0971-4693/94 Euro 10.00
Allelopathy Journal 37 (2): 231-240 (2016) *International Allelopathy Foundation* 2016
Tables: 3, Figs : 5

Algicidal activities of *Cladophora fracta* on red tide-forming microalgae *Heterosigma akashiwo* and *Gymnodinium breve*

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(Received in revised form: February 11, 2016)

ABSTRACT

The effect of algicidal allelochemicals in *Cladophora fracta* (O.F.Muller ex Vahl) Kutzing, on the growth of two microalgae, *Heterosigma akashiwo* (Hada) Hada *ex* Y. Hara *et* Chihara and *Gymnodinium breve* Davis was investigated. .. The aqueous extract as well as the ethyl acetate extract of the ethanol extract, strongly inhibited the growth of both the microalgae. Using GCMS, the active substances in the ethyl acetate fraction were identified. as DIBP (1, 2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester), DBP (1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester) and DOA (Hexanedioic acid, bis(2-ethylhexyl) ester) .

Keywords: Algicidal activity, algicidal allelochemical, *Cladophora fracta*, *Gymnodinium breve*, *Heterosigma akashiwo*, micro algae, red tide.

INTRODUCTION

Harmful algal blooms (HAB) are of global occurrence caused by various microalgal species, which cause severe economic losses to aquaculture and have major environmental and human health impacts (3,6,8,11,12,16,19,20,22,24). Red tides are due to the massive blooms of harmful microalgae in marine waters (8). Blooms are formed due to natural processes such as poor circulation, upwelling relaxation and river flow and anthropogenic loadings leading to eutrophication (20).

Physical, chemical and biological measures can control red tide (2, 15) but these result in disastrous environmental consequences (8). Allelopathy, direct or indirect, is a new effective method examined to control algal blooms (7). With specific biodegradable characteristics, macroalgal allelochemicals offer an environmental friendly and ecologically acceptable method for HAB control (1). The inhibitory effects of *Ulva pertusa* and *Gracilaria lemaneiformis* on *Heterosigma akashiwo* have been reported (27). The methanol extracts of *U. pertusa*, *Corallina pilulifera*, *Ishige foliacea* and *Endarachne binghamiae* are reported to inhibit the growth of harmful microalga *Cochlodinium polykrikoides* (8). Besides, the dry tissue powder and water extract of *C. pilulifera* are also reported to be inhibitory (8). *U. pertusa* inhibits the growth of microalgae such as *Prorocentrum micans*, *H. akashiwo*, *Alexandrium tamarense* and *Prorocentrum*

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donghaiense (9,10,25). The fresh tissue, dry powder and aqueous extracts of the macroalgae, *U. pertusa*, *C. pilulifera* and *Sargassum thunbergii* are also reported to drastically decrease the growth of two microalgae namely *H. akashiwo* and *A. tamarense* (26).

In China, the red tide occurs frequently because of accelerated eutrophication in coastal waters and between 2000 to 2005, thirty to eighty red tides were reported each year (29). *Gymnodinium breve* and *H. akashiwo* are common red tide-causing microalgae, which cause massive death of aquatic organisms by secreting the neurotoxic shellfish ichthyotoxins (4, 26, 28). *Cladophora fracta* is a common filamentous marine macroalga, but little is known about its allelopathic effects on harmful microalgae.

This study aimed at determine the inhibitory potential of *C fracta* on the red tide-causing microalgae *H. akashiwo* and *G. breve* and to characterise the algicidal allelochemicals produced by this marine algae.

MATERIALS AND METHODS

Materials

The microalgae H. akashiwo and *G. breve* were provided by the Institute of Hydrobiology, Chinese Academy of Sciences. These were grown in 2,000 mL conical flasks containing 1,500 mL of f/2 medium (5) under 12:12 LD cycle with a light density of 2,500 ± 500 Lux at 25 ± 1℃. All cultures were shaken slightly 3 times daily and rearranged randomly.

C. fracta was collected from prawn ponds in Hongdao District, Qingdao province, China in June 2014. The seaweed was cleaned with distilled water, air-dried at room temperature (24± 1℃) for 6 d and then pulverized in an electric grinder.

Extraction and isolation of algicidal allelochemicals

The aqueous extract was prepared as per the modified method of Wang *et al*. (28). Fifty g of powdered *C. fracta* were extracted with 1L distilled water at room temperature (24± 1℃) for 48 h in dark**.** The mixture was filtered through a nylon filter (0.45μm) to obtain the aqueous extract.

