

Evaluation of the potential biological toxicities of aqueous extracts from red tide phytoplankton cultures in *in vitro* and *in vivo* systems

Daekyung Kim¹, Seon-Heui Cha¹, Emiko Sato², Yoshimi Niwano², Masahiro Kohno², Zedong Jiang³, Yasuhiro Yamasaki³, Yukihiko Matsuyama⁴, Kenichi Yamaguchi³, and Tatsuya Oda^{3*}

¹*Jeju Center, Korea Basic Science Institute (KBSI), Smart Building 1F, Jeju Science Park, 2170-2, Ara-dong, Jeju-Si, Jeju Special Self-Governing Province 690-756, Korea*

²*New Industry Creation Hatchery Center, Tohoku University, 6-6-10 Aoba, Aramaki, Aoba-ku, Sendai, Miyagi 980-8579, Japan*

³*Division of Biochemistry, Faculty of Fisheries, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan*

⁴*Seikai National Fisheries Research Institute, 1551-8, Taira-machi, Nagasaki, Nagasaki 851-2213, Japan*

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*連絡者の氏名: 小田達也

所属機関及び住所: 長崎大学水産学部
〒852-8521 長崎市文教町 1-14

電話番号: 095-819-2831

E-mail: t-oda@nagasaki-u.ac.jp

ABSTRACT– The biological toxic potentials of aqueous extracts from the dinophycean flagellates *Gymnodinium impudicum* and *Alexandrium affine* and the raphidophycean flagellate *Chattonella ovata* were examined in both *in vitro* and *in vivo* systems. Interestingly, the extract from *A. affine* was the only one that showed potent cytotoxicities towards HeLa, Vero, and Neuro-2a cells in a concentration-dependent manner. Mice given intraperitoneal injections of the extracts revealed that none of the extracts exhibited serious toxicities in mice. However, temporal body weight loss was observed in the mice injected with the extract from *A. affine* during the early stage, and the dramatic enlargement of spleens was also observed in the mice on the 7th day after injection. Since *A. affine* extract showed potent hemolytic activity *in vitro* towards mouse erythrocytes, hemolytic anemia may be a possible mechanism responsible for the splenomegaly in the mice injected with *A. affine* extract. Similar marginal effects were observed in the mice injected with the extract from *C. ovata*; however, no significant toxic or detrimental effects were detected in the mice injected with the extract from *G. impudicum*. These results suggest that the extract from *G. impudicum* may not be contaminated with detectable levels of biologically hazardous compounds and may be relatively safe compared with the other two extracts.

Keywords: *Alexandrium affine*, *Chattonella ovata*, *Gymnodinium impudicum*, red tide phytoplankton, radical scavenger, toxicity

INTRODUCTION

Red tide, often called harmful algal blooms (HABs), can cause mass mortality of natural and aquacultured fish or shellfish, which not only results in serious economic loss of marine resources but also contributes to pollution of the coastal areas. Several species of marine plankton have been identified as causative organisms of HABs (Hallegraeff, 1993; Honjo, 1994). Among them, raphidophycean flagellates *Chattonella* spp. are one of the most frequently appearing noxious red tide phytoplankters in Japanese coastal waters, and they are highly toxic to fish, especially yellowtail (*Seriola quinqueradiata*). Although the underlying mechanisms by which *Chattonella* spp. exert ichthyotoxic effects are still controversial, suffocation due to gill tissue dysfunction is thought to be the direct cause of fish death (Endo *et al.*, 1985, 1988). It has been reported that a decrease in the partial pressure of oxygen in arterial blood was the earliest physiological event in yellowtail exposed to *Chattonella marina*. In addition, the gill surface was covered with excessive mucus substances (Ishimatsu *et al.*, 1996; Hishida *et al.*, 1997). Studies have also demonstrated that *Chattonella* spp. generate reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, under normal culture conditions (Oda *et al.*, 1992ab, 1994, 1997; Tanaka *et al.*, 1992, 1994; Shimada *et al.*, 1993). In a recent study, we found that *C. marina* and *Chattonella ovata* continuously produce ROS, such as superoxide anions, under normal growth conditions (Kim *et al.*, 2007). Reactive oxygen species are generally considered to be toxic to living organisms, and they cause potentially deleterious effects on biological systems by damaging proteins, lipids, and nucleic acids (Halliwell and Gutteridge, 1984; Slater, 1984; Fridovich, 1986; Oda *et al.*, 1989; Miller *et al.*, 1990). Thus, the ROS generated by *Chattonella* spp. might be one of the causative factors responsible for the toxic effects. For example, ROS generated by *C. ovata* exhibited toxic effects on cultured fish under laboratory conditions (Hiroishi *et al.*, 2005), and *Heterosigma akashiwo*, which also belongs to the raphidophycean flagellates, has been reported to have a ROS-mediated toxic effect on rainbow trout (Yang *et al.*, 1995). In addition, it has been found that *C. marina*, *H. akashiwo*, and *C. ovata*, all of which belong to the raphidophycean flagellates, produce potent radical scavengers against superoxide anions (Kim *et al.*, 2000; Sato *et al.*, 2007). Indeed, the aqueous extracts from *C. marina* and *H. akashiwo* showed scavenging activity against superoxide anions generated enzymatically by a hypoxanthine (HPX)-xanthine oxidase (XOD) system. The production of superoxide scavenging substances by the flagellates is thought to be a self-defense mechanism against self-production of ROS. For marine organisms to survive in the sea, it is expected that they would possess antioxidants to protect themselves from oxidative stress. It has recently been reported that the red tide dinoflagellates *Cochlodinium polykrikoides* and *Karenia mikimotoi* generate superoxide anions, as determined by the cytochrome *c* reduction assay and the chemiluminescence method, respectively (Kim *et al.*, 1999; Yamasaki *et al.*, 2004). Hence, it has been postulated that dinophycean red tide flagellates also have an ability to scavenge superoxide anions. Because the ability to scavenge superoxide anions is supposed to function as a self-defense mechanism against superoxide anions generated by the flagellates themselves, we expected that the substances responsible for superoxide anion scavenging were novel antioxidant agents that could be made available for nutraceuticals, cosmeceuticals, and veterinary products. Based on this background, we have recently screened for antioxidant substance(s) generated by a wide variety of red tide

