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Algicidal activities of secondary metabolites of marine macroalgal-derived endophytic fungi*

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Abstract Red tide occurrs frequently and causes significant damage to the environment and human health. As a result, development of new efficient and environment friendly red-tide microalgae inhibitors has gained increasing attention in recent times. Algicolous endophytic fungi with unique habitats are promising sources for active agents owing to their abundant secondary metabolites and distinguished activities. In this study, the algicidal activities of 49 marine macroalgal-derived endophytic fungi against phytoplankton *Alexandrium tamarense*, *Prorocentrum donghaiense*, *Heterosigma akashiwo*, and *Chattonella marina* were examined using 96-well microplate. Four fungal strains, including *Aspergillus wentii* (pt-1), *A. ustus* (cf-42), and *A. versicolor* (dl-29, pt-20), exhibited potent algicidal activities. A total of 32 pure compounds isolated from these fungi were noted to possess different degrees of algicidal activities. Of those, 11 compounds comprising five anthraquinones, two terpenoids, and four steroids showed high 24-h inhibition rates for the four red tide algae, with 24 h EC_{50} values ranging from 0.01 to 14.29 μg/mL. Among them, compound **1** (1,5-dihydroxy-3-methoxy-7-methylanthraquinone) presented the strongest activity against *H. akashiwo*, and could decrease its chlorophyll *a* (Chl *a*) and superoxide dismutase contents and increase the soluble protein, malondialdehyde, and peroxidase contents. These results suggested that the identified anti-algal compound might inhibit the growth of red tide algae by weakening photosynthesis (reducing Chl *a* content), destroying cell membrane, and damaging the antioxidant system.

Keyword: algicidal activity; endophytic fungus; secondary metabolite; marine alga

1 INTRODUCTION

Red tide caused by the outbreak of some planktonic algae and protozoans under certain conditions could significantly change the structure of marine organisms (Song et al., 2003), damage marine fishery, marine aquaculture, and coastal tourism, and even lead to human poisoning and death (Zhou and Zhu, 2006). Besides, red tide could result in heavy losses to the local ecological environment and economic development. Therefore, the use of red tide algae inhibitors has been one of the important contingency measures because of their rapid effects on inhibiting the growth of red tide microalgae (Murray-Gulde et al., 2002). Allelochemicals have attracted significant attention owing to their species-specific effect and ecological safety (Jin and Dong, 2003).

In certain habitat, macroalgal-derived endophytic fungi could produce multifarious secondary metabolites, which are considerably different from the substances produced by other organisms (Ji and Wang, 2016). Rateb and Ebel (2011) showed that the number of new compounds obtained from alga-endophytic fungi accounted for 21% of all the new compounds from marine fungi. These compounds,

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including sesquiterpenes, diterpenoids, steroids, polyketides, and alkaloids, have significant antibacterial, antioxidant, anti-plasmodium, and anticancer activities. However, majority of the studies on secondary metabolites of algal-derived endophytic fungi have focused on medicinal activities rather than algicidal activities (Ji and Wang, 2016). Moreover, most of the algicidal bioassays have examined *Chlorella fusca* (Osterhage et al., 2000; Krohn et al., 2005), the common marine microalgae, but the red tide algae have rarely been considered (Chen et al., 1996). Based on the available reports (Ji and Wang, 2016), it may be hypothesized that some of the secondary metabolites of endophytic fungi isolated from marine macroalgae could inhibit red tide algae. Herein, the algicidal activities of secondary metabolites isolated and identified from the marine macroalgal-derived endophytic fungi are described against red tide algae *Alexandrium tamarense*, *Prorocentrum donghaiense*, *Heterosigma akashiwo*, and *Chattonella marina*.

2 MATERIAL AND METHOD

2.1 Material

Alexandrium tamarense and *P. donghaiense* were kindly provided by Xiamen University, China, *H. akashiwo* was kindly provided by Harbin Institute of Technology, Weihai, China, and *C. marina* was isolated from Yantai seaside by Dr. YANG Cuiyun, Yantai Institute Coastal Zone Research, CAS (Chinese Academy of Sciences). The algae were cultured in a growth chamber containing f/2 medium at 20°C under 14 h:10 h light-dark (LD) cycle with a light density of 2 000 lx.

The fungal strains, preserved in Yantai Institute Coastal Zone Research, CAS, were isolated from the fresh tissue of surface-sterilized marine macroalgae collected from the seaside of China. All the strains were cultured in potato dextrose broth (PDB) at 28°C in a growth chamber.

2.2 Preparation of crude extract of secondary metabolites

The initial fungal cultures were maintained on potato dextrose agar (PDA) plate. Subsequently, pieces of mycelia were cut into small segments and aseptically inoculated into 1 000-mL Erlenmeyer flasks containing 300 mL of PDB, and static fermentation was performed for 30 days at room temperature (25°C). After 30 days, the entire culture (300 mL) was filtered through cheesecloth to separate the mycelia. The culture broth (300 mL) was extracted three times with EtOAc to afford extract I after removal of the solvent by evaporation (40°C) at reduced pressure. The dried and powdered mycelia were extracted three times with a mixture of $CHCl_3$ and MeOH (1:1, v/v) and concentrated by evaporation at reduced pressure to yield the gum. Then, the gum was partitioned between H_2O and EtOAc. The EtOAc soluble part was collected and evaporated at reduced pressure to afford extract II. As the thin-layer chromatography (TLC) profiles of the two extracts were nearly identical, they were combined to obtain the crude extract.

