Bioactivities of algicidal C18 hydroxy unsaturated fatty acid isolated from the red alga *Tricleocarpa jejuensis* and its synthesized propargylic derivative

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Abstract

(±)-(*E*)-12-hydroxyoctadec-10-enoic acid (compound A) was isolated from the red alga *Tricleocarpa jejuensis* in our previous study as a potent algicidal compound against red tide microalga *Chattonella antiqua* (*Ochrophyta, Raphidophyceae*). Interestingly, its propargylic derivative, 12-hydroxyoctadec-10-ynoic acid (compound B), which was obtained as a synthetic intermediate of compound A, showed an even more extensive algicidal activity. In this study, the effects of compound A and B on the major causative species of harmful algal blooms (HABs) such as *Chattonella marina* (*Ochrophyta, Raphidophyceae*), *Heterocapsa circularisquama,* and *Karenia mikimotoi* (*Miozoa, Dinophyceae*) in addition to *C. antiqua* were investigated. Both compounds exhibited toxic effects on these red tide flagellates in a concentration dependent manner with LC_{50} < 10 μg/mL, and the activities of compound B were much stronger than compound A. Zooplankton rotifer (*Brachionys plicatilis*) was relatively resistant to the compounds, and the lethal effects of *H. circularisquama* and *K. mikimotoi* on rotifers were reduced in the presence of compound A and B, suggesting their potential usage in the mitigation strategy for HABs. Consistent with the toxicities on the HAB species, compound B showed stronger cytotoxicity on HeLa, XC, and U937 cells than A. Since compound B but not compound A showed haemolytic activity on rabbit erythrocytes, specific action of compound B on cell membrane may be responsible for the potent cytotoxicity. Furthermore, compound B exhibited higher antibacterial effect on *Staphylococcus aureus* than compound A, while both were not effective on *Enchiridia coli*. Our results suggest that compound A and B are promising multifunctional candidates with potential to develop not only as anti-red tide microalgae but also as tumor cell-killing and antibacterial agents. This is the first report

indicating that replacing a double bond in the hydroxy unsaturated fatty acid with a triple bond results in much augmentation of the bioactivities.

Keywords: *Tricleocarpa jejuensis*; C18 hydroxy unsaturated fatty acid; Red tide phytoplankton; Algicidal activity; Antibacterial activity; Cytotoxicity

1. Introduction

Marine macroalgae have received much attention as potential rich sources of compounds with a wide variety of biological activities including anti-bacterial [1], anti-tumor [2], anti-oxidant [3], anti-inflammatory [4], and anti-algal [5] activities. Since the allelopathic interactions between macloalgae and microalgae have been well documented [6,7], there are extensive studies focused on the practical usage of macroalgae to control harmful algal blooms (HABs) caused by undesirable toxic microalgae species [8]. HABs are one of the most serious threat to marine environment, which can cause tremendous damage on coastal areas associated with massive mortality of natural and aquacultured fish and shellfish, and even with public health problem depending on the causative species [9-11]. Regarding HAB control strategy, macroalgae have many advantages such as the abundant natural sources, low cost, and relatively environmentally friendly features in addition to the allelopathic effects on HABs [5,6,12,13]. In fact, diverse chemical compounds with growth inhibitory effects on HAB species have been found in several marine macroalgal species. Such compounds discovered so far are polyunsaturated fatty acid related compounds (PUFAs) [14-17], glycerolipids [18-20], terpenoids [8,21-24], and phenolics [8,24].

Under these circumstances, we recently examined 17 species of macroalgae including 9 Rhodophyta, 6 Phaeophyta, and 2 Chlorophyta collected from the coastal region of Nagasaki Prefecture, Japan, based on the cytolytic activity against the raphidophycean flagellate *Chattonella antiqua*, and found that the red alga *Tricleocarpa jejuensis* had potent toxic compounds on *C. antiqua* [25]. NMR, IR and mass spectral analyses revealed that the active compounds are mixture of four isomers of C18 hydroxy unsaturated fatty acids; (±)-(*E*)-9-hydroxyoctadec-10-enoic acid, (±)-(*E*)-10-hydroxyoctadec-8-enoic acid, (±)-(*E*)-11-hydroxyoctadec-12-enoic acid, and (±)-(*E*)-12-hydroxyoctadec-10-enoic acid. The structures were confirmed by comparison of the NMR and MS data with their authentic compounds obtained by unambiguous syntheses [25]. During the processes of the chemical syntheses, several intermediate derivatives were obtained. Interestingly, one of such derivatives (±)-12-hydroxyoctadec-10-ynoic acid (compound B) induced cell lysis of *C. antiqua* with even more immediate action of mode than the parental (*E*)-12-hydroxyoctadec-10-enoic acid (compound A), suggesting that replacing of a double bond in the hydroxy unsaturated fatty acid with a triple bond leads to the augmentation of the bioactivity. Hence, it is considered that compound A and B are good pair molecules, which may provide new insight into structure-activity relationship of bioactive fatty acids isolated marine macroalga. To further evaluate the bioactivities and the specificity of these fatty acids, in this study, we conducted comparative studies regarding the effects of compound A and B on four HAB species, three mammalian tumor cell lines, rabbit erythrocytes, and Gram positive and negative bacteria.

