octadeca-6,9,12,15-tetraenoic acid from *Nemacystis decipiens* as an algicidal source against *Heterocapsa circularisquama*

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Abstract

*Heterocapsa circularisquama* as a red tide plankton to cause destruction problem such as releasing toxin in commercially farmed shellfish and other fisheries production. Recently, seaweed has been reported to explore as algicidal substances for decreasing survivability of red tide species. Fortunately, the Phaeophyceae algae of *Nemacystis decipiens* showed potent algicidal agent of octadeca-6,9,12,15-tetraenoic acid as much as 6.09% of the total fatty acid composition. The algicidal activity of 5 µg/ml of octadeca-6,9,12,15-tetraenoic acid from *N. decipiens* showed moderate of mortality cells of *H. circularisquama* between 30 and 69%. It is suggested that octadeca-6,9,12,15-tetraenoic acid from *N. decipiens* is useful mitigation agent to remove harmful effects of *H. circularisquama*.

Keywords: *Heterocapsa circularisquama*, octadeca-6,9,12,15-tetraenoic acid, *Nemacystis decipiens*

INTRODUCTION

*Heterocapsa circularisquama* as a red tide plankton to cause destruction problem such as releasing toxin in commercially farmed shellfish and other fisheries production (Kamiyama and Arima, 1997; Matsuyama et al., 1995; Yamamoto and Tanaka, 1990). Nagai et al. (1996) also reported that *H. circularisquama* as Harmful Algal Blooms (HABs) kills bivalves due to the production toxin that repress bivalve feeding. Fukuyo et al. (2002) showed that red tide species are a big problem to marine culture industry and human health. In blooming condition, red tide species can block the sunlight that other organisms need to live and produce toxin that may harm to shellfish and marine organisms. Bae et al. (1999) explained various chemical, physical, biological and methods are known to control red tide species effectively. However, Jeong et al. (2000) reported that many are clearly unacceptable due to environmental side effects and poor benefit or cost ratios.

Furthermore, Matsuyama et al. (1997) suggested that toxin of *H. circularisquama* is considered to be a protein-like substance localized on the cell surface. Sato et al. (2002) also reported that *H. circularisquama* has photosensitizing hemolytic toxin which can be easily extracted into ethanol. Recently, marine algae have been reported to explore algicidal substances for decreasing survivability of red tide species. Alamsjah et al. (2008) found that the green macroalgae *Ulva fasciata* had significantly algicidal substances against several microalgae of the red tide species. These algicidal substances of *U. fasciata* consist of polyunsaturated fatty acid (PUFA) as hexadeca-4,7,10,13-tetraenoic acid, octadeca-6,9,12,15-tetraenoic acid and α-linolenic acid. Nagayama et al. (2003) have also investigated toxic effect of the brown algae *Ecklonia kurome* on red tide species and several phlorotannins were isolated as the active principles. Jin and Dong (2003) showed that comparative studies on the algicidal effects of different strains of *U. pertusa* on HAB species, such as *Heterosigma akashiwo* and *Alexandrium tamarense*. Nan et al. (2004) also reported that algicidal interaction between *U. pertusa* and microalgae. Wang et al. (2006) showed that effect of green algae *U. pertusa* and red algae *Gracilaria lemaneiformis* on the growth of *H. akashiwo* in co culture. In this study, I turned attention to...
explore the marine alga of *Nemacystis decipiens* for investigating the algicidal substances.

**Materials and methods**

Macroalgae *N. decipiens* were collected from intertidal areas of the coast. All samples were brought to the laboratory in plastic bags containing sea water to prevent evaporation, and then washed with distilled water to separate potential contaminants. For convenient use of the samples, the *N. decipiens* collected were air dried for 5 days at room temperature and then ground to powder using a blender.

