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Screening marine algae from China for their antitumor activities

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Abstract

Thirty-nine species of marine algae collected from the coast of China were screened for their antitumor activities, and eight species *Leathesia difformes, Polysiphonia urcedata, Scytosiphon lomentarius, Gloiopeliis furcata, Punctaria latifolia, Symphyocladia latiuscula, Rhodomela confervoides* and *Ulva pertusa* showed potent cytotoxic activities. Three, *Rhodomela confervoides*, *Scytosiphon lomentarius* and *Gloiopeliis furcata*, were used for further investigation. More than 30 compounds were isolated and purified, and 14 bromophenols, 1 steroid and 1 carotene were identified by advanced spectroscopic methods including IR, MS, and NMR techniques. Amongst the 16 identified compounds, 7 showed vigorously selective activities against KB, Bel7402 and A549 cancer cells, and 6 bromophenols were new compounds.

Abbreviations: IR, infrared spectroscopy; MS, mass spectroscopy; NMR, nuclear magnetic resonance.

Introduction

Drug discovery has been developed greatly in the process of screening large numbers of pure organic compounds or crude extracts to provide new lead. And large-scale screening will continue to play an important role in the procedure of developing new drugs. Marine algae have been historically an exceptionally rich source of pharmacologically active metabolites with antineoplastic, antimicrobial and antiviral effects (Faulkner, 2000; Tziveleka et al., 2003). Random screenings were effective to have found marine algae with various biological activities (Gerwick et al., 1994; Harada et al., 1997), and many of these reports have been reviewed (Cannell et al., 1993; Nekhoroshev, 1996). In addition, some natural products previously ascribed to marine invertebrate animals were proved to be algal secondary metabolites (Scheuer, 1990).

The antitumor activity was one of the most important activities in marine drugs, and lots of algae and their metabolites showed potent cytotoxic activities (Fuller et al., 1994; Harada et al., 2002; Mayer and Gustafson, 2003; Sheu et al., 1997). These metabolites have played an important role in leading to new pharmaceutical compounds for antitumor drugs (Luesch et al., 1999; Patterson et al., 1994; Yoo et al., 2002). Several representative antitumor compounds from algae, such as Halomon, had been developed to the clinical phase (Egorin et al., 1996).

Bromophenol compounds have proved to be characteristic natural products in marine organisms including macroalgae, *Polysiphonia, Myagropsis, Sargassum, Rhodomela* (Flodin and Whitfield, 2000), marine invertebrate and the common seafood (Chung et al., 2003). The accumulated metabolites often showed various potentially biological activities including antibacterial, antioxidant and α-glucosidase inhibitor activities (Choi et al., 2000), but there were no reports of their antitumor activities. They were also special substances for marine fish and shrimp to possess the seafood flavor (Whitfield et al., 1999).

The coast of China has a rich algal flora, with more than 2590 species of seaweed recorded (Huang, 1994). And certain marine algae, such as *Ecklonia kuroma* and *Sargassum fusiforme*, have been used in traditional Chinese herbal medicine in treatment of cancer. But only a few new natural products have been isolated from algae in the South China Sea (Su et al., 1997). None relate to antitumor screening and new antineoplastic compounds of the marine algae in North China. In this study, eight species of algae with antitumor activities were screened out, and the cytotoxic activities of the seven compounds from the three candidate algae were reported for the first time. The aim of the paper was to discover new marine resource for antitumor medicine and to demonstrate that marine algae, which is distributed broadly in China, were potential candidate sources.

Materials and methods

Materials

Thirty-nine species of marine algae for screening were collected from the coast of Qingdao and Weihai, China, in May 2000. Those used for isolating antitumor compounds were collected at the same place and the same season in the next year and were identified by Prof. B.M. Xia, and were deposited at the Institute of Oceanology, Chinese Academy of Sciences.