2.3 Algicidal activities of crude extracts and pure compounds

Rapid bioassay (Schrader et al., 1997) was used to evaluate the algicidal activities of the crude extracts of secondary metabolites from the algal-derived endophytic fungi. In brief, the stock solution of the crude extract was dissolved in 100% DMSO (technical-grade) and added (2 μL per well) to each well of the microplate with 198 μL microalgal culture containing with medium in a well, respectively. The final volume of each wells were 200 μL. The plates were placed in a growth chamber at 20°C and 14 h: 10 h LD cycle. After 24 h, the live algal cell number was counted using blood cell counting chamber. Three replications were used for each treatment and the control (2 μL DMSO per well). The 24-h inhibition rates (IRs) were calculated as follows:

$$\text{Inhibition rate (IR)}=(N_{CK}-N_T)/N_{CK}\times 100\%,$$

where N_{CK} is the live algal cell number of the control group; N_T, live algal cell number of the treatment group.

The crude extracts with high 24-h IRs were considered to have high algicidal activities. The 24-h IRs of the pure compounds isolated from these crude extracts (Liu, 2012; Liu et al., 2012, 2013, 2014; Miao et al., 2012; Sun et al., 2013) were determined according to the above-mentioned procedure, and the 24-h inhibition concentration (24 h EC_{50}) of the compounds with high IR was measured based on the method developed by Schrader et al. (1997).

2.4 Inhibition mechanisms of compounds against *H. akashiwo* growth

As *H. akashiwo* generally presented higher growth

rate and cell density than the other three algae examined, the effects of compounds with highest algicidal activities on *H. akashiwo* were examined to explore the underlying mechanisms of growth inhibition of red tide algae.

The *H. akashiwo* cells in logarithmic phase were treated with DMSO (control), 40% 24 h EC_{50} of compound **1** (0.25 μg/mL), and 80% 24 h EC_{50} of compound **1** (0.50 μg/mL), respectively. After 24 h, the cell membrane integrity, Chl *a* content, soluble protein content, and malondialdehyde (MDA) content were investigated, and the activities of superoxide dismutase (SOD) and peroxidase (POD) were tested. All the experiments were performed in triplicate with control.

2.5 Cell membrane integrity

Characterization of microalgae as viable or nonviable at single-cell level is essential to determine the effects of antialgal agents. Impermeability of the cell membrane to nucleic acid dyes such as propidium iodide (PI) is one of the criteria for "viability" (Shapiro, 2008). Fluorescent dyes such as fluorescein diacetate (FDA) can only stain intact cell membrane, whereas macromolecular fluorescent dyes such as PI can infiltrate into the cell only when the cell membrane is damaged. In the present study, the fluorescence intensity of the algal cells in the treatment and control groups stained with FDA and PI, respectively, were assayed using flow cytometry (FCM).

The *H. akashiwo* cells in each group were stained with 10 μmol/L PI (Sigma, P4170) and 10 mg/L FDA (Sigma, F7378), respectively, and the cell membrane integrity was measured using FCA. Healthy cells took up FDA to convert fluorescein, which presented emission peak at 515–545 nm when excited at 488 nm. In contrast, cells with damaged cell membrane were stained with PI, which exhibited an emission peak at 600–620 nm when excited at 488 nm. The results obtained were expressed as relative fluorescence intensity.

2.6 Chl *a* content

The Chl *a* content in the algal cells was measured by spectrophotometric method as described by Wintermans and De Mots (1965). In brief, the harvested algal cells were resuspended in 95% ethanol solution and stored at 4°C in dark for 24 h. Then, the mixture was centrifuged at 4 000×*g* for 10 min at room temperature, and the supernatant was collected and its absorbance was measured at 665 and 649 nm by using a spectrophotometer. The Chl *a* content was calculated as follows:

Chl *a*=13.7×A665–5.76×A649.

2.7 Soluble protein and MDA contents, and SOD and POD activities

A total of 30 mL of the harvested algal cells were centrifuged at 1 500×*g* for 10 min at room temperature, and the supernatant was discarded. Subsequently, 2 mL of 0.07% physiological saline were added to the cells and mixed well and centrifuged again. This procedure was repeated twice, and the cells were ground in a homogenizer in ice bath to break the cell wall. The cell debris was dissolved in 2 mL of 0.07% physiological saline and stored at -80°C. The soluble protein and MDA contents as well as SOD and POD activities were determined using assay kits (Jiancheng Biotech, Nanjing, China) according to the manufacturer's instructions.

2.8 Statistical analysis

One-way ANOVA and Duncan's test with P=0.05 were performed using SPSS software to examine the effects of different concentrations of the identified compounds on soluble protein content, MDA content, SOD activity, and POD activity in *H. akashiwo* cells.