2. Materials and Methods

2.1. Preparation of compound A and B

Compound A ((±)-*E*-12-hydroxyoctadec-10-enoic acid) and B ((±)-12-hydroxyoctadec-10-ynoic acid) were synthesised as described previously [25]. The structures of compound A and B are shown in Fig. 1. The solutions of these compounds in dimethyl sulfoxide (DMSO) at 4 mg/mL were used throughout the experiments.

2.2. Phytoplankton species and rotifer

Heterocapsa circularisquama (NIES-3621), *Karenia mikimotoi* (NIES-2411), and *Chattonella antiqua* (NIES-1) were obtained from the National Institute for Environmental Studies, Environmental Agency, Japan. *Chattonella marina was* isolated from Kagoshima Bay, Japan. These phytoplankton species were maintained at 26 °C in 100 mL Erlenmeyer flasks containing 50-60 mL of modified seawater medium (SWM-3) at a salinity of 25 [26] under a 12:12 h photoperiod using a cool-white fluorescent lamp (200 ± 5 μmol m^{-2} s^{-1}). The modified SWM-3 containing Tris-HCl buffer system was autoclaved for 15 min at 121 °C before use. The cell numbers of the cultures were counted microscopically using a haemocytometer (Erma Inc., Tokyo, Japan). The cultures at late exponential growth phase were used throughout the experiments. The rotifers *Brachionys plicatilis* were kindly provided by Dr. A. Hagiwara (Faculty of Fisheries, Nagasaki University, Japan) and were maintained with *Nannochloropsis oculata* (*Ochrophyta, Eustigmatophyceae*) as

described previously [27]. All the cultures were conducted using sterilized instruments.

2.3. Algicidal activity

To each of phytoplankton cell suspension in 96-well plates, varying concentrations of samples were added (100 μL/well). After 4 h cultivation under the usual phytoplankton culture conditions, the survival rate of each phytoplankton was determined as described previously [15]. In the assay, the initial cell density of *C. antiqua*, *C. marina*, *H. circularisquama*, and *K. mikimotoi* were adjusted as approximately 2-3 × 10^4 cells/mL, respectively.

2.4. Rotifer exposure experiment

The rotifer exposure to HAB species or compound A and B were carried out in 48-well plates (Becton-Dickinson) as described previously [27]. Ten rotifers in each well were incubated with varying concentrations of the samples or HAB species in modified SWM-3 (500 μL/well) at 26 °C in the light for 4 h. Then, the surviving individuals, which were defined as those actively swimming in each well, were counted using a stereomicroscope.

2.5. Cytotoxicity

HeLa (human epithelia carcinoma), XC (rat sarcoma), and U937 (human histiocytic lymphoma) cells were obtained from American type culture correction.

HeLa and XC cells were cultured in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 10 μg each of adenosine, guanosine, cytidine, and thymidine per ml of medium, penicillin (100 IU/mL), and streptomycin (100 μg/mL). U937 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Cytotoxicities of the samples were evaluated by MTT assay, which detects mitochondrial succinate dehydrogenase present in living cells. In brief, 1~4 x 10^4 cells/well in a 96-well plate in the medium were incubated with varying concentrations of the samples in the culture medium for 24 h at 37 °C, and then conducted MTT assay as described previously [28].

2.6. Haemolytic activity

Fresh rabbit blood was obtained from Nippon Bio-Test Laboratories (Tokyo, Japan). Erythrocytes were washed three times with PBS and the final erythrocyte pellet was suspended in PBS to make 2% (v/v) suspension. Triplicate 50 μL aliquots of serial two-fold dilutions of the samples were added to round-bottom 96-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA). To the wells containing 50 μL of samples in PBS, 50 μL of a 2% (v/v) suspension of erythrocytes were added, after which the well plates were gently shaken. After incubation for 4 h at 26 °C in the light, the plates were centrifuged at 900 × *g* for 10 min at 4 °C. Aliquots (50 μL) of the supernatant were withdrawn from the wells and transferred to flat-bottom 96-well plates (Becton-Dickinson). The amount of haemoglobin released was determined by measuring absorbance at 560 nm using a microplate reader (MPR-A4i, TOSOH Co.,

LTD., Tokyo, Japan). Negative controls (zero haemolysis) and positive controls (100% haemolysis) were included as erythrocytes suspended in PBS alone and in PBS containing 1% v/v Triton X-100, respectively.