Antitumor screening

The collected algae were rinsed first with seawater, and then with distilled water to remove all the epiphytes. Air-dried samples (15.0 g) were soaked in 100 mL methanol for 2 days at room temperature twice, then filtered and evaporated under reduced pressure below 40 °C. The dried extracts were subjected to antitumor assay with MTT protocol (Han, 1997). Of the three cell lines used, two were cancer cells, human oral epidermoid carcinoma KB and human colon cancer line HT-29, and the other was human normal cells NIH-3T3 used for control. The optical density (OD) of the wells was measured with a microplate reader at 550 nm as reference. The growth inhibition rate was calculated by the following equation: Inhibition rate $= (OD_{control} - OD_{treated})/OD_{control} \times 100\%$. IC_{50} was defined as the drug concentration that resulted in 50% growth inhibition.

Effects of extraction

Ten species of algae with abundant biomass, *Polysiphonia urcedata*,*Gloiopeliis furcata*,*Scytosiphon lomentarius*, *Gracilaria vervucosa*, *Sargassum thunbergii*, *Dictyopteris divaricata*, *Ulva pertusa*, *Grateloupia filicina*, *Enteromorpha liza* and *Grateloupia turuturu* were used to test for the effects of extraction. Each algal sample (15.0 g) was extracted by different solvents: methanol, ethanol, ethyl acetate (EtOAc), acetone, hexane, dichloromethane (DCM) and chloroform, respectively. The extracts were subjected to the antitumor assay by MTT method described earlier.

Compounds isolation

The air-dried samples (14.4 kg) were soaked in methanol for 3×48 h, and then the extracts were concentrated in vacuum at low temperature, and were partitioned between EtOAc and H_2O. The EtOAc phase (594.6 g) was further fractionated by silica gel column chromatography with stepped gradient from petroleum ether to petroleum ether/acetone 1:1, then from chloroform/methanol 5:1 to methanol. With various silica gel column chromatography methods together with Sephadex LH_{20}, recrystallization and reverse-phase preparative HPLC, pure compounds were isolated from petroleum ether/acetone 2:1 fraction to chloroform/methanol 3:1 fraction.

Structure identification

Compounds were identified by the spectrum analyses of ^{1}H NMR, ^{13}C NMR, DEPT, HMBC, HMQC, H-Hcosy, MS and IR. 1D- and 2D-NMR spectra were obtained at 300 and 75 MHz for ^{1}H and ^{13}C NMR, respectively, on an Inova 300 MHz spectrometer in acetone-d_6 with solvent peaks as references. EIMS and HREIMS data were measured with a Micromass Autospec-Ultima ETOF spectrometer. IR spectra were recorded as KBr disks on a Nicolet Impact 400 FT-IR Spectrophotometer. Melting points were determined on a XT-4 micro melting point apparatus.

Antitumor assay

The identified compounds were subjected to antitumor assay, and 2.0 mg of sample was used in the MTT assay. Three human cancer cell lines used were KB, hepatocellular carcinoma Bel7402 and lung cancer A549, while normal human HELF cell line was used

as the control. The MTT assay protocol was the same as the above.

Results

Algal screening

During the screening process of the crude extracts, 39 species of algae were tested for their antineoplastic activities using the KB and HT-29 cell lines (Table 1). Seven showed selective antitumor activities against both KB and HT-29 cells: four Rhodophyta (*Symphyocladia latiuscula*, *Rhodomela confervoides*, *Polysiphonia urcedata*,*Gloiopeliis furcata*) and three Phaeophyta (*Leathesia difformes, Punctaria latifolia*, *Scytosiphon lomentariu*). *Ulva pertusa* showed cytotoxicity to both tumor and normal cells.

Among all the algae, *Leathesia difformes* showed the most potential selective activity with the IC_{50} of 12.6 μg mL^{-1}against KB cells, the IC_{50} of 40.6 μg mL^{-1}against HT-29 cells. The extracts of *Polysiphonia urcedata* showed the most prominent cytotoxic activity against HT-29 cells ($IC_{50} = 26.0\,\mu$g mL^{-1}). *Symphyocladia latiuscula*, *Rhodomela confervoides* and *Punctaria latifolia* had also showed remarkable inhibition of KB cells and extracts of the *Symphyocladia latiuscula,* and *Rhodomela confervoides* had showed cytotoxic activities towards HT-29 cells.