3 RESULT

3.1 Algicidal activities of crude extracts

Except for some crude extracts that promoted algal growth, most of the crude extracts obtained showed varied algicidal activities (Table 1). None of the *H. akashiwo* cells could survive treatment with 50 μg/mL crude extracts from strains cf-8, cf-25-1, cf-32-1, dl-29, dl-34, pt-20, qd-20, qd-21, and yt-17. Similarly, treatment with 2.5 μg/mL crude extracts from strains cf-32-2, dl-4, lyg-8, pt-20, qd-20, qd-21, and yt-5 resulted in the death of all *C. marina* cells. Furthermore, 400 μg/mL crude extracts from strains cf-36-1, cf-25-1, lyg-11, cf-41-1, yt-21, cf-44-1, lyg-8, and hd-5 showed strong antialgal activities against *A. tamarense* with IR values higher than 70%, while 200 μg/mL crude extracts from strains yt-11, cf-32-1, cf-28-2, dl-29, na-5, pt-1, cf-42, yt-8, and yt-13 presented strong antialgal activities against *P. donghaiense* with IR values higher than 50%.

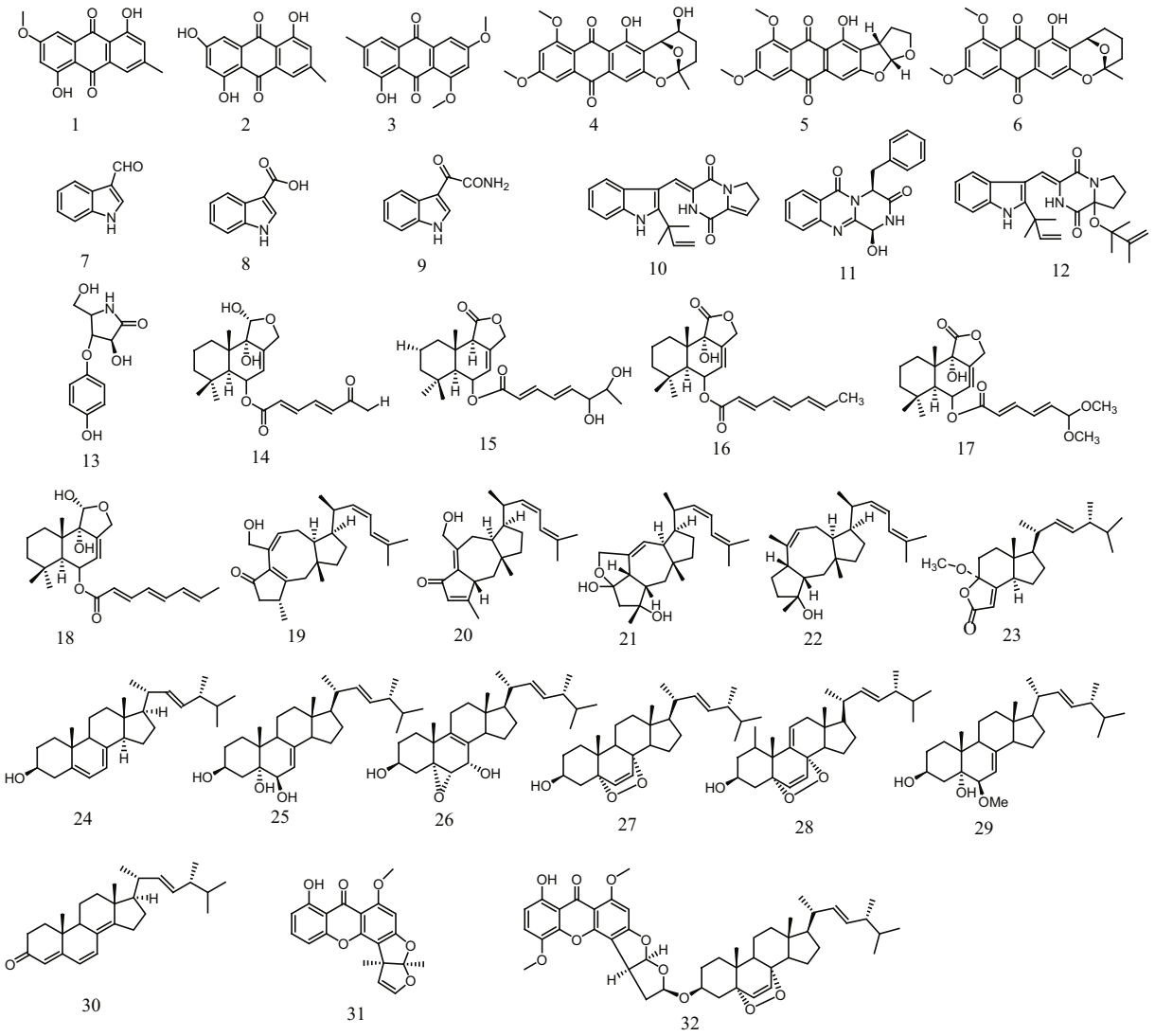

Fig.1 Structures of the compounds isolated from the secondary metabolites crude extracts of fungal strains dl-29, pt-1, pt-20, and cf-42

Compounds **1**-**6**, **7**-**13**, **14**-**22**, **23**-**30**, and **31**-**32** are anthraquinones, alkaloids, terpenoids, steroids, and others, respectively.