Screening for algicidal activity were performed on *N. decipiens*, whereas macroalgae tissues were subjected to methanol extraction following the method previously described by Jeong *et al.* (2000) with minor modifications. Each 100 mg of dried sample of *N. decipiens* was soaked in 5 ml of methanol at room temperature for 1 day and filtered through no. 2 filter paper under reduced pressure. This extraction procedure was repeated three times, and the extracts were combined.

Axenic red tide species of *H. circularisquama* were obtained from the National Institute for Environmental Studies of Japan. These red tide species were cultured aseptically in f/2 medium (Guillard’s Sigma) at 20°C, 40µmol/m²/s using 40 W white fluorescent lamps (Toshiba) with 12L: 12D cycle and sub cultured after 30 days. Prior to the experiments, the red tide species were sub cultured for 7 days.

Methanol extract solution (10µl) was added to each 1 ml of *H. circularisquama* cell suspension of a cell density of 3 x 10⁴ cells/ml. After 4 hours, the survivability and mortality of the cells were calculated under microscopic observation (x 20). All experiments in this study were done separately at least in triplicate, and aseptic techniques were employed in all experimental steps.

Algicidal activity was calculated using the formula: algicidal activity = (dead cells/living and dead cells) x 100%. All data in this study were tested by means of the ANOVA-test (p<0.05).

Measurement of fatty acid composition were performed by the dried sample of *N. decipiens* (10 g) was extracted using 150 ml of methanol (Wako Pure Chemical) for 3 days. The extraction procedure was repeated twice and the extracts were combined. The 50 mg of the crude extract was esterified by exposure to 2.5 ml of 3% HCl/MeOH overnight. After concentrating under reduced pressure, the residue was taken up in CH₂Cl₂ and washed with 5% NaHCO₃ solution. The CH₂Cl₂ layer was separated and dried over Na₂SO₄. After concentration, the residue was passed through a short column on silica gel (63-230 µm, 500 mg) using CH₂Cl₂ as the liquid solvent. Finally, the methyl ester fraction was diluted in 1 ml of hexane and analyzed using a GC-2014 gas chromatograph (Shimadzu) equipped with a capillary column (CP-Sil 88 for FAME fused silica WCOT, 50 m x 0.25 mm i.d., 0.2 µm film thickness, Chrompack, Hewlett-Packard) and a flame ionization detector (FID). The detector and the injector temperature were maintained at 300°C. The initial oven temperature was programmed at 170°C for 15 min, and then, was allowed to rise to 230°C at a rate of 5 °C/min, and was kept at the final temperature for 5 min. Nitrogen was used as the carrier gas at a flow rate of 24 ml/min. A standard fatty acid methyl ester mixture C8-C22 and C14-C22 (Supelco) was used for identification of the peaks.

Extraction and isolation of algicidal compounds (octadeca-6,9,12,15-tetraenoic acids) from *N. decipiens* was performed based on Alamsjah *et al.* (2005). The methanol extract of macroalgae was partitioned into hexane, 50% methanol, and water soluble fractions. The active hexane-soluble fraction was separated by silica gel chromatography, followed by reversed phase (ODS) chromatography and finally reversed-phase HPLC monitoring the algicidal activity against *H. circularisquama*.

**Results**

*Nemacystis decipiens* showed the high average ratios of fatty acid composition with palmitic (C16:0), linoleic (C18:2), myristic (C14:0), behenic (C22:0), α-linolenic (C18:3), cis-9,12,15), and octadeca-6,9,12,15-tetraenoic (C18:4) acids. Interestingly, algicidal compounds/dry tissue was measured as much as 80.38 mg/100g, whereas chlorophyll a of *N. decipiens* was 0.18 g/100g (Table 1).