Effects of extraction

Two tumor cell lines and one normal NIH-3T3 cell line were used for exploring the extracting effects (Table 2). Ethanol was proved to be the best solvent, next was chloroform and the third was methanol. The ethanol extracts and chloroform extracts of *Polysiphonia urcedata*, the ethanol extracts of *Scytosiphon lomentarius* and the hexane extracts of *Dictyopteri divaricata* had strong selective cytotoxic activities against KB cells ($IC_{50} < 4.40\,\mu$g mL^{-1}). The ethanol extracts of *Scytosiphon lomentarius* had mighty activities against HT-29 cells ($IC_{50} = 1.49\,\mu$g mL^{-1}).

Isolation and identification

From the preceding results, seven kinds of algae with powerful selectively antitumor activities were selected. But considering the biomass, three algae, *Rhodomela confervoides*, *Scytosiphon lomentarius* and *Gloiopeliis furcata* were chosen to isolate and purify antitumor compounds. Amongst

Table 1. $IC_{50}$of methanol extracts of marine algae toward tumour and normal cells.

Algae	KB	HT-29	NIH-3T3
Rhodophyta			
Symphyocladia latiuscula	36.2	43.3	>50
Rhodomela confervoides	37.0	40.5	>50
Polysiphonia urcedata	40.0	26.0	42.36
Chondria tenuissima	42.7	>50	>50
Gloiopeliis furcata	35.7	38.2	>50
Ceramium japonicum Okam	>50	49.3	>50
Ceramium kondoi	>50	>50	>50
Gracilaria sjoestedtii Kylin	>50	>50	>50
Gracilaria vervucosa	>50	>50	>50
Ceramium boydenii Gepp	>50	>50	>50
Porphyra yezoensis	>50	>50	>50
Ahnfeltiopsis flabelliformis	>50	>50	>50
Grateloupia filicina	>50	>50	>50
Chondrus ocellatus	>50	>50	>50
Corallina officinales	>50	>50	>50
Grateloupia turuturu Yamada	>50	>50	>50
Chrysymenin wrightii	>50	>50	>50
Heterosiphonia japonica	>50	>50	>50
Hyalosiphonia caespitcsa	>50	>50	>50
Gelidium amansii Lamx	>50	>50	>50
Chlorophyta			
Ulva pertusa	29.9	14.9	23.8
Enteromorpha liza	>50	>50	>50
Codium fragile	>50	>50	>50
Phaeophyta			
Leathesia difformes	12.65	40.60	>50
Punctaria latifolia	38.99	45.42	>50
Scytosiphon lomentariu	45.3	32.3	48.7
Leathesia sp.	>50	>50	>50
Sargassum thunbergi	>50	>50	>50
Sargassum kjelimanianum	>50	>50	>50
Myelophycus simplex	>50	>50	>50
Undaria pinnatifida	>50	>50	>50
Sargassum fusiforme	>50	>50	>50
Laminaria japonica	>50	>50	>50
Ectocarpus confervoides	>50	>50	>50
Desmarestia viridis	>50	>50	>50
Chorda filum	>50	>50	>50
Dictyopteris divaricata	>50	>50	>50
Calpomenia sinuosa	>50	>50	>50
Punctaria plantaginea	>50	>50	>50

Note. Values are given in (μ gmL^{-1}).

Table 2. Antitumor activity (IC_{50}) of the algal extracts using different solvents.