flagellates (Sato *et al.*, 2007). Our further examinations using an electron spin resonance (ESR)-spin trapping method coupled with a steady-state kinetic analysis revealed that the aqueous extracts from *Gymnodinium impudicum*, *Alexandrium affine*, and *C. ovata* have the ability to scavenge superoxide anions and hydroxyl radicals directly. The superoxide anion and hydroxyl radical scavenging potentials of all of the extracts were comparable to that of L-ascorbic acid and mannitol, respectively (Niwano *et al.*, 2007). Interestingly, we found that the scavenging activities of the extract from *G. impudicum* against superoxide anions and hydroxyl radicals were increased by heat exposure at 100°C and 200°C, respectively (Niwano *et al.*, 2007). Although the reason for the increased activity of the aqueous extract from *G. impudicum* is not clear, the heat-resistant effect of the extract from *G. impudicum* could be a desirable characteristic for an antioxidant. In addition to the direct harmful effects of HABs on various marine organisms, some causative species of HABs can also become a potential threat to human health because of the production of toxins that can accumulate in shellfish and other marine organisms. Dinoflagellates are well known for their ability to produce a wide-variety of hazardous compounds (Shimizu, 1995). Therefore, this study examined the potential biological toxicities of the extracts from three flagellate species in which potent antioxidant activities were discovered in our previous studies.

MATERIALS AND METHODS

Reagents

Reagents were purchased from the following sources:

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) from Sigma-Aldrich (St. Louis, MO, USA); fetal bovine serum (FBS) from Life Technologies Corp. (Carlsbad, CA, USA); manganous disodium ethylenediaminetetraacetate, trihydrate [Mn (II)-EDTA], ethylenediamine-N, N, N', N'-tetraacetic acid, iron (III), sodium salt, trihydrate [Fe (III)-EDTA], and ethylenediamine-N, N, N', N'-tetraacetic acid, disodium salt, dihydrate (EDTA · 2Na) from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Establishment of culture isolates of phytoplankters

Establishment of the clonal cultures of phytoplankters isolated from Japanese coastal waters was conducted under observation with an inverted microscope (Nikon Model TE300, Tokyo, Japan) using a micropipette (\varnothing 50-100 μ m). The isolated cell or chain was rinsed four or five times by filtered seawater (0.22 μ m pore size; GSWP, Millipore, MA, USA) and placed in a culture tube filled with 5 ml of culture medium, which was sterilized at 75°C for 1 hr before inoculation. Each isolated cell or chain was cultured at 30 practical salinity units with an illumination of approximately 100 μ mol/m²/s, with a cycle of 12 h light and 12 h dark provided by cool-white fluorescent lamps. The incubation temperature was set at 24°C. The stock culture of each isolate was maintained by serial transfer to fresh medium until its use in the experiments.

Culture of isolated phytoplankters

All equipment and glassware were soaked in 1 N HCl for a few days to remove substances such as phosphate-metal complexes, which might attach to the glass walls. Next, they were finely rinsed with re-distilled water and precombusted at 190°C for 1 h. To obtain a maximum gross of phytoplankters, each culture medium was modified as described below. For the raphidophycean red tide flagellate *C. ovata*, 170 mg of NaNO₃, 15.6 mg of NaH₂PO₄ · 2H₂O, 0.84 mg of Fe-EDTA, 0.69 mg of Mn (II)-EDTA, 0.24 mg of CoCl₂ · 6H₂O, 1 µg of H₂SeO₃, 2 mg of EDTA · 2Na and 1 ml of vitamin mixture (0.1 µg of thiamine, 0.1 µg of Ca-pantothenate, 10 ng of *p*-aminobenzoic acid, 1 ng of biotin, 5 µg of inositol, 3 µg of thymine, 1 ng of vitamin B₁₂, and 2 ng of folic acid) were added to 1,000 ml of glass-filtered natural seawater and heated at 75°C for 2 h. For the dinophycean red tide flagellates *G. impudicum*, *A. affine*, and *A. tamiyavanichii*, 45 mg of NaNO₃, 4 mg of NaH₂PO₄ · 2H₂O, 0.21 mg of Fe (III)-EDTA, 0.17 mg of Mn (II)-EDTA, 0.12 mg of CoCl₂ · 6H₂O, 0.5 µg of EDTA · 2Na and 1 ml of vitamin mixture (0.1 µg of thiamine, 0.1 µg of Ca-pantothenate, 10 ng of *p*-aminobenzoic acid, 1 ng of biotin, 5 µg of inositol, 3 µg of thymine, 1 ng of vitamin B₁₂, and 2 ng of folic acid) were added to 1,000 ml of glass-filtered natural seawater and heated at 75°C for 2 h. Next, 125 ml of medium and 0.4 ml of 2% (w/v) streptomycin solution were added to a 250 ml culture flask, and the 2 ml of stock culture of the isolated strain was inoculated. Cultures were conducted at 22°C under 120 µmol/m²/s illumination with a cycle of 16 h light and 8 h dark for 10-14 days. The final yield of each culture reached approximately 3 × 10⁴ cells/ml. Cells of phytoplankters were counted with a Sedgewick-Rafter chamber under the light microscope. Unless otherwise noted, flagellates in the exponential growth phase were used throughout the experiments.