Algicidal substances in *C. fracta* were separated from an ethanolic extract as per the modified method of Wang *et al*. (28). The isolation procedure is shown in Fig. 1.

For this, the ethanol extract was prepared by suspending 50g of dried powder of *C. fracta* in 1 L 95% ethanol (v/v) at room temperature (24± 1℃) for 48 h and then filtered. The Ethanol was evaporated at 50℃ under reduced pressure using a spin steaming instrument (EYELA, N-1100D-W). The dry residue was mixed with 50 mL distilled water and centrifuged (3,024× G, 4℃).

The supernatant was first mixed with 50 ml of light petroleum in a separatory funnel and shaken for 5 min. After standing for a few min, the lower phase was separated and mixed with 50 ml n-butyl alcohol and shaken thoroughly. After standing, the upper n-buty alcohol phase (fraction A) was separated. The lower aqueous phase was mixed with 50 ml of ethyl acetate (50 mL) and allowed to stand. The upper ethyl acetate phase (Fraction B) was separated from the lower aqueous phase (fraction C).

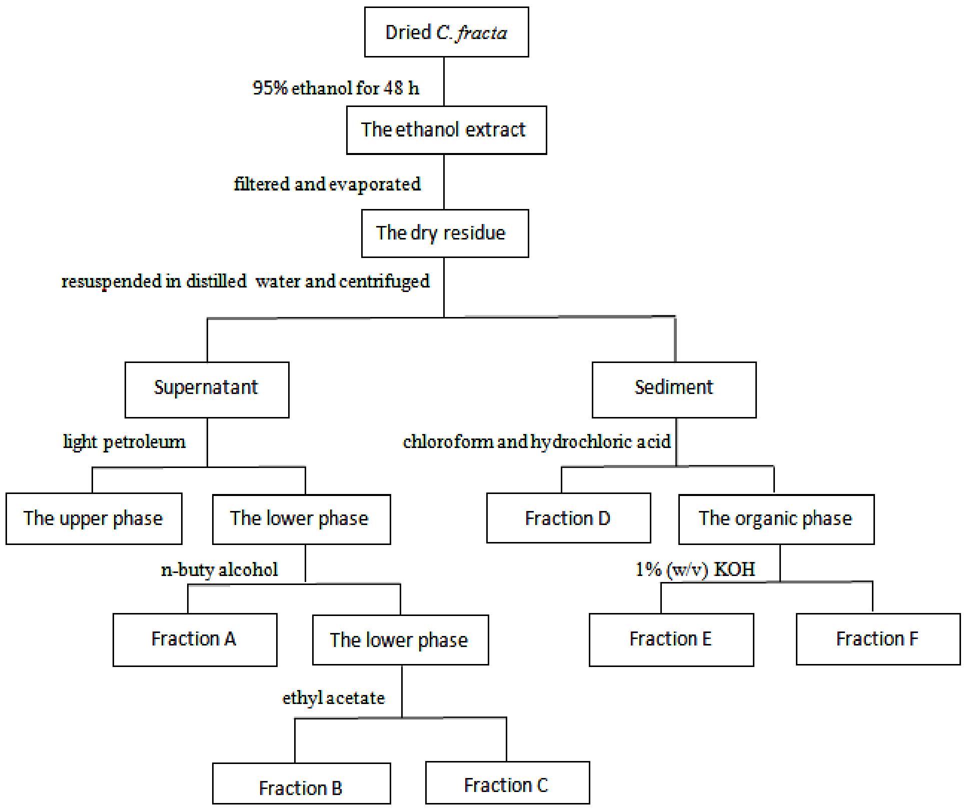


Figure 1. The isolation procedure of fractions in *C. fracta*

The insoluble residue from ethanol extraction, was suspended in 50 mL chloroform and 50 mL 2%(V/V) hydrochloric acid. After shaking and standing for a while, the upper organic phase was separated from the lower aqueous phase (fraction D).

To the upper organic phase, 50 mL 1% (w/v) KOH was added and allowed to stand for a while for separating the aqueous phase (fraction E) and organic phase (fraction F).

All the organic phases were completely air-dired in dark at room temperature (24± 1℃) and then dissolved in Ethanol. After drying at 50℃ under reduced pressure using a spin steaming instrument, Fractions A, B and F were re-suspended separately in 50 ml distilled water each. After drying at 55℃ in electric oven, fractions C, D and E were also re-suspended separately in 50 ml distilled water each. All the 6- fractions re-suspended separately in 50 ml distilled water each, were used in subsequent bioassay to measure their algicidal activities.