2.7. Antibacterial activity

Staphylococcus aureus (NBRC12732) and *Escherichia coli* (NBRC13898) were obtained from NITE Biological Resource Centre (Tsukuba, Japan). Nutrient agar was used to maintain the strains. Bacterial strains were cultured for 18 h at 37 °C in nutrient broth medium. The cells were harvested and washed with phosphate-buffered saline (PBS) by centrifugation at 15,000×g for 10 min at 4 °C. The cell pellets were diluted to the appropriate cell density with PBS, and immediately used for the experiments. Bacterial cultures were incubated with varying concentrations of samples for 4 h at 37 °C in triplicate. An aliquot of each reaction mixture was withdrawn and suitably diluted with PBS and inoculated in triplicates into nutrient agar medium. After 48 h of incubation at 37 °C, the numbers of colonies formed were counted to determine the colony forming unit (CFU). The survival rate was calculated by dividing the CFU at time 4 h by the CFU at time 0 h.

2.8. Statistical analysis

All the experiments were repeated at least three times. The results were expressed as a mean ± standard deviation (SD), and the data was analyzed by a paired t-test to determine significant differences. A value of $p < 0.05$ was considered statistically

significant.

3. Results and Discussion

3.1. Effects of compound A and B on four HAB species

Compound A is one of the four isomeric C18 hydroxy unsaturated fatty acids found in a methanol extract of *Tricleocarpa jejuensis*, which exerts algicidal activity against the red tide phytoplankton *C. antiqua* with >80% mortality after 24 h incubation at a concentration of 20 μg/mL [25]. To further evaluate the algicidal activity of compound A, its concentration-dependent effects on *C. marina*, *K. mikimotoi*, and *H. circularisquama* in addition to *C. antiqua* were examined. From the dose response curves (Fig. 2), the 50% lethal concentration (LC_{50}) of compound A against *C. antiqua*, *C. marina*, *K. mikimotoi*, and *H. circularisquama* were estimated to be 3.31, 5.64, 5.70, and 1.68 μg/mL, respectively under the experimental conditions used in this study (Table 1). Interestingly, the cytotoxicity of compound B on these HAB species was evidently more potent than compound A (Fig. 2), and its LC_{50} against *C. antiqua*, *C. marina*, *K. mikimotoi*, and *H. circularisquama* were estimated to be 0.8, 2.98, 1.63, and 0.69 μg/mL, respectively (Table 1). These results suggest that replacing a double bond in the compound A with a triple bond can lead to enhanced algicidal activity. Previous numerous studies have reported that certain free fatty acids produced by algae exhibit toxic effects on various aquatic organisms including phytoplankton [14-17, 29-31]. Regarding toxic mechanism of free fatty acids, Wu et al. have found that leakage of intracellular potassium (K^+) occurred as a result of plasma

membrane damage after exposure to deleterious concentrations of fatty acids, and unsaturated fatty acids induced higher level of K^+ leakage than saturated ones [32]. Among the fatty acids tested (lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, and α-linolenic acid), α-linolenic acid induced the highest level of K^+ leakage from chlorophyte *Monoraphidium contortum*, and the cytotoxicity and K^+ leakage were well correlated [32]. Other studies also found that the toxicity of fatty acids increased with an increase in the degree of unsaturated double bonds [14,33]. In our previous study, linoleic acid and α-linolenic acid were isolated from green alga *Ulva lactuca* during the course of screening various macroalgae for the algicidal agents against *Heterosigma akashiwo* (*Ochrophyta, Raphidophyceae*) [34]. Comparative study on the cytotoxic effects of linoleic acid and α-linolenic acid on various red tide phytoplankton species (*C. marina, H. akashiwo, Alexandrium tamarense, Alexandrium taylori, Gymnodinium impudicum, and H. circularisquama* (*Miozoa, Dinophyceae*)) demonstrated that the LC_{50} values of α-linolenic acid were lower than those of linoleic acid, suggesting that number of unsaturated double bond of fatty acid is important structural element involved in the cytotoxic effect in agreement with other study [14]. The cytotoxic activities of these unsaturated fatty acids were highly species-specific. The LC_{50} values of linoleic acid and α-linolenic acid against *C. marina* were estimated to be 22.35 and 3.22 μg/mL, respectively, whereas no significant toxic effects were observed on *H. circularisquarma* up to 1000 μg/mL [34]. In contrast to linoleic acid and α-linolenic acid, *C. marina* and *H. circularisquarma* showed nearly equal susceptibility to compound A and B (Fig. 2 and Table 1). Probably, hydroxylated unsaturated fatty acids like compound A and B may be able to exert potent cytotoxicity

on broader phytoplankton species. This notion may be supported by the study in which Aliotta et al. found that unsaturated fatty acids were oxidized to form hydroxy compounds and exhibited anti-algal activity [35]. Taken together with our results obtained in this study and previous findings suggest that unsaturated double bond and hydroxy group are important structural elements influencing the cytotoxic activity of fatty acids against microalgae, and replacing the double bond with a triple bound can lead to further potentiation of the activity. To the best of our knowledge, this is the first report indicating that propargyl group can contribute to the enhancement of the algicidal activity of bioactive fatty acids.