Chlorophyll determination

We measured the chlorophyll content of each flagellate culture. Each culture was harvested by centrifugation (15,000 rpm, 5 min), and the supernatant was carefully removed. An aliquot of 98% dimethylformamide was added to the precipitate and stored below 5°C for 24 h (Suzuki and Ishimaru, 1990). After centrifugation (15,000 rpm, 5 min), chlorophyll content in the supernatant was spectrofluorometrically determined at 665 nm with excitation at 460 nm using a multiwell scanning spectrophotometer (Multiskan Spectrum, Thermo Electron Co., Vantaa, Finland).

Preparation of aqueous extract

After adjusting the chlorophyll concentration to 60 µg/ml with pure water, the cells were ruptured by pipetting followed by 10 times repeated ultrasound treatments for 1 min each at 4°C. The treated suspension was then centrifuged at 15,000 rpm for 5 min, and the supernatant was used as the aqueous extract.

Culture of mammalian cell lines

HeLa (human epithelial carcinoma), Vero (African green monkey kidney), and Neuro-2a (mouse neuroblastoma) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). HeLa and Vero cells were cultured in α -minimal essential medium (α -MEM) supplemented with 10% fetal bovine serum (FBS), 10 μ g/ml of adenosine, guanosine, cytidine, and thymidine, penicillin (100 μ g/ml), and streptomycin (100 μ g/ml) in 5%CO₂ at 37°C. Neuro-2a cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 10 μ g/ml of adenosine, guanosine, cytidine, and thymidine, penicillin (100 μ g/ml), and streptomycin (100 μ g/ml) in 5% CO₂ at 37°C.

Measurement of cytotoxicity

The cytotoxicity of each extract was assessed with the MTT assay. Cells were detached from the flasks by treatment with trypsin (0.5 mg/ml)/EDTA (0.2 mg/ml) in phosphate-buffered saline (PBS) at 37°C for 5 min. Detached cells in the growth medium were seeded into flat-bottomed 96-well plates at a density of 2×10^5 cells/ml in a final volume of 100 μ l/well and incubated at 37°C for 24 h. Adherent cells in the 96-well plates were washed twice with PBS and incubated with serially diluted extract in the medium with or without 10% FBS at 37°C for 24 h prior to incubation with MTT (final 10%) for another 0.5 h. After aspiration of the medium, dimethylsulfoxide was added to dissolve the MTT formazan reaction product, and the optical density was measured at 570 nm using a multiwell scanning spectrophotometer (Multiskan Spectrum, Thermo Electron Co., Vantaa, Finland).

Bioassay for PSP toxins using Neuro-2a cells

Neuro-2a cells maintained in culture flasks were detached from the flasks by treatment with trypsin (0.5 mg/ml)/EDTA (0.2 mg/ml) in PBS at 37°C for 5 min. Detached cells were suspended in RPMI 1640 supplemented with 10% FBS, seeded into flat-bottomed 96-well plates at a density of 2×10^5 cells/ml in a final volume of 100 μ l/well, and incubated at 37°C for 24 h. Adherent Neuro-2a cells in 96-well plates were washed twice with PBS, and 90 μ l of RPMI 1640 medium without 10% FBS, which contained the sodium channel activator veratridine (final 0.05 mM) and Na⁺/K⁺ ATPase inhibitor ouabain (final 0.5 mM), were added to each well with the extracts containing 10 μ g chlorophyll. After 24 h incubation at 37°C, the cell viability of each well was examined by the MTT assay as described above. This bioassay is based on the protective effects of sodium channel blockers, such as paralytic shellfish poisoning (PSP) toxins, on the cytotoxicity induced by the cooperation of veratridine and ouabain in Neuro-2a cells. PSP toxin-like activity can be estimated from the extent of the protective effects of the samples (Jellett *et al.*, 1992).

Measurement of hemolytic activity

The hemolytic activity of each extract towards mouse erythrocytes was measured as described

previously (Kuroda *et al.*, 2005). In brief, freshly collected heparinized mouse blood was washed three times with PBS, and made to a final concentration of 4% (v/v) in PBS. Triplicate 50 μ l aliquots of serial twofold dilutions of each extract in PBS were added to the wells of round-bottom 96-well microplates (Falcon). To each well, the same volume of 4% (v/v) suspension of erythrocytes in PBS was added, and the plate was gently shaken. After incubation for 2 h at 26°C, the plate was centrifuged at $900 \times g$ for 10 min. Fifty microliter of supernatant from each well was transferred to a flat-bottom 96-well microplate and the released hemoglobin absorbance at 570 nm was measured with a multiwell scanning spectrophotometer (Multiskan Spectrum, Thermo Electron Co., Vantaa, Finland). Zero hemolysis (blank) and 100% hemolysis controls were determined using erythrocytes suspended in PBS alone and 1% Triton X-100 in PBS, respectively.

Animals

Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee. Specific pathogen-free male ddY mice (6 weeks old) were purchased from Kyudo (Kumamoto, Japan). The mice were acclimated for 1 week in cages in a room under controlled temperature ($23^{\circ}\text{C} \pm 1^{\circ}\text{C}$) and humidity ($55\% \pm 5\%$). Commercial rodent diet (CE-2, Clea, Tokyo, Japan) and water were provided *ad libitum* throughout the duration of the experiment.