All the 6 fractions were then dissolved in Ethanol was evaporated at 50 C, resuspended in 50 ml of distilled water and used in the subsequent bioassay to measure their algicidal activities.

The aqueous solutions of the dried fractions found effective in bioassay(Fr.B), was analyzed using a GC-MS Agilent computerized system consisting of a 6890 gas chromatograph fitted with a HP-5MS capillary column (30 m× 0.25 mm ×0.25mm id) and coupled with a Agilent 5973N quadrupole mass spectrometer. Injected volume was 1 μL. The oven temperature was programmed at 100℃ for 2 min, then increased to 120℃ at a rate of 5℃/min and kept at 120℃ for 2 min. Thereafter it was increased to 260 ℃ at 10 ℃/min, and kept at 260 ℃ for 25 min. Helium was used as the carrier gas with a flow rate of 1 mL/min. Mass fragments of the fraction were compared with the mass fragmentation data contained in NIST 08. The algicidal allelochemicals 2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester (DIBP), 1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester (DBP) and Hexanedioic acid, bis (2-ethylhexyl) ester (DOA) were identified and compared with synthetic products (J&K Scientific Industries Inc., PRC) used to estimate their algicidal activities.

Bioassay

For bioassay, microalgae in exponential phase of growth (grown for 3 days in 2L flasks containing 1500 ml f/2 medium) were treated with the aqueous extract and six fractions (A-F) at a final concentrations equivalent to 2.5 g air dry macroalga L^{-1}. The final concentrations of standard synthetic chemicals (DIBP, DBP and DOA) added to the algal cultures were 0, 1.6, 2.5, 4, 6.3 and 10 $mg \cdot L^{-1}$. All treatments were in triplicate.

Growth was measured by determining the algal cell number microscopically using a 0.1 mL counting chamber.

To determine the effect of chemicals and extracts on microalgal growth, samples were withdrawn from the cultures at 24h intervals (0, 24, 48, 72, and 96 h) after the addition of the extracts or chemicals (21) and inhibition was calculated as :

$$I\ (\%) = (N_0 - N_S)/N_0 \times 100,$$

Where N_0: Algal cell density in treatment without algicidal substance ($ind \cdot L^{-1}$); N_S: algal cell density in the treatment with algacidal substance ($ind \cdot L^{-1}$). (21)

Statistical analysis

The results are expressed as mean ± SD. One-way analysis of variance (ANOVA, SPSS version 13.0) followed by LSD's multiple range test was used to examine whether there were any significant differenced among treatments. Statistical significance was established at $P < 0.05$. Median effect concentration (EC_{50}) was obtained by PROBIT program of SPSS. Excel 2007 was used for graphical presentations.

RESULTS AND DISCUSSION

The effect of aqueous extracts of *C. fracta* on *H. akashiwo* and *G. breve*

The cell density of both *H. akashiwo* and *G. breve* decreased significantly at 24 h after exposure to the aqueous extract of *C. fracta* ($P< 0.05$) (Fig. 2) and did not increase further while in the control cell densities increased steadily with culture time.

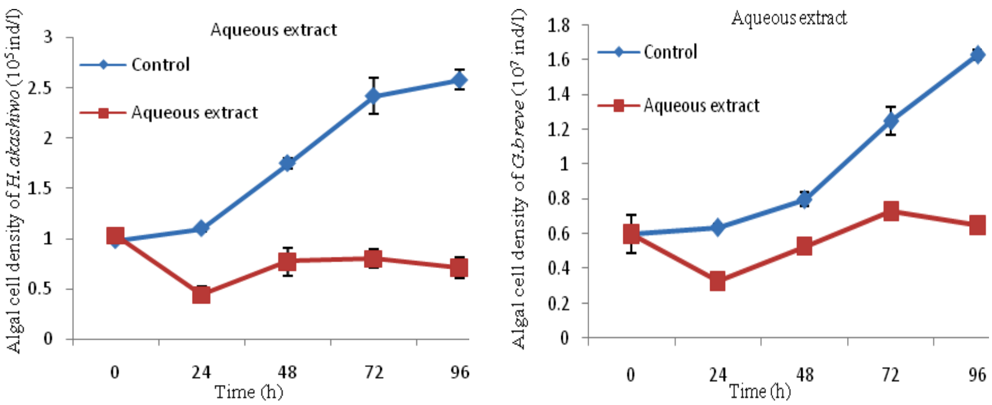


Figure 2. Changes in cell density of *H. akashiwo* and *G. breve* exposed to aqueous extract of *C. fracta*

The cell density dropped at 24 hour. Indicating cell lysis caused by the allelochemicals in the aqueous extract. However, the growth of microalgae recovered slightly thereafter suggesting either slow degradation of the allelochemcials or slow development of resistance.