3.2. Protective effects of compound A and B on rotifer exposed to H. circularisquarma and K. mikimotoi

Herbivorous zooplankton, such as rotifer and copepod, have been used to evaluate toxic potential of HAB species [36-38] and several dinoflagellates exhibited lethal effects on the rotifer *Brachionus plicatilis* [39-40]. We previously found that the rotifer is highly susceptible to *H. circularisquarma* and *K. mikimotoi* [27,40]. The toxic effects of the strains of *H. circularisquarma* and *K. mikimotoi* used in this study were confirmed, and 10 or 8 out of 10 rotifers exposed to *H. circularisquarma* at 2×10^4 cells/mL or *K. mikimotoi* at 2×10^3 cells/mL died after 4 h incubation, respectively (Table 2). As compared to *H. circularisquarma* and *K. mikimotoi*, rotifer was relatively resistant to compound A and B (Fig. 3), and the LC_{50} values were estimated to be 56.99 and 39.76 μg/mL, respectively (Table 1), which were more than 10 times higher than those against *H. circularisquarma* and *K. mikimotoi* (Table 1). In the presence of

compound A (20 μg/mL) or B (10 μg/mL) at the concentration toxic enough to the dinoflagellates but not so much detrimental to rotifer, significant increases in the survival rates of rotifers exposed to *H. circularisquarma* and *K. mikimotoi* were observed (Table 2). The results suggest that compound A or B can be used to suppress or mitigate the toxicity of these HAB species.

3.3. Cytotoxic effects of compound A and B on three cancer cell lines

Certain fatty acids show cytotoxic effects on cancer cells with different degrees depending on the structures [41], and some of them have been recognized as effective bioactive molecules in the treatment of tumors [42,43]. To gain further insight into the specificity of the cytotoxic mechanism of compound A and B and their potential as effective anti-tumor agents, the effects of compound A and B on three tumor cell lines were examined. As shown in Fig. 4, both compounds showed cytotoxic effects on HeLa, XC, and U937 cells in a concentration-dependent manner with different extents depending on the cell lines, and the cytotoxicity of compound B was stronger than compound A similar to the toxicity to HAB species. In contrast to hydroxy unsaturated fatty acids like compound A and B, it has previously found that α-linolenic acid and linoleic acid isolated from green alga *U. lactuca* as potent algicidal agents showed no significant cytotoxic effects on mammalian cell lines including tumor cell lines such as HeLa cells and U937 cells [34]. Although further studies are necessary to clarify the exact cytotoxic mechanism, it is considered that hydroxy group in compound A and B may be an important structural group commonly involved in the cytotoxicity against phytoplankton and tumor cells. Since the cytotoxic effects of compound B on these

tumor cell lines were greater than compound A as observed in the toxicities on microalgae, the triple bound in compound B may contribute to the increase in the cytotoxic activity against mammalian tumor cells as well as to unicellular phytoplankton cells.

3.4. Haemolytic activity of compound A and B

One possible cellular level action mechanisms of compound A and B may be an effect on the membrane integrity of target cells. The damaging effects on cell membrane are easily detected as a haemolytic activity, and cytotoxicity and haemolytic activity of such compounds are well correlated. A mixture of two unsaturated fatty acids isolated from sponge show cytotoxicity on mouse Ehrlich carcinoma cells and causes haemolysis toward mouse erythrocytes with nearly equal 50% effective dose (ED_{50}) [44]. As expected, compound B showed haemolytic activity toward rabbit erythrocytes, but no significant activity of compound A was observed up to 100 μg/mL (Fig. 5). Although further studies are necessary to clarify the exact action mechanisms of compound A and B, the potent haemolytic activity of compound B may partly contribute to the stronger cytotoxicity than compound A. Probably compound B may be able to induce membrane damage or perturbation through the triple bond-mediated specific interaction with the membrane of the target cells.

3.5. Antibacterial effects of compound A and B

Compound A is one of the four isomers of C18 hydroxy unsaturated fatty acids.

Some isomers with same structure of compound A isolated from other natural sources have been reported to show antimicrobial activities. (*E*)-9-hydroxyoctadec-10-enoic acid and (*E*)-10-hydroxyoctadec-8-enoic acid found in a higher plant (*Epichloe typhina*) showed antifungal activity against plant-pathogenic *Cladosporium herbarum* (*Fungi, Ascomycota*) [45]. These isomers were also isolated from the medicinal plant *Alternanthera brasiliana* and its endophytic bacteria, and they exhibited antimicrobial activity against some human pathogenic bacteria [46]. (*E*)-11-hydroxyoctadec-12-enoic acid isolated from the green alga *U. lactuca* showed antibacterial activity against *Streptomyces aureus* and *Escherichia coli* with different extent depending on the bacterial species [47]. Moreover, the antibacterial activities of various fatty acids have been well documented, and there are studies focused on therapeutic potential of fatty acids against infectious diseases [47,48]. Especially certain long-chain unsaturated fatty acids are even bactericidal to important pathogenic microorganisms such as *Helicobacter pylori* [49] and mycobacteria [50]. To see the fundamental aspect of antibacterial activity of compound A and B, in the current study, colony forming assay against gram-positive *S. aureus* and gram-negative *E. coli* were conducted. Compound A and B exerted bactericidal activity against *S. aureus* in a concentration-dependent manner, and compound B was obviously more potent than compound A, whereas no significant toxicity of these compounds on *E. coli* was observed up to 100 μg/mL (Fig. 6). The results suggest that compound A and B may have specificity to gram-positive bacteria. Regarding antibacterial activity of unsaturated fatty acids, it has been reported that gram-positive bacteria are more susceptible than gram-negative bacteria [51,52]. The differences in the sensitivities toward fatty acids between gram-positive and gram-negative bacteria may result from the impermeability of the outer membrane of