Treatment of animals

Mice were randomly assigned into four groups, and each group consisted of ten mice to test fixed concentrations of each flagellate extract. Each mouse received a 2 μ g chlorophyll-equivalent/g body weight dose of extract by intraperitoneal injection using a syringe with a needle on the first day. The control group received only PBS. All mice were observed daily for abnormalities and signs of ill health, and the body weights were measured during the experiments. After 7 days, all mice were sacrificed by decapitation under CO_2/O_2 anesthesia. Organ weights were obtained for the liver, heart, kidney, lung, and spleen. Organs showing macroscopic abnormalities were preserved in a neutral phosphate-buffered 4% formaldehyde solution.

Statistical analysis

The results were expressed as a mean \pm SE, and the data were analyzed using one-way ANOVA followed by a Student's t-test to determine any significant differences. $p < 0.01$ was considered as statistically significance.

RESULTS AND DISCUSSION

To evaluate the biological toxic potentials of the aqueous extracts of marine phytoplankters, we first examined the cytotoxic activities of each extract towards HeLa, Vero, and Neuro-2a cells. We selected HeLa and Vero cells as representative tumor and normal tissue-derived cell lines, respectively. Because neurotoxic substances have often been discovered from marine phytoplankters, especially dinoflagellates, the Neuro-2a cell line was used as a representative neuronal cell line. The extract prepared from *A. affine* showed potent cytotoxicity towards all the cell lines tested in a concentration-dependent manner (Fig. 1). A slight decrease in the cytotoxic activity was observed in the presence of 10% FBS, which suggests that FBS interfered with the activity of the toxic agents. The extracts from *C. ovata* and *G. impudicum*, however, did not show any significant toxic effects on these cell lines even at the highest concentrations tested. No significant PSP-like activity was detected in the extracts (data not shown), which suggests that the cytotoxic effect of the extract of *A. affine* may be due to a direct cell-killing mechanism distinct from sodium channel blockade.

To examine whether or not the extracts have toxic or harmful effects in an *in vivo* system, we administered each extract to mice (2 µg chlorophyll-equivalent/g body weight, i.p.) and observed them for 7 days. Although there was one dead mouse in the group injected with the extract of *A. affine* during the 7 days of observation, all of the mice injected with *C. ovata* or *G. impudicum* extract survived (Fig. 2). The injection of 2 µg chlorophyll-equivalent/g body weight of extract prepared from *A. tamiyavanichii*, which has been confirmed to have a high level of PSP toxins, resulted in ten out of ten mice dying within 24 h after injection (Fig. 2). These results suggest that the extracts prepared from *C. ovata*, *A. affine*, and *G. impudicum* may not contain high levels of PSP-like lethal toxins.

Figure 3 shows the profiles of the changes in the body weights of the mice over the 7 days they were observed after administration of each extract. In the group injected with *C. ovata* extract, a transient decrease in body weight was observed at day 2, but these mice recovered by day 3. Transient body weight loss was also observed in the group injected with *A. affine* extract, and it was more serious than it was in the *C. ovata* group. We did not observe any significant effects on body weight gain following *G. impudicum* extract administration, and the profiles of the changes in body weights were almost the same as the controls.

Liver and spleen weights were extremely higher in the group injected with *A. affine* extract compared with the control group, but there were no significant differences in lungs, kidney, and heart weights between groups (Fig. 4). Enlarged spleens from the *A. affine* group were obvious under macroscopic observation (Fig. 5). A slight enlargement of the spleens of mice injected with *C. ovata* extract was also observed. However, no abnormal symptoms were observed in the organs, including spleen, from the group injected with *G. impudicum* extract. These results clearly demonstrated that the aqueous extract of *A. affine* contained cytotoxic substances that may be distinct from PSP-like toxins, and it caused detrimental effects in mice following intraperitoneal administration. Previous studies have shown that some *Alexandrium* species produce toxins other than PSP toxins, which have been shown to cause noxious effects on surrounding marine living organisms. For instance, Simonsen *et al.* (1995) found that one strain of *A. tamarense* had a low level of PSP toxins but strong hemolytic activity. They suggested that the hemolytic activity was due to

compounds other than PSP toxins because purified PSP toxins showed weak or no hemolytic activity. Eschbach *et al.* (2001) reported potent hemolytic activity for *Alexandrium* species whereas other dinoflagellates, such as *Gyrodinium aureolum*, *Gyrodinium instriatrium*, *Gymnodinium chlorophorum*, and *Gymnodinium varians*, showed only weak or no hemolytic activity. Thus, it seems likely that hemolytic activity is a common biochemical feature of *Alexandrium* species. These findings prompted us to speculate that the dramatically enlarged spleens and enlarged livers in the mice injected with *A. affine* extract may be a result of splenic and hepatic extramedullary hematopoiesis provoked by hemolytic anemia, which may be due to hemolytic agents present in the extract (Cheng and Kazazian Jr., 1976; Kazazian Jr. *et al.*, 1974). To ascertain whether or not this is the case, we examined the hemolytic activity of the extracts. As shown in Fig. 6, *A. affine* extract showed a potent hemolytic activity *in vitro* towards mouse erythrocytes, while no significant hemolytic activity was observed in other extracts under the conditions tested. Thus, the extract of *A. affine* contains hemolytic agent that may be responsible for hemolytic anemia leading to the splenomegaly in the mice injected with *A. affine* extract.