3.2 Algicidal activities of pure compounds

Based on their algicidal activities, TLC profile, and growth profile of the secondary metabolite crude extracts, fungal strains pt-20, pt-1, cf-42, and dl-29 were selected and cultured on a large scale. A total of 32 different compounds (Fig.1), comprising seven alkaloids, six anthraquinones, nine terpenes, eight steroids, and two other compounds, were isolated from the secondary metabolites crude extracts (Miao et al., 2012; Liu, 2012; Liu et al., 2012, 2013, 2014; Sun et al., 2013).

Among them, 11 compounds were considered to have strong algicidal activities (Tables 2 and 3). None of the *C. marina* cells could survive treatment with compounds **1**, **2**, **4**, **5**, **6**, **17**, **21**, **24**, **26**, and **30** at a final concentration of 5 μg/mL (Table 2), and their 24 h EC_{50} values ranged from 0.01 to 1.50 μg/mL (Table 3). Compounds **2**, **4**, and **5**, at a final concentration of 40 μg/mL, showed strong algicidal activities against *A. tamarense* with IR values higher than 95% (Table 2), and their 24 h EC_{50} values were in the range of 2.37–5.24 μg/mL (Table 3). Similarly, at a final concentrations of 2.5 μg/mL, compounds **1**, **2**, **4**, **17**, **24**, and **26** showed intense algicidal activities against *H. akashiwo* with IR values higher than 95% (Table 2), and their 24 h EC_{50} values ranged from 0.63 to 1.80 μg/mL (Table 3). The IRs of compounds **1** and **27** against *P. donghaiense* were both higher than 90% at a concentration of 20 μg/mL (Table 2), and their 24 h EC_{50} values were 4.24 and 14.29 μg/mL, respectively (Table 3).

Table 1 IRs (%, means±S.D.) of the crude extracts at concentrations of 2.5, 400, 50, and 200 μg/mL, respectively, against *C. marina*, *A. tamarense*, *H. akashiwo*, and *P. donghaiense*

Strain No.	*C. marina*	*A. tamarense*	*H. akashiwo*	*P. donghaiense*	Strain No.	*C. marina*	*A. tamarense*	*H. akashiwo*	*P. donghaiense*
cf-8	47.03±12.35	9.73±4.12	100.00±0.00	22.59±12.80	dl-6	-	3.90±2.91	-14.28±11.54	35.48±8.53
cf-25-1	49.32±5.81	74.76±8.90	100.00±0.00	18.82±16.16	hd-5	-	94.18±5.82	53.06±2.89	30.11±9.86
cf-27-1	69.86±5.48	26.63±6.73	86.39±9.43	48.39±11.63	kyg-1	-	65.06±2.06	97.28±2.36	44.36±3.42
cf-28-1	41.55±15.09	-36.87±4.12	98.64±2.36	41.94±12.80	lyg-11	-	76.70±11.65	14.28±5.77	2.42±1.14
cf-28-2	46.12±10.37	30.11±0.00	95.92±7.07	51.62±8.38	lyg-8	100.00±0.00	82.53±17.47	98.64±2.36	39.52±10.26
cf-30-2	54.79±5.81	-68.90±8.24	24.69 ±8.66	30.66±5.59	na-5	76.71±5.81	-13.57±4.12	91.84±8.16	54.04±10.26
cf-31-1	-	45.64±6.72	18.37±2.89	29.03±10.58	pt-1	-	35.93±8.24	55.10±0.96	56.45±6.84
cf-32-1	-	53.41±17.47	100.00±0.00	51.62±14.51	pt-20	100.00±0.00	37.88±6.72	100.00±0.00	13.64±7.82
cf-32-2	100.00±0.00	57.29±3.36	97.28±4.71	20.16±3.42	qd-19	33.33±12.95	-10.66±8.24	86.39±10.27	24.21±7.39
cf-35-1	69.60±7.75	3.90±4.12	83.67±2.89	38.72±7.39	qd-20	100.00±0.00	35.93±8.24	100.00±0.00	44.36±10.26
cf-36-1	35.16±8.37	74.76±18.72	95.92±7.07	35.49±7.39	qd-21	100.00±0.00	47.58±8.24	100.00±0.00	33.88±10.07
cf-41-1	-	76.70±15.41	94.56±9.43	-1.60 ±4.84	qd-23	39.73±3.87	2.93±1.37	46.94±12.24	24.21±7.39
cf-42	56.16±15.50	26.23±8.90	97.96±2.89	61.30±3.42	qd-24	56.16±7.75	-63.08±8.24	93.20±2.36	43.55±11.29
cf-44-1	37.90±3.16	80.59±14.66	95.92±4.08	37.90±5.70	qd-6	34.25±7.25	18.46±17.47	91.84±11.54	27.42±8.06
cf-44-2	-	53.41±8.24	98.64±2.36	30.66±9.45	rc-4	-	65.06±0.00	-8.84 ±9.43	26.88±9.45
cf-45-1	-	35.93±8.24	24.49±8.66	37.10±8.06	yt-1	-	38.85±14.56	76.87±16.50	26.62±17.15
cf-47-2	-	-26.19±8.90	90.48±2.36	15.34±3.42	yt-11	82.65±11.07	65.06±0.00	93.20±2.36	50.01±15.55
dl-26	96.35±4.19	35.93±16.47	89.12±8.50	44.62±17.77	yt-13	53.42±11.62	-14.54±2.74	79.59±5.77	94.62±3.36
dl-29	52.05±1.94	18.46±4.20	100.00±0.00	53.23±14.34	yt-15	60.73±8.81	53.41±8.24	93.20±2.36	36.02±9.17
dl-34	-	45.64±17.79	100.00±0.00	25.01±3.42	yt-16	99.09±1.58	33.02±4.12	97.28±4.71	25.00±13.71
dl-36	31.51±11.62	61.17±8.90	85.71±6.12	45.17±7.39	yt-17	-	-42.69±4.12	100.00±0.00	20.98±12.18
dl-37	49.77±1.58	26.63±6.73	97.28±4.71	23.66±16.94	yt-21	56.16±3.87	79.62±4.12	25.17±10.27	29.57±16.16
dl-39	26.03±15.25	57.29±3.36	3.40±6.23	37.11±9.68	yt-5	100.00±0.00	50.50±12.35	91.84±4.08	35.48±1.61
dl-4	100.00±0.00	38.84±4.12	98.64±2.36	34.95±9.17	yt-8	99.09±1.58	53.41±8.24	93.20±4.71	62.37±6.72
dl-5	32.42±15.09	-86.37±8.24	94.56±6.23	15.34±3.42					

