to use the fungal extract in treating microbial infection and cancer cells at such lower concentrations. Admittedly further studies of application aspect of these crude extracts in animal model are required.

DISCUSSION

It is well known that chemical compounds possessing potent biological activity may not be useful in pharmacological preparations if they are hemolytic. The results contribute to screening of fungal extracts, aiming to select bio-pharmaceutically potential candidates for further studies on application aspects. Besides, hemolysis and antimicrobial activity have been used to search for surfactant-producing bacteria [13], therefore the present result may be applied in further research of the specific bacterial group.

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REFERENCES

[1] Montaser, R., and Luesch, H., 2011. Marine natural products: a new wave of drugs?. *Future Medicinal Chemistry*, **3**(12), 1475–1489.

[2] Kong, D. X., Jiang, Y. Y., and Zhang, H. Y., 2010. Marine natural products as sources of novel scaffolds: Achievement and concern. *Drug Discovery Today*, **21**(15), 884–886.

[3] Bugni, T. S., and Ireland, C. M., 2004. Marine-derived fungi: a chemically and biologically diverse group of microorganisms. *Natural Product Reports*, **21**(1), 143–163.

[4] König, G. M., Kehraus, S., Seibert, S. F., Abdel-Lateff, A., and Müller, D., 2006. Natural products from marine organisms and their associated microbes. *ChemBioChem*, **7**(2), 229–238.

[5] Knutsen, S. H., Myslabodski, D. E., Larsen, B., and Usov, A. I., 1994. A modified system of nomenclature for red algal galactans. *Botanica Marina*, **37**(2), 163–170.

[6] Ponce, N. M., Pujol, C. A., Damonte, E. B., Flores, M. L., and Stortz, C. A., 2003. Fucoidans from the brown seaweed Adenocystis utricularis: Extraction methods, antiviral activity and structural

studies. *Carbohydrate Research*, **338**(2), 153–165.

[7] Fleurence, J., 1999. Seaweed proteins: biochemical, nutritional aspects and potential uses. *Trends in Food Science & Technology*, **10**(1), 25–28.

[8] Dawczynski, C., Schubert, R., and Jahreis, G., 2007. Amino acids, fatty acids, and dietary fibre in edible seaweed products. *Food Chemistry*, **103**(3), 891–899.

[9] Le Hau, N., Ly, B. M., Van Huynh, T., and Trung, V. T., 2015. New records of marine algae in Vietnam. *Ocean Science Journal*, **50**(2), 221–229.

[10] Van Nguyen, T., Le, N. H., Lin, S. M., Steen, F., and De Clerck, O., 2013. Checklist of the marine macroalgae of Vietnam. *Botanica Marina*, **56**(3), 207–227.

[11] Zhang, Y., Mu, J., Feng, Y., Kang, Y., Zhang, J., Gu, P. J., Wang, Y., Ma, L. F., and Zhu, Y. H., 2009. Broad-spectrum antimicrobial epiphytic and endophytic fungi from marine organisms: isolation, bioassay and taxonomy. *Marine Drugs*, **7**(2), 97–112.

[12] Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., and Boyd, M. R., 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. *JNCI: Journal of the National Cancer Institute*, **82**(13), 1107–1112.

[13] Romanenko, L. A., Uchino, M., Kalinovskaya, N. I., and Mikhailov, V. V., 2008. Isolation, phylogenetic analysis and screening of marine mollusc-associated bacteria for antimicrobial, hemolytic and surface activities. *Microbiological research*, **163**(6), 633–644.