gram-negative bacteria since the outer membrane of gram-negative bacteria can function as an effective barrier against hydrophobic substances such as fatty acids [53-54]. More importantly, our results suggest that having triple bond instead of double bond in the hydroxy unsaturated fatty acid can lead to the increase in the bactericidal activity in addition to the algicidal and the tumor cell cytotoxicity.

4. Conclusions

Comparative studies on the bioactivities of C18 hydroxy unsaturated fatty acid (compound A) and its synthesized propargylic derivative (compound B) revealed that compound B was much stronger than compound A in terms of the algicidal, tumour cell-killing, and bactericidal activities. This is the first finding that replacing a double bond in hydroxy unsaturated fatty acid with a triple bond can result in significant increase in the multiple bioactivities. Regarding structure-activity relationship of unsaturated fatty acids, our results suggest that triple bond is an important structural element influencing the bioactivities in addition to the chain length, the degree of unsaturation, and the functional group such as hydroxy group. Since the LC_{50} values of compound A and B against HAB species are much lower than those against tumor cells and bacteria, the hydroxy unsaturated fatty acids may be able to exhibit highly specific cytotoxicity toward marine microalgae, and are promising agents to control HABs or mitigate their impacts.

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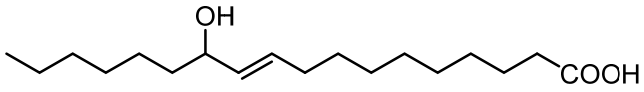
Table 1 The LC_{50} (50% lethal concentration) of compound A and compound B on various species after 4 h of treatment.

Species	LC_{50} (μg/mL)	
	Compound A	Compound B
Phytoplankton		
C. antiqua	3.31	0.80
C. marina	5.64	2.98
H. circularisquama	5.70	1.63
K. mikimotoi	1.68	0.69
Zooplankton		
B. plicatilis	56.99	39.76
Tumor cell lines		
XC cells	84.07	46.31
U937 cells	45.02	40.52
HeLa cells	75.86	42.51
Bacteria		
E. coli	>100	>100
S. aureus	79.31	11.01

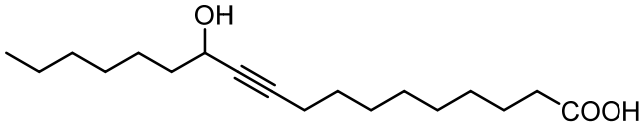
Table 2 Effects of *K. mikimotoi* (2.0×10^3 cells/mL) and *H. circularisquama* (2.0×10^4 cells/mL) on rotifers in the presence or absence of compound A (20 μg/mL) or B (10 μg/mL).

Exposure condition	Number of live rotifers/exposed	Exposure condition	Number of live rotifers/exposed
Control (medium only)	10/10	Control (medium only)	10/10
Exposure to *H. circularisquma*	0/10	Exposure to *K. mikimotoi*	2/10
+ Compound A	8/10	+ Compound A	8/10
+ Compound B	9/10	+ Compound B	4/10

After 4 h of cultivation, the number of living rotifers were counted as described in the text.



Compound **A**: (*E*)-12-Hydroxyoctadec-10-enoic acid



Compound **B**: 12-Hydroxyoctadec-10-ynoic acid

Fig. 1. The chemical structure of compound A; (*E*)-12-Hydroxyoctadec-10-enoic acid and compound B; 12-Hydroxyoctadec-10-ynoic acid.