"-" means no further experiments were conducted because of the weak algicidal activities in preliminary tests.

A total of 11 compounds with lower EC_{50} values were screened out, including five anthraquinones (compounds **1**, **2**, **4**, **5**, **6**), two terpenoids (compounds **17**, **21**), and four steroids (compounds **24**, **26**, **27**, **30**). It can be observed from Table 3 that compound **1** (1,5-dihydroxy-3-methoxy-7-methylanthraquinone) had the highest algicidal activity against *C. marina*, *H. akashiwo*, and *P. donghaiense*, and had higher algicidal activity against *A. tamarense*.

3.3 Inhibitory mechanisms of compound 1 against *H. akashiwo* growth

3.3.1 Cell membrane integrity

As shown in Fig.2 and Table 4, the integrity of the algal cell membrane was destroyed by compound **1**. The relative fluorescence intensity was 35 466 for the control group stained with FDA, whereas the values decreased to 1 528 and 1 173 for the 40% 24 h EC_{50} and 80% 24 h EC_{50} treatment group, which were only 4.31% and 3.31% of that of the control group, respectively. In contrast, the relative fluorescence intensity for the control group stained with PI was 2 775, whereas the values increased to 3 562 and 18 129 for the 40% 24 h EC_{50} and 80% 24 h EC_{50} treatment group, respectively. These results clearly showed that the integrity of the algal cell membrane was severely destroyed by compound **1** even at the concentration of 40% 24 h EC_{50}.

3.3.2 Chl *a* content

The Chl *a* content in the algal cells significantly decreased with the increasing concentration of compound **1** (Fig.3). Treatment with compound **1** at a concentration of 80% 24 h EC_{50} resulted in a partial reduction in the Chl *a* content, which was 11.65%