The aqueous extracts of *U. pertusa*, *C. pilulifera* and *Sargassum thunbergii* strongly inhibits the growth of *H. akashiwo* (26) and aqueous extracts of *Entermorpha clathrata* and *U. pertusa* also reported to strongly inhibits the growth of *G. breve* (28). Our results showed that aqueous extracts of *C. fracta* inhibited the growth of *H. akashiwo* and *G. breve*, but continuous release of degradable allelochemicals from the tissue of *C. fracta* is perhaps essential for inhibiting the microalgal growth.

The effects of Ethanol fractions of *C. fracta* on *H. akashiwo* and *G. breve*

The effect of different fractions **(A-F)** on the growth of the two microalgae is shown in Fig. 3.Within 24h, the cell densities of *H. akashiwo* exposed to fraction **B** decreased and the cell number continuously decreased while the other fractions did not have any significant effect. Similarly with *G.breve,* fraction **B** decreased cell number at 24 h but recovered slightly thereafter. These findings show that fraction B (the ethyl acetate extract) had the potential to inhibit the growth of both the microalga. Methanol extracts of the seaweed *U. pertusa*, *C. pilulifera*, *I. foliacea* and *E. binghamiae* are reported to inhibit the growth of the harmful microalgae *Cochlodinium polykrikoides* (8). The ethyl acetate extract of *U. pertusa* is reported to strongly inhibit the growth of *G. breve* (28). Our studies show that among the fractions tested, only ethyl acetate fraction (fraction B) strongly inhibited the growth of both *H. akashiwo* and *G. breve* suggesting that this fraction perhaps contains the algicidal allelochemicals of *C. fracta*.

Identification of active substances from the ethyl acetate fraction (Fr.B)

Using GC-MS, three compounds were identified from the ethyl acetate extract (Fr.B) (Table 1) and their structures are presented in Fig. 4. The relative content of DOA in dried Fraction B is 83.57%, DIBP 12.76% and DBP 3.67%.

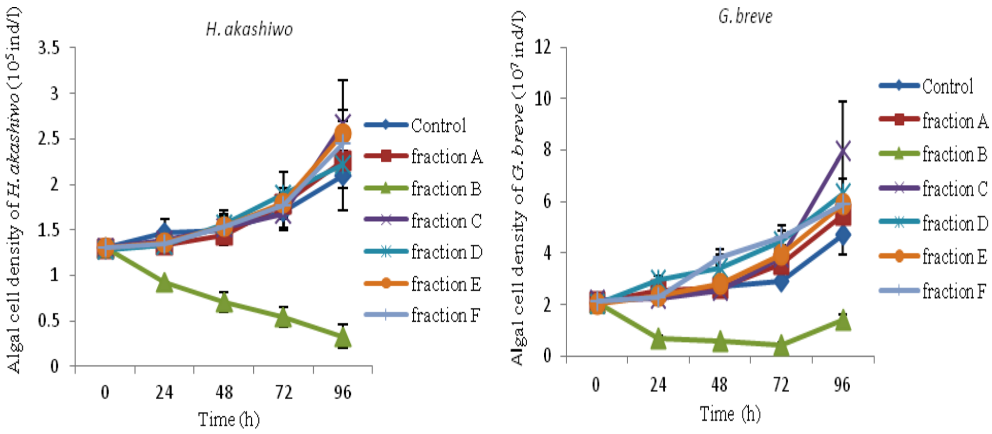
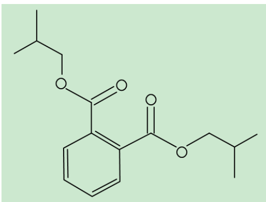


Figure 3. Changes in cell density of *H. akashiwo* and *G. breve* exposed to fractions A-F

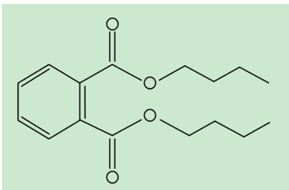
Table 1. Composition of ethyl acetate fraction (Fr.B) of *C. fracta*