Algae	Solvent	KB	HT-29	NIH-3T3
Polysiphonis urcedata	Methanol	40.0	26.0	42.3
	Ethanol	<0.50	>50	>50
	EtOAc	>50	>50	>50
	Acetone	14.3	34.3	42.2
	Hexane	23.5	11.7	41.5
	DCM	>50	>50	>50
	Chloroform	2.02	>50	>50
Scytosiphon lomentarius	Methanol	45.3	32.3	48.7
	Ethanol	12.6	1.49	35.3
	EtOAc	>50	17.2	25.9
	Acetone	>50	>50	>50
	Hexane	49.7	>50	>50
	DCM	>50	>50	>50
	Chloroform	>50	>50	>50
Sargassum thunbergii	Methanol	>50	>50	>50
	Ethanol	>50	>50	>50
	EtOAc	>50	>50	>50
	Acetone	27.2	>50	>50
	Hexane	>50	>50	>50
	DCM	>50	>50	>50
	Chloroform	25.0	>50	>50
Ulva pertusa	Methanol	29.9	14.93	23.89
	Ethanol	22.2	>50	44.1
	EtOAc	33.9	47.4	43.1
	Acetone	44.8	27.4	44.9
	Hexane	>50	44.8	>50
	DCM	42.7	40.5	42.9
Gloiopeliis furcata	Methanol	35.7	38.2	>50
	Ethanol	11.8	>50	>50
	EtOAc	39.3	12.2	>50
	Acetone	41.7	>50	>50
	Hexane	>38.3	>40.0	>50
	DCM	>23.4	>50	>50
	Chloroform	>19.8	>50	>50
Gracilaria vervucosa	Methanol	>50	>50	>50
	Ethanol	>50	>50	>50
	EtOAc	22.9	>50	>50
	Acetone	>50	>50	>50
	Hexane	>50	>50	>50
	DCM	>50	>50	>50
	Chloroform	>50	>50	>50
Dictyopteris divarticata	Methanol	>50	>50	>50
	Ethanol	>50	>50	>50
	EtOAc	>50	>50	>50
	Acetone	>50	>50	>50
	Hexane	4.40	>50	>50
	DCM	47.1	42.8	>50
	Chloroform	21.6	30.4	>50
Grateloupia filicina	Methanol	>50	>50	>50
	Ethanol	47.5	45.7	>50
	EtOAc	25.9	>50	>50
	Acetone	>50	46.5	34.9
	Hexane	27.3	49.7	20.9
	DCM	48.6	41.0	13.0
Enteromorphaliza	Methanol	>50	>50	>50
	Ethanol	>50	>42.4	>50
	EtOAc	48.6	>50	>50
	Acetone	>43.7	>50	>42.4
	Hexane	>50	>50	>50
	DCM	>50	>42.3	>48.9
	Chloroform	>48.3	>47.4	>50
Grateloupia turuturu	Methanol	>50	>50	>50
	Ethanol	>50	>50	>50
	EtOAc	46.1	>50	>50
	Acetone	>50	>50	>50
	Hexane	>50	>50	>50
	DCM	>50	>50	>50
	Chloroform	>50	>50	>50

Note. Values are given in μg mL^{-1}.

the 30 compounds isolated, some pigment and steroids, such as (3R,3′R)-3,3'-dihydroxy-β-carotene (Zeaxanthin, **15**) and fucosterol (**16**), were identified from *Scytosiphon lomentarius* and *Gloiopeliis furcata*. Fourteen bromophenols were identified from the *Rhodomela confervoides*. They are 3,4-dibromo-5-(ethoxymethyl)-1,2-benzenediol(**1**),2,3-di bromo-4,5-didroxy benzyl alcohol(**2**), 2,3-dibromo-4,5-dihydroxy-benzaldehyde (**3**), 3-bromo-4-[2,3-dibromo-4,5-dihydroxyphenyl]methyl-5-(methoxymethyl)-1,2-benzenediol(**4**),3-bromo-4-[2,3-dibromo-4,5-dihydroxyphenyl]methyl-5-(hydroxymethyl)-1,2-ben zenediol(**5**),3-bromo-4-[2,3-dibromo-4,5-dihydroxy phenyl]methyl-5-(ethoxymethyl)-1,2-benzenediol(**6**), 3,4-dibromo-5(methoxymethyl)-1,2-benzenediol (**7**), 3-bromo-4,5-dihydroxy-benzaldehyde (**8**), 3-bromo-4,5-dihydroxy-benzoic acid methyl ester(**9**), 2-methyl-3(2,3-dibromo-4,5-dihydroxy)-phenyl-propylaldehyde (**10**), 2- methyl-3(2,3- dibromo-4,5-dihydroxy)-

phenyl-propnal methyl hemiacetal (**11**), 3-bromo-4,5-bis(2,3-dibromo-4,5-dihydroxybenzyl)-1,2-benzenediol (**12**), 4,4'-methylenebis(5,6-dibromo-1,2-benzenediol) (**13**), and bis(2,3-dibromo-4,5-dihydroxybenzyl)ether (**14**), resp ectively. Among them, **5**, **6, 9**, **10**, **11**, and **12** were new compounds, and their structure identifications were published previously (Fan et al., 2003).