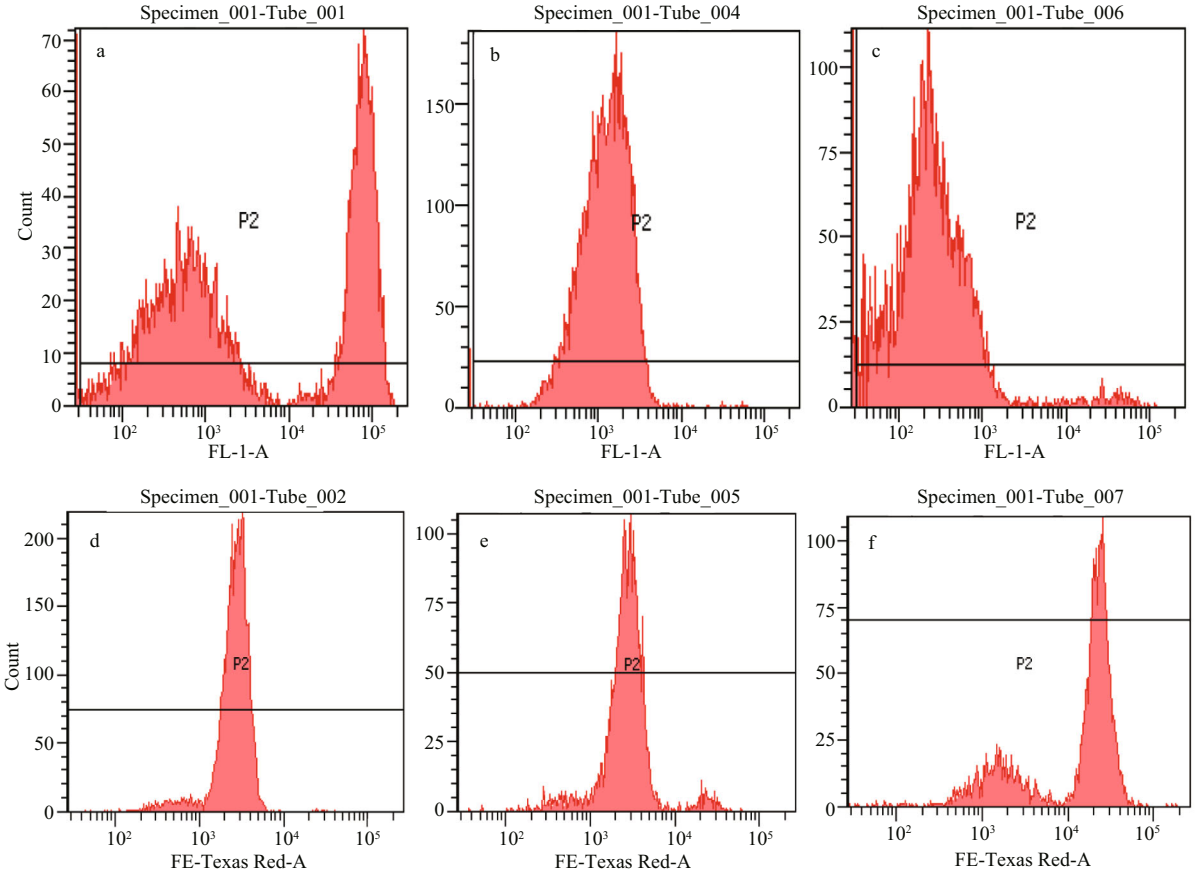

Fig.2 FCM spectra of *H. akashiwo* cells

a. control stained with FDA; b. cells treated with compound **1** at 40% 24 h EC_{50} and stained with FDA; c. cells treated with compound **1** at 80% 24 h EC_{50} and stained with FDA; d. control stained with PI; e. cells treated with compound **1** at 40% 24 h EC_{50} and stained with PI; f. cells treated with compound **1** at 80% 24 h EC_{50} and stained with PI.

Table 2 IRs of pure compoundsat concentrations of 5, 40, 2.5, and 20 μg/mL, respectively, against *C. marina*, *A. tamarense*, *H. akashiwo*, and *P. donghaiense* (%, means±S.D.)

Compound No.	*C. marina*	*A. tamarense*	*H. akashiwo*	*P. donghaiense*	Compound No.	*C. marina*	*A. tamarense*	*H. akashiwo*	*P. donghaiense*
1	100.00±0.00	90.16±9.84	100.00±0.00	100.00±0.00	**17**	100.00±0.00	63.49±3.37	100.00±0.00	29.17±6.50
2	100.00±0.00	96.83±5.50	98.31±2.94	9.12±7.45	**18**	98.00±3.46	47.53±0.00	35.60±5.87	53.12±11.26
3	-	93.44±7.51	62.72±12.79	53.13±13.62	**19**	99.40±1.04	94.36±2.84	72.89±10.58	69.79±4.78
4	100.00±0.00	98.41±2.75	100.00±0.00	46.87±5.41	**20**	15.34±9.96	68.85±7.51	-	7.29±12.63
5	100.00±0.00	100.00±0.00	93.22±11.74	29.69±6.63	**21**	100.00±0.00	36.06±4.92	-	44.79±18.79
6	100.00±0.00	77.78±7.27	-	53.12±11.27	**22**	70.40±12.01	66.50±4.77	36.45±10.78	89.58±10.98
7	-	36.06±9.84	-	-	**23**	82.40±3.02	66.67±12.6	49.16±10.17	89.58±10.98
8	-	70.49±4.92	-	82.29±15.41	**24**	100.00±0.00	2.18±3.78	98.31±2.94	89.58±9.55
9	-	3.82±6.82	-	4.69±4.83	**25**	-	22.22±6.74	-	51.04±10.98
10	25.63±8.66	14.29±4.78	-	67.71±13.01	**26**	100.00±0.00	-14.29±6.74	96.61±2.94	69.80±10.06
11	22.81±10.48	55.56±16.72	-	16.67±18.31	**27**	49.61±13.83	26.22±4.92	-	90.62±3.13
12	-	39.68±33.45	-	59.37±8.27	**28**	37.21±13.91	57.37±15.03	-	87.50±5.41
13	-	40.98±13.91	-	26.04±17.77	**29**	99.60±0.69	47.62±6.73	-22.01±15.25	75.00±16.54
14	-	6.54±0.00	49.16±16.74	75.00±5.41	**30**	100.00±0.00	58.19±8.40	88.14±16.34	55.21±15.73
15	-	10.65±10.65	-	-	**31**	-	49.17±12.38	-	85.94±2.21
16	99.60±0.69	28.57±0.00	25.44±7.77	48.44±6.63	**32**	-	90.16±9.84	-	23.96±13.01

"-" means no further experiments were conducted because of the weak algicidal activities in preliminary tests.

lower than that noted in the control (P<0.05). These results indicated that the active compounds in the secondary metabolites of algal-derived endophytic fungi could reduce the Chl *a* content in the algal cells.

3.3.3 Soluble protein and MDA contents, and SOD and POD activities

Figure 4a illustrates that both 40% and 80% 24 h EC_{50} of compound **1** significantly increased the soluble protein contents in *H. akashiwo* cells (P<0.05).