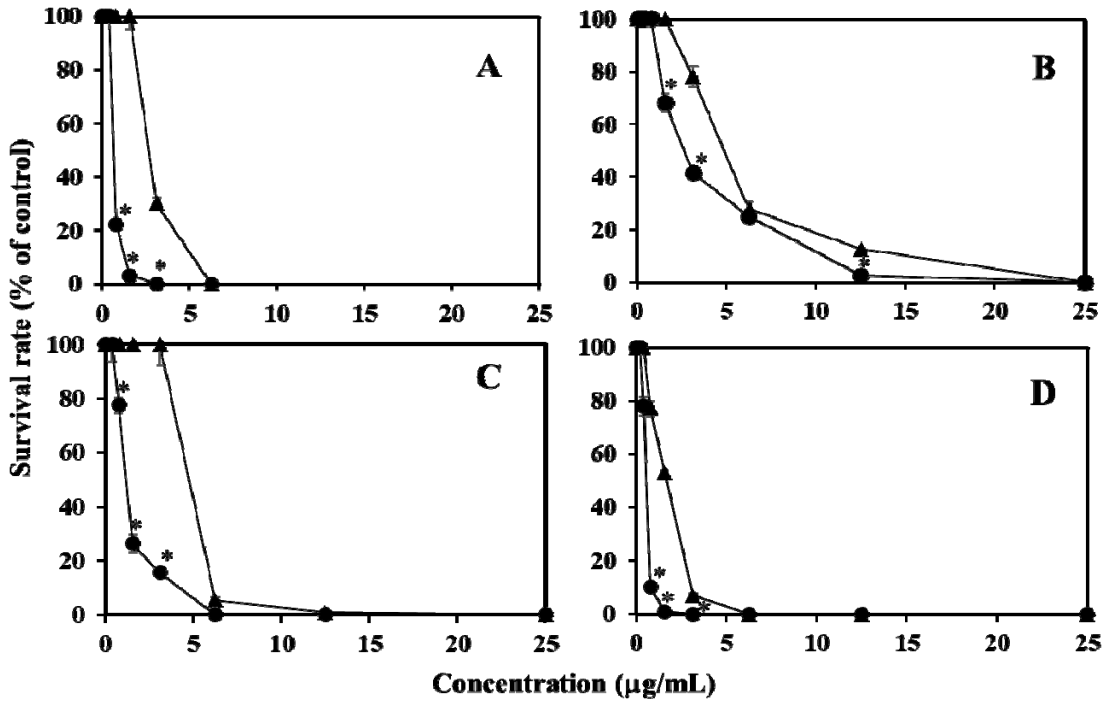


Fig. 2. The effects of compound A (▲) and compound B (●) on *C. antiqua* (A), *C. marina* (B), *H. circularisquama* (C), and *K. mikimotoi* (D). To the cell suspension of each phytoplankton species in 96-well plates, the indicated concentrations of compound A or B were added. After 4 h incubation under the phytoplankton culture conditions, the cell viabilities were determined as described in the text. Points are average of triplicate measurements, and bars indicate the standard deviation. Asterisks indicate significant differences between compound A and B ($p < 0.05$).

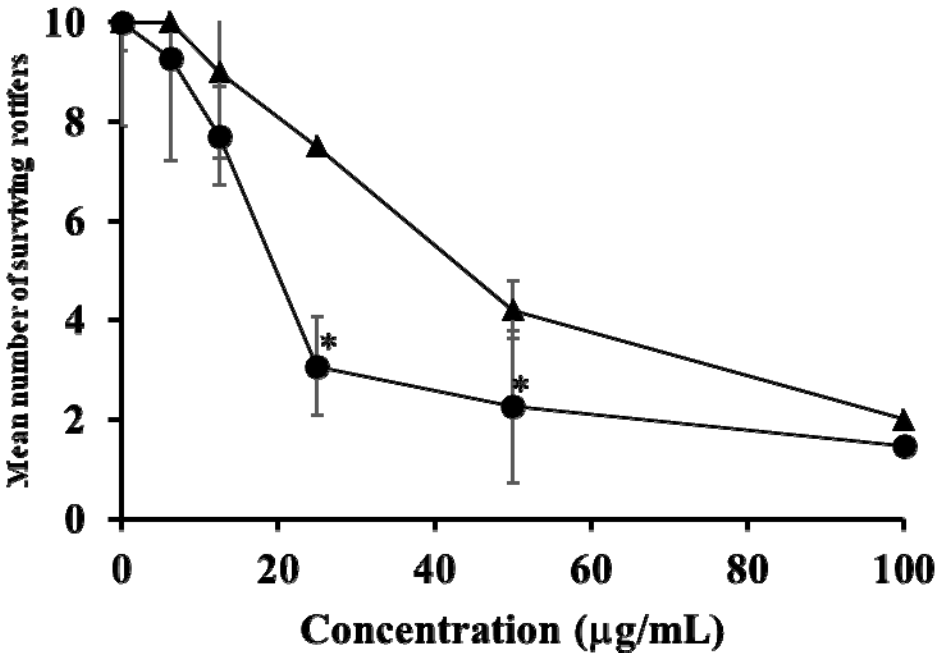


Fig. 3. The effects of compound A (▲) and compound B (●) on rotifer (*Brachionus plicatilis*). To the rotifers in 48-well plates (10 rotifers/well), the indicated concentrations of compound A or B were added. After 4 h incubation under the phytoplankton culture conditions, the viabilities of rotifers were determined as described in the text. Points are average of triplicate measurements, and bars indicate the standard deviation. Asterisks indicate significant differences between compound A and B ($p < 0.05$).