Structural determination of octadeca-6,9,12,15-tetraenoic acid from *N. decipiens* was proved by NMR δH (400MHz, CDCl₃): 0.97 (t, 3H, J = 7.6 Hz, -CH₂CH₃), 1.42 (quint., 2H, J = 7.6 Hz, -CH=CH₂), 1.61-1.71 (m, 2H, 3-H), 2.03-2.12 (m, 4H, -CH=CH-CH₂- x 2), 2.35 (t, 2H, J = 7.6 Hz, -CH₂-COOH), 2.77-2.87 (m, 6H, -CH=CH-CH₂-CH=CH- x 3), 5.28-5.43 (m, 8H, -CH=CH- x 4), 6.65-7.59 (br, 1H, COOH). Based on NMR δC (75MHz, CDCl₃) also showed 14.4 (-CH₃), 20.6 (-CH₂CH₃), 24.4 (-CH₂-CH₂COOH), 25.6 (-CH=CH-CH₂=CH=CH), 25.7 (-CH=CH-CH₂=CH=CH), 26.9 (-CH=CH-CH₂=CH=CH), 30.0 (-CH=CH=CH₂=CH=CH), 33.8 (-CH₂-COOH), 127.0 (-CH=CH-), 127.8 (-CH=CH-), 128.0 (-CH=CH-), 128.2 (-CH=CH-), 128.2 (-CH=CH-), 128.5 (-CH=CH-), 129.5 (-CH=CH-), 132.0 (-CH=CH-), 178.8 (COOH). EIMS m/z: 276 (M⁺, base), 247, 220, 207, 180, 161, 147, 135, 119, 108, 93, 79, 67, 55, 41. HRMS m/z (M⁺): Calculated for C₁₈H₃₂O₂: 276.2089 (Figure 1 and 2). The algicidal activity of 25 and 5 µg/ml of octadeca-6,9,12,15-tetraenoic acid from *N. decipiens* showed moderate of mortality cells of *H. circularisquama*, i.e. between 30 and 69% (Table 2). In this study, octadeca-6,9,12,15-tetraenoic acid was also found significantly
Table 1. Fatty acid composition (% of the total composition), the active compounds of algicidal/dry tissue (mg/100g), chlorophyll a (g/100g) from *Nemacystis decipiens*

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Mean ± SD</th>
</tr>
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<tbody>
<tr>
<td>10:0</td>
<td>nd</td>
</tr>
<tr>
<td>14:0</td>
<td>8.51 ± 0.00</td>
</tr>
<tr>
<td>14:1, cis-9</td>
<td>nd</td>
</tr>
<tr>
<td>16:0</td>
<td>50.96 ± 0.00</td>
</tr>
<tr>
<td>16:4</td>
<td>nd</td>
</tr>
<tr>
<td>18:0</td>
<td>5.89 ± 0.00</td>
</tr>
<tr>
<td>18:1, trans-9</td>
<td>nd</td>
</tr>
<tr>
<td>18:1, cis-9</td>
<td>0.67 ± 0.00</td>
</tr>
<tr>
<td>18:2, cis-9,12</td>
<td>12.34 ± 0.00</td>
</tr>
<tr>
<td>18:3, cis-9,12,15</td>
<td>6.30 ± 0.00</td>
</tr>
<tr>
<td>18:4</td>
<td>6.09 ± 0.00</td>
</tr>
<tr>
<td>20:1</td>
<td>1.11 ± 0.00</td>
</tr>
<tr>
<td>22:0</td>
<td>8.11 ± 0.00</td>
</tr>
<tr>
<td>22:1, cis-13</td>
<td>nd</td>
</tr>
<tr>
<td>PUFA: FA</td>
<td>0.25 ± 0.00</td>
</tr>
<tr>
<td>Algicidal compounds/dry tissue</td>
<td>80.38 ± 0.00</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>0.18 ± 0.00</td>
</tr>
</tbody>
</table>

PUFA: polyunsaturated fatty acid; FA: fatty acid; nd: not detected

Table 2. Algicidal activity of octadeca-6,9,12,15-tetraenoic acid from *Nemacystis decipiens* against *Heterocapsa circularisquama*. Each concentration of octadeca-6,9,12,15-tetraenoic acid was added to f/2 medium inoculated with approximately 3 x 10^4 cells/ml of micro alga for 4 hours. A “+” symbol indicates more than 70% of mortality cells; “±” symbol indicates moderate of mortality cells, i.e. between 30 and 69%; “-” symbol indicates less than 30% of mortality cells