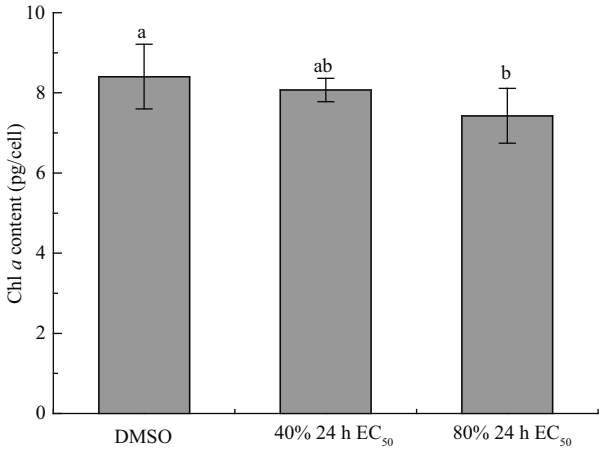

Fig.3 Chl *a* content in *H. akashiwo* cells treated with DMSO (control), 40% 24 h EC_{50} of compound 1, and 80% 24 h EC_{50} of compound 1

Table 3 24 h EC_{50} values of the compounds with strong algicidal activities (μg/mL)

Compound No.	*C. marina*	*A. tamarense*	*H. akashiwo*	*P. donghaiense*
1	0.17	/	0.63	4.24
2	0.44	5.24	1.22	/
4	0.01	2.37	1.39	/
5	0.17	3.21	/	/
6	0.41	/	/	/
17	1.50	/	1.35	/
21	0.57	/	/	/
24	1.18	/	1.80	/
26	1.09	/	1.03	/
27	/	/	/	14.29
30	1.39	/	/	/

"/" means no further experiments were conducted because of the weak algicidal activities in preliminary tests.

Table 4 Relative fluorescence intensity after PI and FDA staining

Sample	FDA stained	PI stained
Control	35 466	2 775
40% EC_{50}	1 528	3 562
80% EC_{50}	1 173	18 129

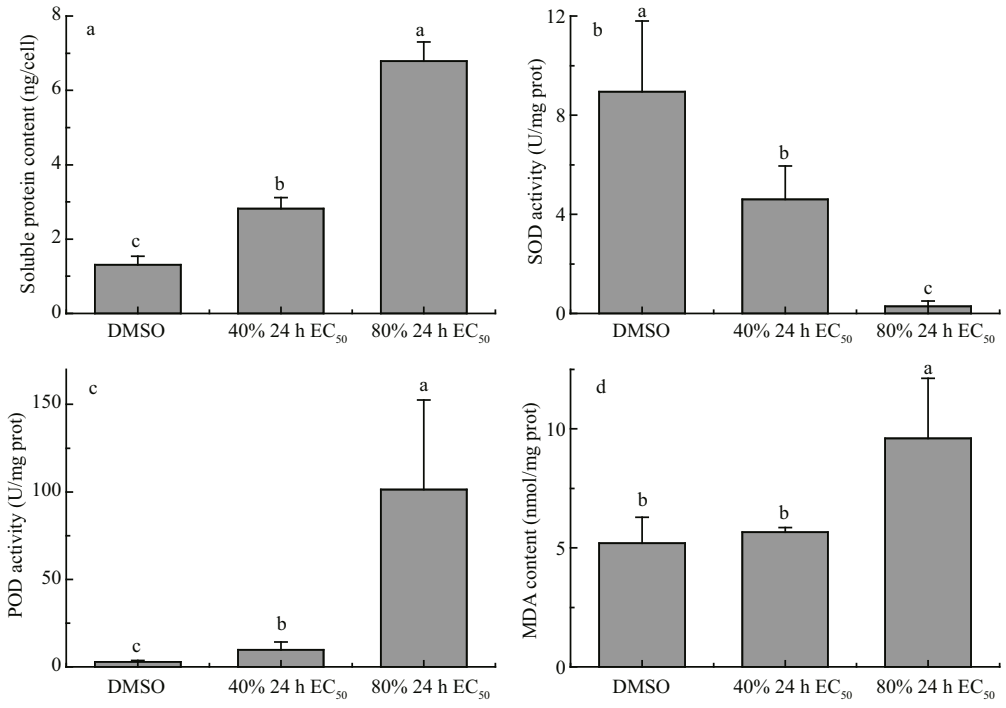

Fig.4 Soluble protein content (a), SOD activity (b), POD activity (c), and MDA content (d) in *H. akashiwo* cells treated with DMSO (control), 40% 24 h EC_{50} of compound 1, and 80% 24 h EC_{50} of compound 1

When compared with the control, the SOD activity in *H. akashiwo* cells was significantly reduced ($P<0.05$) by 48.6% and 96.8% following treatment with 40% and 80% 24 h EC_{50} of compound **1**, respectively (Fig.4b). In contrast, the POD activity was significantly increased ($P<0.05$) by 2.5 and 35.2 times in *H. akashiwo* cells following treatment with 40% and 80% 24 h EC_{50} of compound **1**, respectively (Fig.4c). Figure 4d shows that the MDA content in *H. akashiwo* cells increased with the increasing concentration of compound **1**, and that the MDA content in *H. akashiwo* cells treated with 80% 24 h EC_{50} of compound **1** was significantly higher than that in the control ($P<0.05$).