Cytotoxic assay

All identified compounds were further analyzed for their antitumor activities, and the results are listed in Table 3. Seven compounds (**4**, **8**, **9**, **10**, **11**, **14**, **15**) showed selective cytotoxic activity against KB cells. Four of them (**4**, **8**, **9, 15**) showed selective cytotoxic activity against Bel7402 cells. Compounds **8** and **9** showed selective antitumor activity against KB, Bel7402 and A549 cells.

Discussion

The antitumor compounds might be metabolites produced during the life process under a particular environment, and various activities might be explored in the same alga when collected in different living environment and different life phase (Patterson et al., 1984). Therefore, it was very important to elucidate the harvested place and season of the samples. Combined with Numata's results, the extracts of *Sargassum thunbergii* and *Gelidium amansii* showed inhibition activities towards P388, but no antitumor activities against KB and HT-29 cells (Numata et al., 1991).

The selective antitumor activity with no side effects was vital for a candidate drug to be a clinical medicine, and not all algal metabolites were found to have such selective antitumor activities, and so normal cells were usually used as the control in the screening course. In this study, the extracts of the *Ulva pertusa*, which was usually considered as an antitumor Chinese medicine, showed cytotoxic activity to both normal and tumor cells. So it shouldn't be considered as a good candidate for isolating antitumor compounds even if it had strong activities against tumor cells and the largest biomass at the Chinese coast.

Amongst the eight species of algae with potential antitumor activities, *Rhodomela confervoides* was the

Table 3. Cytotoxic activity of the identified compounds.

	Compounds	KB	Bel7402	A549	HELF
1	3,4-dibromo-5-(ethoxymethyl)-1,2-benzenediol	4.27	3.94	—	2.15
2	2,3-dibromo-4,5–didroxybenzyl alcohol	2.80	4.02	3.14	2.80
3	2,3-dibromo-4,5–dihydroxy-benzaldehyde	7.32	9.54	8.14	7.32
4	3-bromo-4-[2,3-dibromo-4,5-dihydroxyphenyl]methyl-5 -(methoxymethyl)-1,2-benzenediol	*2.68*	*1.56*	—	3.88
5	3-bromo-4-[2,3-dibromo-4,5-dihydroxyphenyl]-methyl-5-(hydroxymethyl)-1,2- benzenediol	–	–	–	–
6	3-bromo-4-[2,3-dibromo-4,5-dihydroxyphenyl]-methyl-5-(ethoxymethyl)-1,2-benzenediol	6.73	7.31	9.81	3.57
7	3,4-dibromo-5 (methoxymethyl)-1,2-benzenediol	6.26	3.33	7.08	2.65
8	3-bromo-4,5-dihydroxy-benzaldehyde	*8.71*	*5.36*	*7.56*	–
9	3-bromo-4,5-dihydroxy -benzoic acid methyl ester	*3.09*	*3.18*	*3.54*	6.39
10	2-methyl-3(2,3-dibromo-4,5-dihydroxy)-phenyl propylaldehyde	*1.89*	6.58	4.16	4.27
11	2-methyl-3(2,3- dibromo-4,5-dihydroxy)-phenyl-propnal methyl hemiacetal	*2.52*	7.95	9.92	4.12
12	3-bromo-4,5-bis (2,3-dibromo-4,5-dihydroxybenzyl)-1,2-benzenediol	–	–	–	–
13	4,4′methylenebis (5,6-dibromo-1,2-benzenediol)	6.69	3.27	7.57	6.69
14	bis(2,3-dibromo-4,5-dihydroxybenzyl) ether	*4.19*	7.94	–	7.41
15	Zeaxanthin	*8.31*	*7.06*	–	–
16	Fucosterol	–	–	–	–

Note.: Values are given in μg mL^{-1}. "–" indicates IC$_{50}$ > 10.0, μg mL^{-1}.

most suitable to be developed into a natural medicine. On one hand, it is easy to cultivate, and so sufficient material could be obtained to isolate enough antitumor compounds. On the other hand, the main compounds showed the most prospective cytotoxic activities, which prompted us to research and develop them into a clinic drug.

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