It is conceivable that such hemolytic agents may also be involved in the cytotoxicity of *A. affine* extract. Because we have previously found hemolytic and cytotoxic activities in the extract of *Chattonella* (Kuroda *et al.*, 2005), one can speculate that such agents may be partly responsible for the temporal weight loss and moderate splenomegaly observed in the mice injected with *C. ovata* extract. Further studies are required to clarify the exact mechanism of splenomegaly caused by *A. affine* extract as well as an identification of the causative compounds responsible for this effect.

The most important finding in the present study was the fact that we did not detect any biologically toxic or harmful aspect of the extract from *G. impudicum* in either an *in vitro* or an *in vivo* system. Many species of red tide phytoplankters, especially dinoflagellates, are known to produce various toxins, such as the diarrhetic shellfish poisons (DSPs) or PSP toxins. These algal toxins can often accumulate in shellfish and finfish, and the consumption of contaminated seafood poses serious human health risks. Therefore, when it comes to the development of useful bioactive compounds derived from red tide phytoplankters, it is essential to ascertain whether the extracts from phytoplankters are contaminated with biologically hazardous compounds. Under these circumstances, our results provide supportive evidence for the safety of the extract from *G. impudicum* as a source of useful antioxidants.

G. impudicum has been shown to produce antioxidant substances under simple culture conditions without any specialized stimuli. Hence, based on the previous findings and the results obtained in this study, the extract from *G. impudicum* is a promising novel candidate for industrialization. Indeed, it may be useful as an additive for nutraceuticals, cosmeceuticals and veterinary products.

In addition, ROS are supposed to be involved in ichthyotoxic mechanisms (Yang *et al.*, 1995; Hiroishi *et al.*, 2005), and the radical scavenging substance produced by red tide flagellates could be a novel agent for the remedy of flagellate-mediated ichthyotoxicity. The relatively non-toxic properties and heat-resistance of the extract from *G. impudicum* could be particularly desirable characteristics for a commercial agent.

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Figure legends

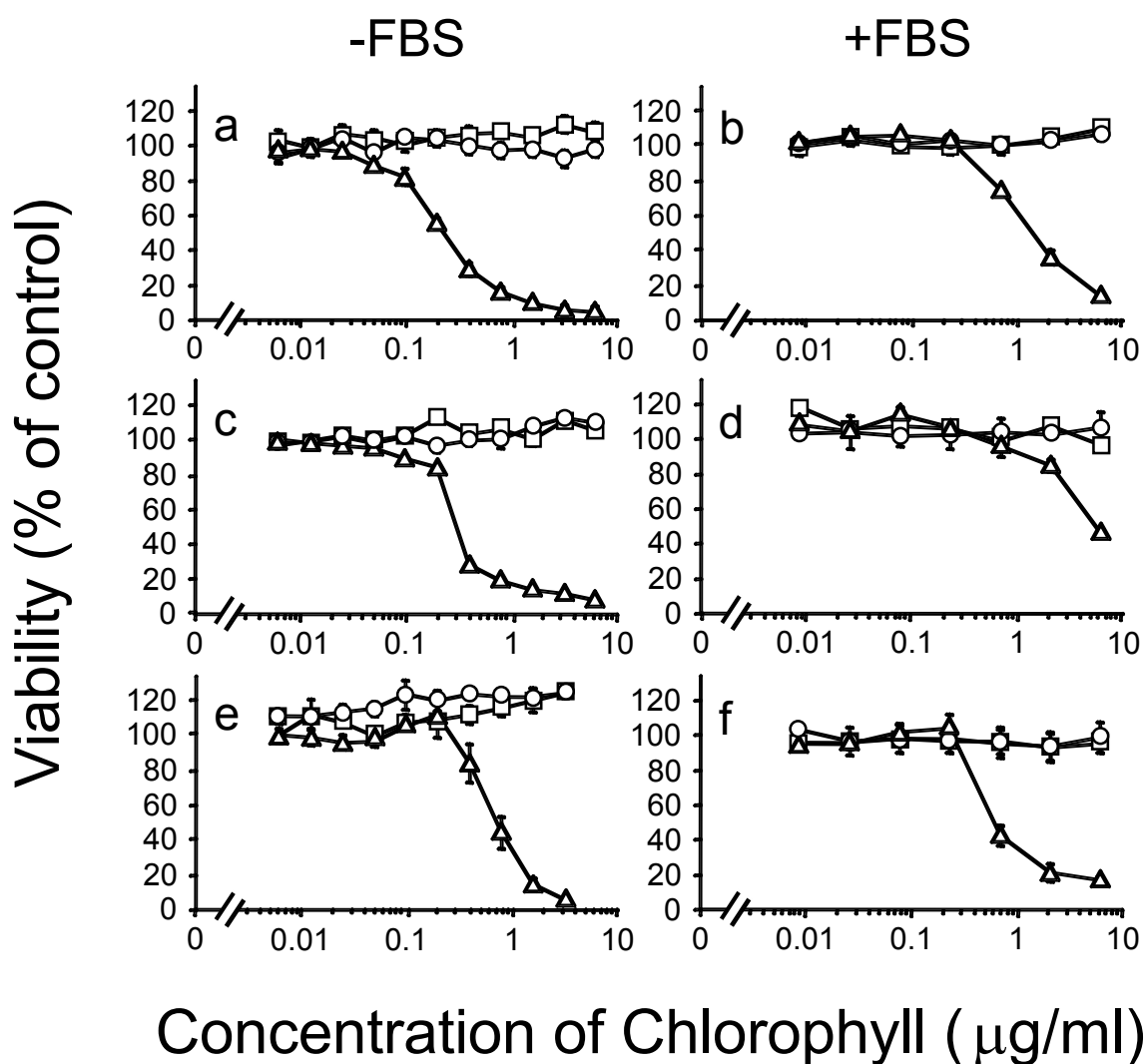


Fig. 1.