Rt (min)	Compounds	Relative amount (%)*
18.36	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester (**DIBP)**	12.76
19.42	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester **(DBP)**	3.67
23.30	Hexanedioic acid, bis(2-ethylhexyl) ester **(DOA)**	83.57

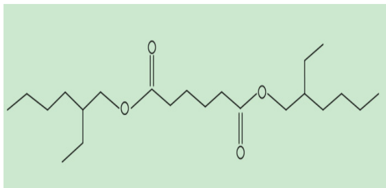
Rt: Retention time * Relative to the total



1, 2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester (DIBP)



1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester (DBP)



Hexanedioic acid, bis (2-ethylhexyl) ester (DOA)

Figure 4. Chemical structures of identified compounds from ethyl acetate fraction(Fr B) of *C. fracta*

The presence of DIBP in *U. pertusa* (28), DBP in *Ageratina adenophora* (30) and *U. pertusa* has been reported. The presence of DOA in the macro alga *C. fracta* is however being reported for the first time.

Comparison of the inhibitory effects of DIBP, DBP and DOA

The inhibitory effect of the three identified chemicals (Synthetic) on the growth of *H. akashiwo* and *G. breve* is shown in Fig 5. All three showed strong inhibitory effects on the growth of *H. akashiwo* and *G. breve* and the extent of inhibition was concentration

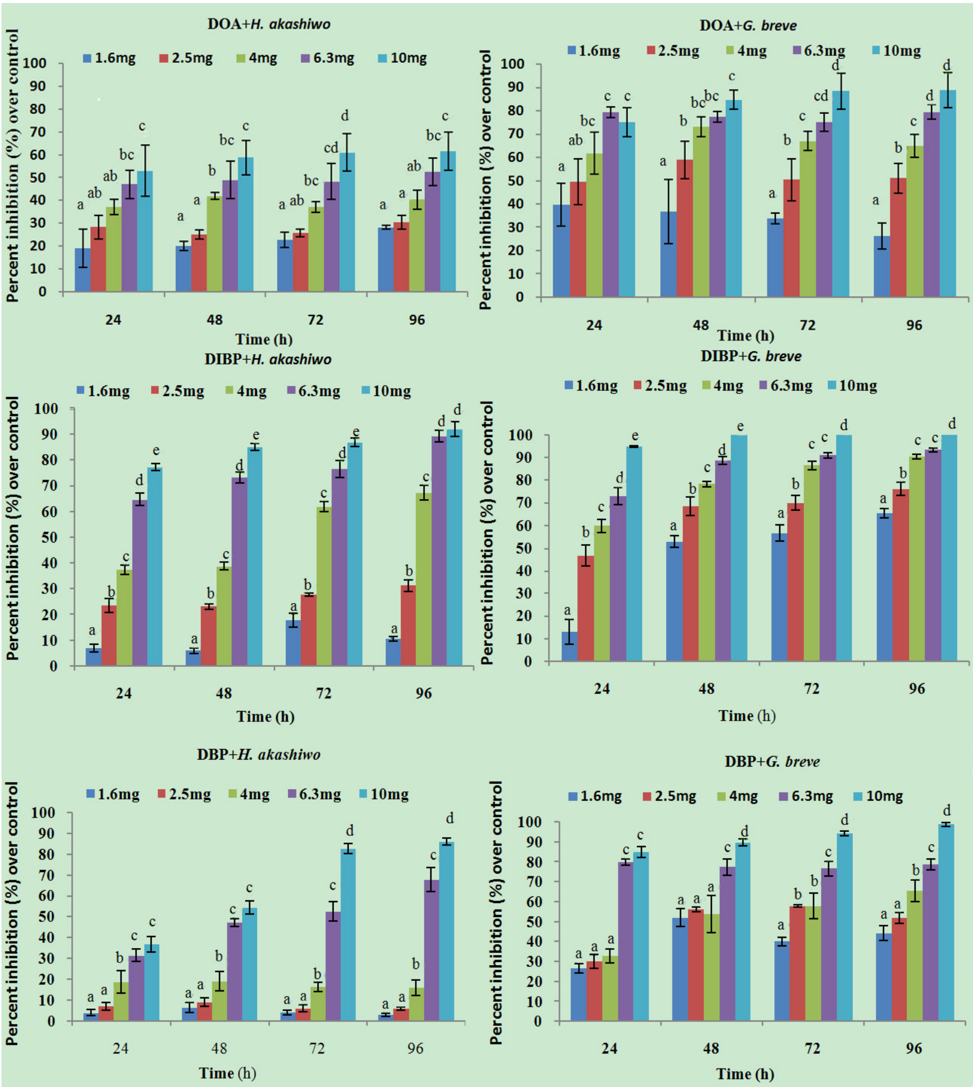


Figure 5. Inhibition (%) of *H. akashiwo* and *G. breve* by active substances. Different letters (abcde) indicate significant difference between treatments (*P* <0.05).