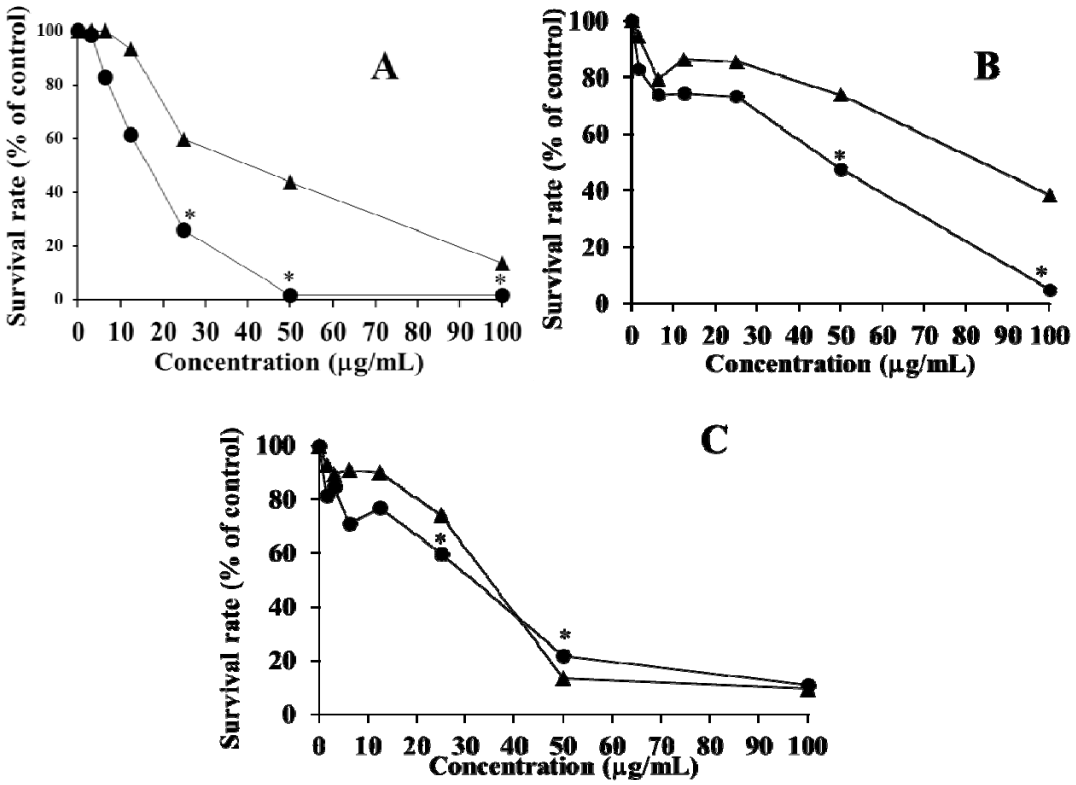


Fig. 4. The effects of compound A (▲) and compound B (●) on HeLa cells (A), XC cells (B), and U937 cells (C). To the cells in 96-well plates, the indicated concentrations of compound A or B were added. After 24 h incubation under culture conditions, the survival rates of the cells were determined by MTT assay. Points are average of triplicate measurements, and bars indicate the standard deviation. Asterisks indicate significant differences between compound A and B ($p < 0.05$).

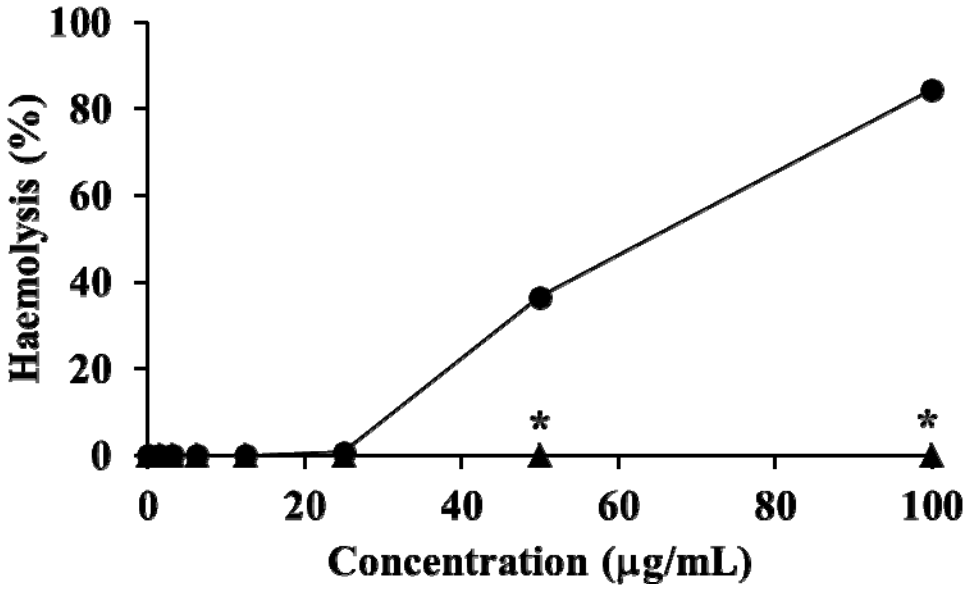


Fig. 5. Haemolytic activities of compound **A** (▲) and compound **B** (●) against rabbit erythrocytes. To the indicated concentrations of compound A or B in PBS in 96-well plates, the rabbit erythrocytes in PBS were added. After incubation for 4 h at 26°C, the extents of haemolysis were measured as described in the text. Points are average of triplicate measurements, and bars indicate the standard deviation. Asterisks indicate significant differences between compound A and B ($p < 0.05$).