The cytotoxic effects of the extracts from *Gymnodinium impudicum*, *Alexandrium affine*, and *Chattonella ovata* on Neuro-2a (a, b), HeLa (c, d), and Vero (e, f) cells. Adherent cells (2×10^4 cells/well in 96-well plates) were incubated with the indicated concentrations of each extract from *G. impudicum* (○), *A. affine* (△), and *C. ovata* (□) in α -MEM for HeLa and Vero cells, and RPMI 1640 for Neuro-2a cells with (b, d, f) or without (a, c, e) 10% FBS at 37°C. After 24 h, the viabilities of the cells were examined by the MTT assay as described in the text. Each value represents the mean of triplicate measurements.

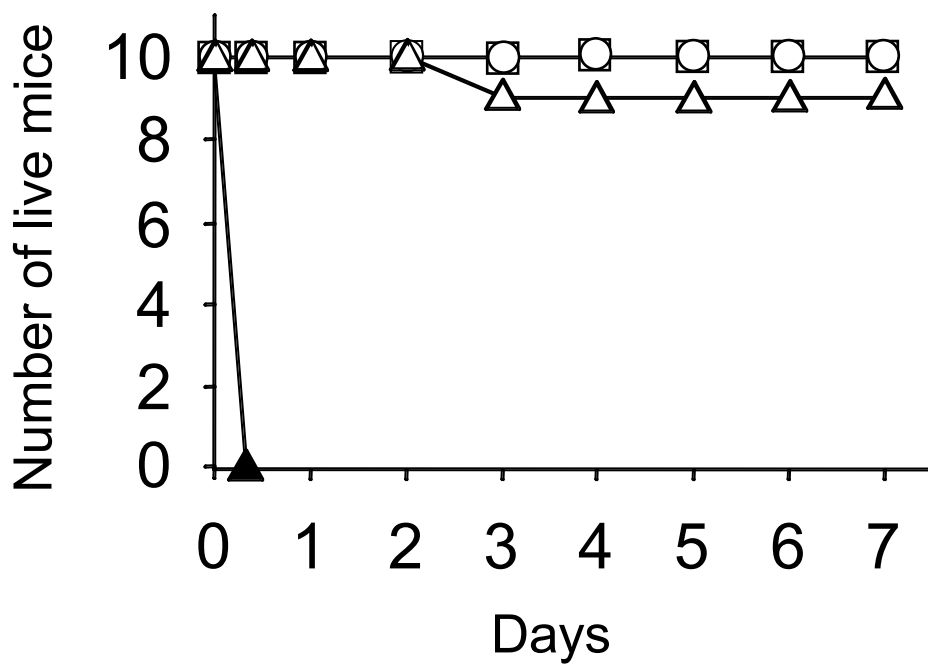


Fig. 2.

The cytotoxic effects of the extracts from *Gymnodinium impudicum*, *Alexandrium affine*, *Chattonella ovata*, and *A. tamiyavanichii* on ddY mice. Fixed concentrations (2 µg chlorophyll equivalent/g body weight) of the extracts from *G. impudicum* (○), *A. affine* (△), *C. ovata* (□), or *A. tamiyavanichii* (▲) in 0.4 ml of PBS were injected intraperitoneally at day 0, and the mice were observed for 7 days. Control mice (●) were injected with the same volume of PBS.