dependant. While at lower levels some recovery was seen which may be due to either resistance to development or biodegradation, at higher concentrations, there was no recovery. It is reported that DIBP and DBP induce the production of reactive oxygen species (ROS) in algal cells. Excess ROS inhibits the activities of Superoxide Dismutase and Catalase, leading to lipid oxidation, algal cells destruction and death (28). The mechanism of growth inhibition by DOA needs investigation.

The EC_{50} values of DIBP, DBP and DOA on *G. breve* are showed in Table 2. After 24 h exposure, the inhibitory effects of three active substances decreased in the following order: DOA > DIBP > DBP. DIBP exhibited the strongest inhibitory effects followed by DBP after 48 h, 72 h and 96 h exposure.

Table 2. EC_{50} of active substances in ethyl acetate fraction(Fr.B) on *G. breve*

	24 h	95%CI		48 h	95%CI		72 h	95%CI		96 h	95%CI	
	EC_{50}	upper	lower	EC_{50}	upper	lower	EC_{50}	upper	lower	EC_{50}	upper	lower
DOA	2.39	1.77	2.94	2.11	1.61	2.54	2.53	2.10	2.94	2.74	2.36	3.10
DIBP	3.28	2.25	4.45	1.58	0.71	2.22	1.45	1.11	1.74	1.14	0.78	1.44
DBP	3.86	1.28	11.15	1.94	0	3.61	2.31	0.772	3.48	2.23	0.69	3.35

95% CI: 95% confidential interval

Table 3 shows the EC_{50} values of active substances against *H. akashiwo*. Within 48 h, DIBP had the strongest inhibitory effect and DOA was next. However, DBP exhibited stronger inhibitory effects than DOA with prolongation of exposure time.

Table 3. EC_{50} of active substances in ethyl acetate fraction (Fr. B) on *H. akashiwo*

	24 h	95%CI		48 h	95%CI		72 h	95%CI		96 h	95%CI	
	EC_{50}	upper	lower	EC_{50}	upper	lower	EC_{50}	upper	lower	EC_{50}	upper	lower
DOA	7.87	6.09	12.07	6.56	5.36	8.78	6.64	5.38	9.02	5.82	4.65	8.02
DIBP	5.01	4.51	5.60	4.52	4.13	4.96	3.57	3.21	3.96	3.30	2.55	4.13
DBP	13.37	10.32	20.41	8.29	7.14	10.11	6.14	4.77	8.74	5.58	4.25	7.80

EC50 expressed as $mg.L^{-1}$

DIBP and DBP are usually used in plastic industries as plasticizers and belong to Phthalate Acid Esters (PAEs) which are endocrine disrupting chemicals. Studies have shown that DIBP and DBP have reproductive toxicity and developmental toxicity inn animals (13,14,17,18,23).

DOA also is also a kind of plasticizer. From the inhibitory effects of DOA seen on *H. akashiwo* and *G. breve*, we feel that DOA has a promising future in using it as a HAB inhibitor. Further investigations are needed to assess the toxicity of DOA to other aquatic organisms and its environmental safety.

CONCLUSIONS

The macro algae *C. fracta* contains allelopathic compounds which are effective in controlling the growth of the two red tide causing microalgae (*H. akashiwo and G. breve*) . The active substances have been identified as 1, 2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester (DIBP), 1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester (DBP) and Hexanedioic acid, bis (2-ethylhexyl) ester (DOA). The presence of DOA is reported here for the first time and is found to be a strong (Harmful algal blooms) inhibitor.

ACKNOWLEDGEMENTS

The financial provided by National Natural Science Foundation of China (Grant No. 31300393), Marine and Fisheries Research Project of Liaoning Province (Grant No. 201415), China Postdoctoral Science Foundation (Grant No. 2014M551013, 2015T80212), and Program for Tianjin innovative Research Team in University (Grant No. TD12-5018) is gratefully acknowledged.

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