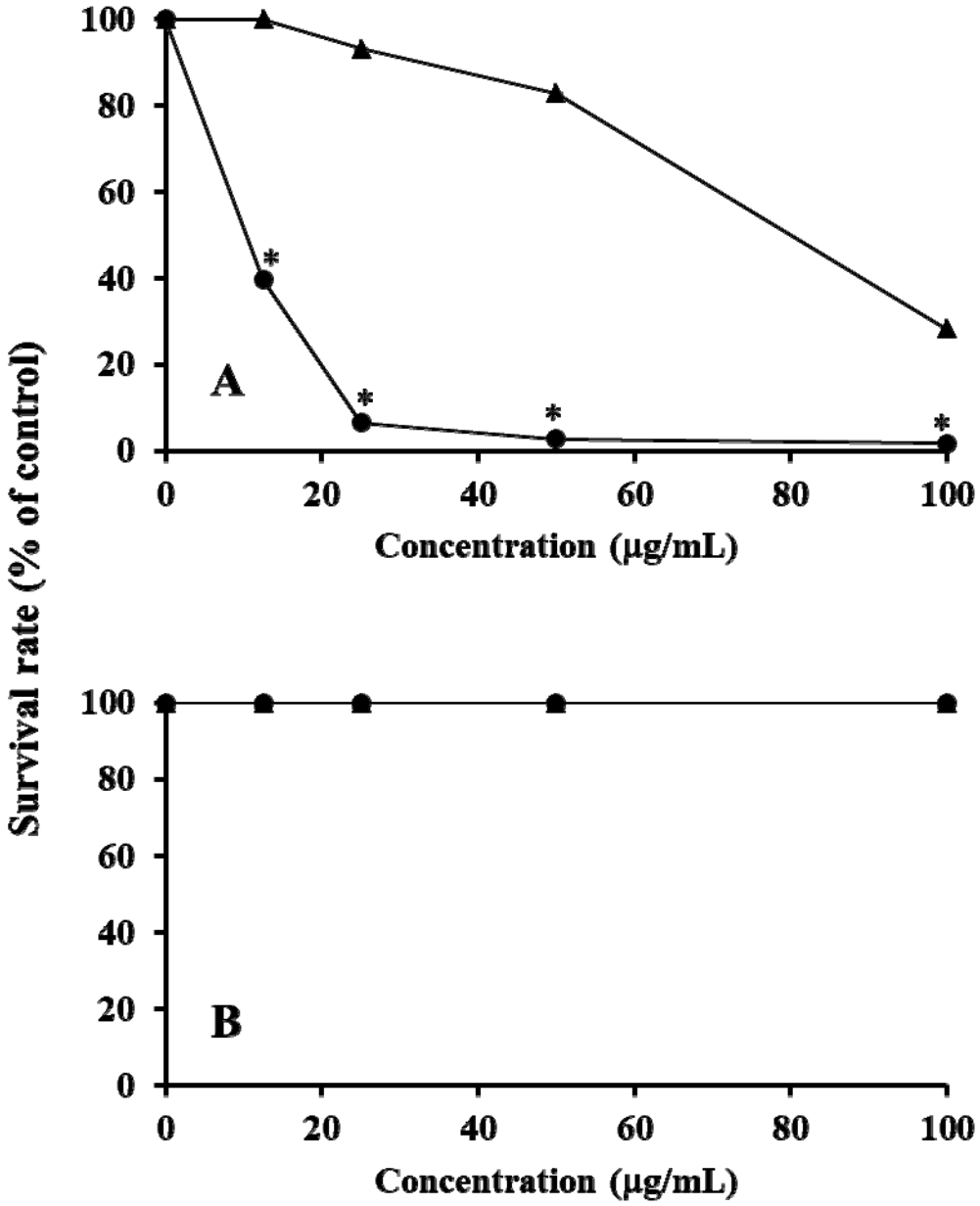


Fig. 6. The effects of compound A (▲) and compound B (●) on *Staphylococcus aureus* (A) and *Escherichia coli* (B). The indicated concentrations of compound A or B were added to the cell suspensions of the bacterial strains in PBS, and incubated at 37°C. After incubation for 4 h, the CFU of each assay mixture was measured as described in the text. Points are average of triplicate measurements, and bars indicate the standard deviation. Asterisks indicate significant differences between compound A and B ($p <$

0.05).