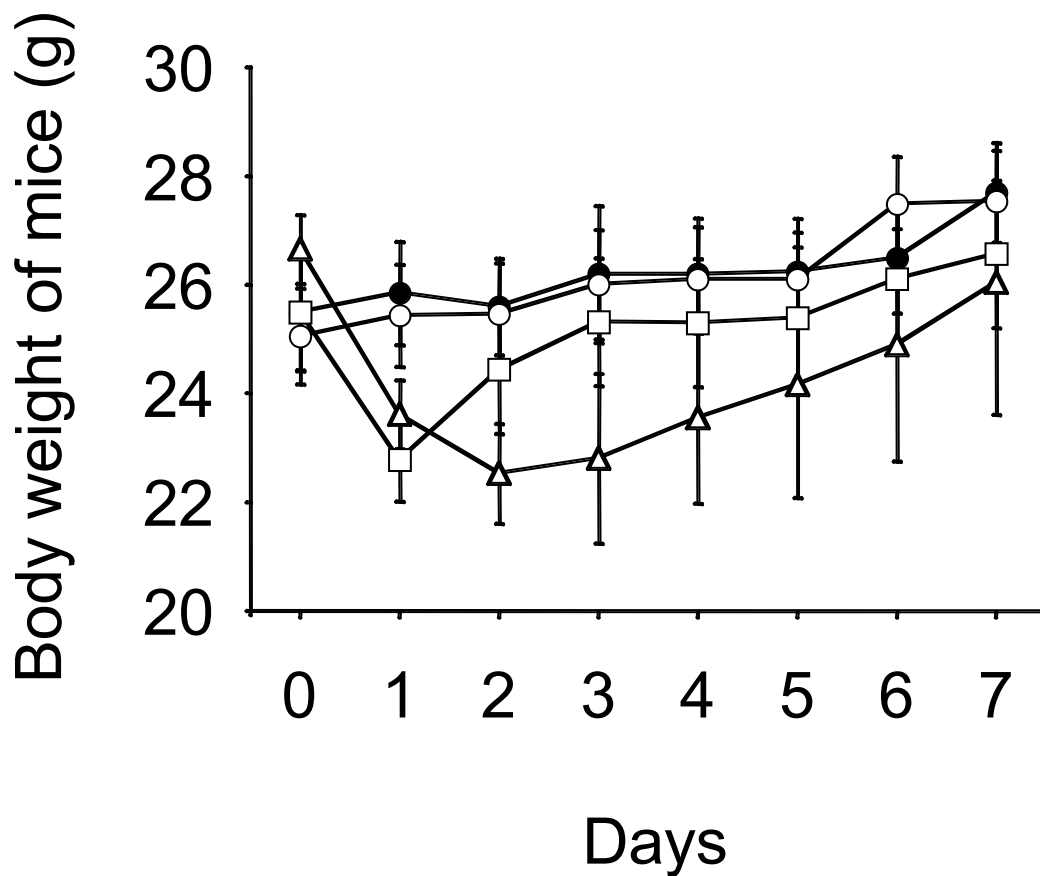


Fig. 3.

Individual changes in the body weights of the mice after intraperitoneal injection of the extracts from *Gymnodinium impudicum*, *Alexandrium affine*, and *Chattonella ovata*. After injection of the fixed concentration (2 μg chlorophyll equivalent/g body weight) of the extracts from *G. impudicum* (b, ○), *C. ovata* (c, □), or *A. affine* (d, △), the body weights of the mice were measured once daily. Control mice (a, ●) were injected with the same volume of PBS. Each point represents the mean values \pm SD. $n=10$.

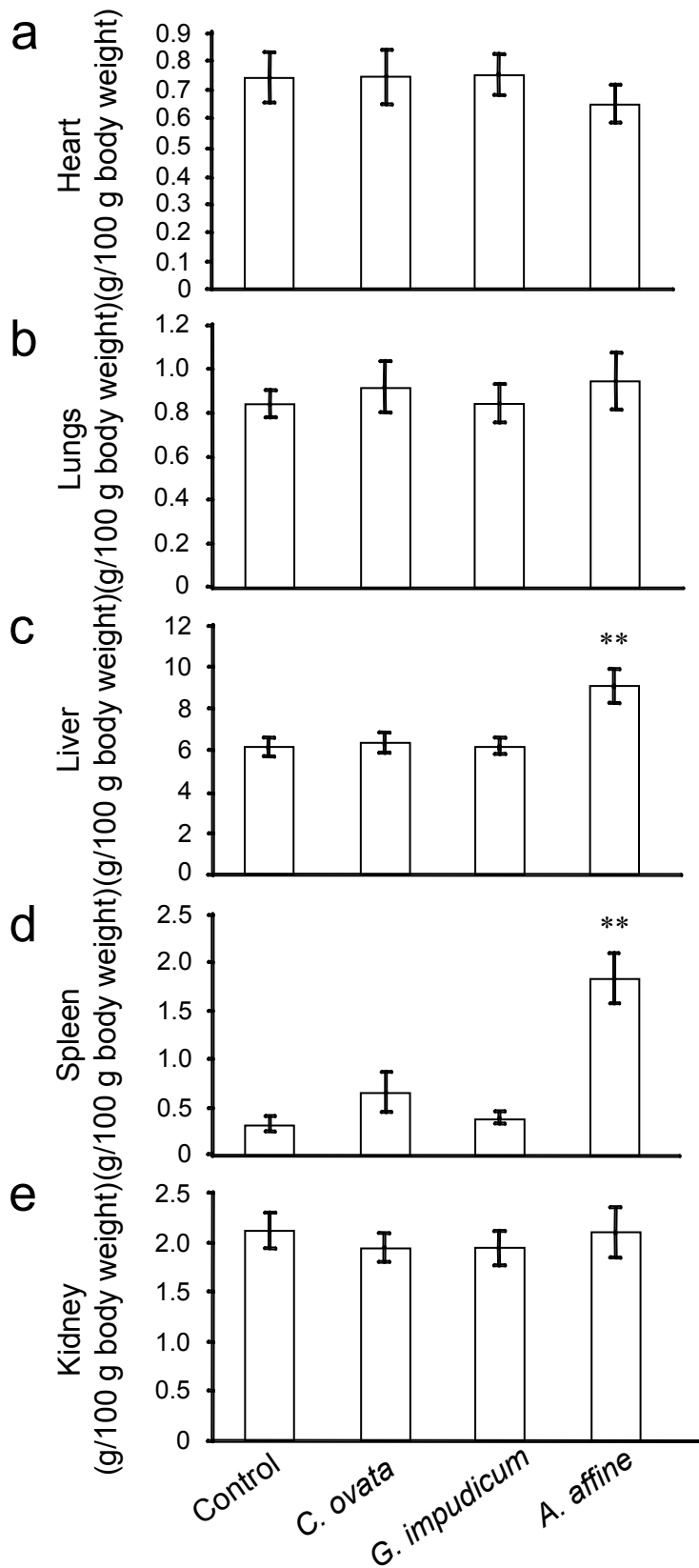


Fig. 4.

Changes in the organ weights of the mice after intraperitoneal injection of the extracts from *Chattonella ovata*, *Gymnodinium impudicum*, and *Alexandrium affine*. After injection of the fixed concentration (2 μ g chlorophyll equivalent/g body weight) of the extracts from *G. impudicum*, *C. ovata*, and *A. affine* at day 0, the mice were sacrificed at day 7, and the weights of heart (a), lungs (b), liver (c), spleen (d), and kidney (e) were measured. Control mice were injected with the same volume of PBS. $n=10$. **: $p < 0.01$.