4 DISCUSSION

When compared with other fungi, endophytic fungi isolated from marine macroalgae are considered to be one of the main sources of natural active substances (Cui et al., 2010; Wang et al., 2011; Sun et al., 2013). Many kinds of pure compounds with high bioactivities, such as alkaloids, anthraquinones, and steroids, have been separated from the secondary metabolites of *Aspergillus* (Miao et al., 2012; Liu, 2012; Liu et al., 2013). In the present study, the four fungal strains with strong algicidal activities against red tide algae belonged to the genus *Aspergillus*: *A. wentii* (pt-1), *A. ustus* (cf-42), and *A. versicolor* (dl-29, pt-20). A total of 11 compounds with strong algicidal activities were isolated from the secondary metabolites of these fungal strains (Tables 2 and 3), and were found to be composed of five anthraquinones (compounds **1**, **2**, **4**, **5**, **6**), four steroids (compounds **24**, **26**, **27**, **30**), and two terpenoids (compounds **17**, **21**). The anthraquinone compounds appeared to have broad-spectrum algicidal activities (Table 3). In recent years, novel anthraquinone compounds with strong antibacterial, antioxidant, and medicinal activities have been continuously isolated, suggesting that algal-derived endophytic fungi might be one of the important sources of active compounds against red tide algae, and that more attention should be paid to *Aspergillus* and anthraquinones. It can be observed from Table 2 that compounds **16**, **18**, **21** showed strong inhibitory activity against *C. marina*, but had weak effects on the other algae examined. Furthermore, compounds **26**, **29** presented strong inhibitory activity against certain algae, while promoted growth of some algal species. These findings indicated that the algicidal activities of compounds might be species-specific because of varied cell structure and cell size (Jin and Dong, 2003; Cantrell et al., 2005).

The exposure to allelochemicals can induce oxidative stress in microalgae cells, thereby affecting microalgal growth and metabolism (Miazek et al., 2014). Chl *a* is the main photosynthetic pigment of *H. akashiwo*, and the active compound could weaken photosynthesis and block growth of this alga by destroying Chl *a* (Fig.3). Soluble protein is an osmotic regulatory substance in cells, which can decrease the damages to the cells caused by stress. Therefore, the soluble protein content is an important indicator of cell membrane injury during metabolism. SOD and POD are two protective enzymes that widely exist in living organisms. Under stress conditions, SOD and POD could remove excessive reactive oxygen species to protect the cells from damage. MDA is the main product of membrane lipid peroxidation and an important indicator of membrane system damage. The MDA content in a cell could reflect the degree of membrane lipid peroxidation (Qian et al., 2009), membrane damage, and amount of reactive oxygen species in the cell (Zhang et al., 2011). In the present study, an increase in the soluble protein content, POD activity, and MDA content in *H. akashiwo* cells treated with compound **1** (Fig.4a, c, d) indicated that the cells had been subjected to stress. Furthermore, a decrease in the SOD activity in *H. akashiwo* cells treated with compound **1** (Fig.4b) showed that the cells were affected by severe stress. This result is in agreement with that reported by Qian et al. (2009). Therefore, it can be concluded that the active compounds could restrain the growth of red tide algae by inhibiting photosynthesis (reducing Chl *a* content), destroying cell membrane, and damaging the antioxidant system.

Interestingly, although the crude extract of strain pt-1 did not show strong algicidal activities (Table 1), compound **1**, which was separated from this crude extract, showed the strongest algicidal activities (Table 3). A possible reason for this observation might be the effect of other compounds in the crude extract, which may have weakened the algicidal activities of compound **1**, or the low content of compound **1** in the crude extract. Therefore, a moderate criterion for screening of the fungal strains could help in detecting more compounds with strong algicidal activities from the secondary metabolites of algal-derived endophytic fungi.

The solubility of the compounds in different solvent is different. DMSO, known as the “universal solvent” in areas of pharmaceutical sciences and cell

biology, could dissolve both polar and nonpolar compounds. Under the same condition, the solubility of these compounds in DMSO is greater than that in water. Therefore, the concentrations of these compounds might not reach the concentrations in the experiment in natural water bodies. However, concentrations of red tide algae under normal conditions are much lower than those in this experiment. And generally, red tide algae at low concentrations would be more susceptible to allelochemicals. Therefore, low concentrations of the compounds might inhibit the growth of the red tide algae in natural water bodies. Furthermore, application of allelochemicals dissolved into DMSO might be used as an emergency treatment measure when red tides broke out, for the low toxicity of this solvent.

5 CONCLUSION

The following conclusions were drawn from this study:

(1) A total of 32 pure compounds were assayed to possess different degrees of algicidal activities.

(2) Eleven of these compounds including five anthraquinones showed high 24-h IRs for the four red tide algae, with 24 h EC_{50} values ranging from 0.01 to 14.29 μg/mL. The anthraquinone compounds appeared to have broad-spectrum algicidal activities.

(3) Active compound could decrease the Chl *a* and SOD contents and increase soluble protein, MDA and POD contents of *H. akashiwo*.

(4) The anti-algal compound might inhibit the growth of red tide algae by weakening photosynthesis (reducing Chl *a* content), destroying cell membrane and damaging the antioxidant system.

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