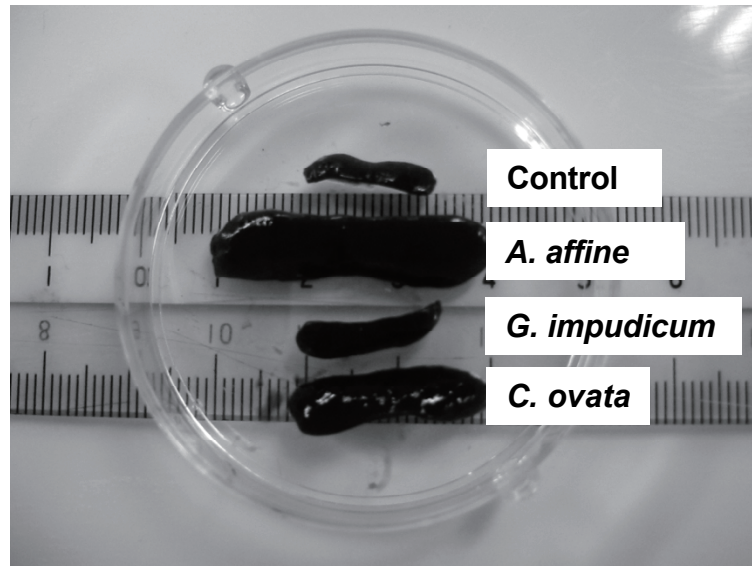


Fig. 5.

The macroscopic appearances of the spleens from the mice injected with the extracts from *Gymnodinium impudicum*, *Alexandrium affine*, and *Chattonella ovata*. After the injection of the extracts, the mice were sacrificed at day 7. Photomicrographs of a representative spleen from each group were taken.

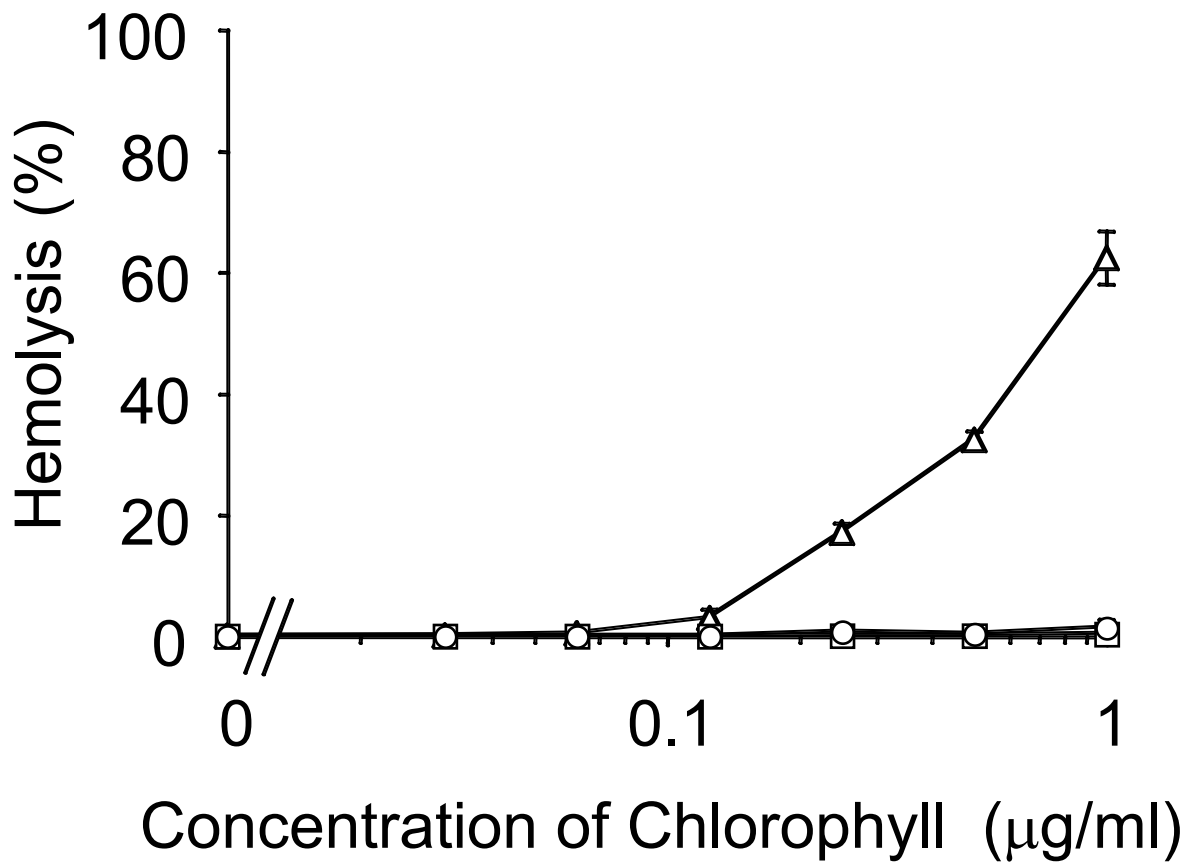


Fig. 6.

The hemolytic activities of the extracts from *Gymnodinium impudicum*, *Alexandrium affine*, and *Chattonella ovata* against mouse erythrocytes. Varying concentrations of each extract were mixed with mouse erythrocytes and incubated 26°C for 2 h, and the extents of hemolysis were measured as described in the text. Each point represents the mean of triplicate measurements. Each bar represents SD.