<table>
<thead>
<tr>
<th>Species</th>
<th>octadeca-6,9,12,15-tetraenoic acid from <em>Nemacystis decipiens</em> (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Heterocapsa circularisquama</em></td>
<td>25   ±   5   ±   1</td>
</tr>
</tbody>
</table>

active against *H. circularisquama*, whereas this compound caused cells of micro alga become immobile, round, swollen, and then cells are ruptured finally (Figure 3).

**DISCUSSION**

Salcedo *et al.* (2012) explained that unlike most dinoflagellate toxins, which are polyketides, the toxin from *H. circularisquama* is a porphyrin derivative, which this toxin is lethal to a variety of marine organisms. Interestingly, *N. Decipiens* showed the algicidal substances of octadeca-6,9,12,15-tetraenoic (C18:4) acids against *H. circularisquama* significantly, whereas this bioactive substance were found to be polyunsaturated fatty acid (PUFA). Vaskovsky *et al.* (1996) and Khotimchenko *et al.* (2002) reported that PUFA are commonly found in macroalgae. Takagi *et al.* (1985) also reported that macroalgae of Chlorophyta such as *U. pertusa* and *Enteromorpha* sp. contain large amount (63% to 72% of the total lipids) of PUFA composed mainly of C16:4, C18:3, and C18:4. In this study, *N. decipiens* showed the percentage of octadeca-6,9,12,15-tetraenoic acid as much as 6.09% of the total composition, and ratio of polyunsaturated fatty acid and fatty acid are 0.25% of the total composition. However content of PUFA:FA ratio from *N. decipiens* was less than 0.5, tissue of marine algae of *N. decipiens* showed potent algicidal activity as much as 80.38 mg/100g. These results are relative enough to depress survivability of microalgae *H. circularisquama*. It is suggested that the algicidal substances of *N. decipiens* may have a broad range of biological activities on *H. circularisquama*, which depend on nature of contents of algicidal activity, type of material and difference of species or strains from macroalgae.

*Heterocapsa circularisquama* cells treated with
octadeca-6,9,12,15-tetraenoic (C18:4) acids become lethal as it observed for the allelopathic PUFA of Cladosiphon okamuranus against H. akashiwo (Kakisawa et al., 1988). Chiang et al. (2004) also showed that the highest toxicity from the green colonial algae Botryococcus barunii was observed with α-linolenic acid, followed by linoleic acid and oleic acid. Similarly, McCraken et al. (1980) identified the fatty acid composition in the toxic Chlamydomonas reinhardii culture distillate as a mixture of saturated, monounsaturated and polyunsaturated fatty acids. Furthermore, Morohashi et al. (1991) determined that
unsaturated fatty acids, such as octadeca-6,9,12,15-tetraenoic (C18:4), were more effective than saturated fatty acids. Although the detailed mechanism of action is unknown, it is supposed that these amphiphatic molecules interact with the cell membrane of *H. circularisquama* and disrupt the osmolarity regulation. Furthermore, Matsuyama et al. (1997) suggested that toxin of *H. circularisquama* is a protein-like substance localized on the cell surface. Kamiyama and Arima (1997) reported that the most probable site of toxicity of *H. circularisquama* is considered to be on the cell surface of *H. circularisquama* and cause of the mortality other marine organisms due to cell contact. Nagai et al. (1996) also showed that the mortality of pearl oysters due to *H. circularisquama* was considered to be caused by direct contact of *H. circularisquama* with the body of oysters. In this case, algidic substances probably destruct membrane permability of cells *H. circularisquama* before cell contact incident so that cells of *H. circularisquama* become immobile, round, swollen, and then cells are ruptured finally.

The main factors generally considered when selecting *H. circularisquama* as harmful algal bloom species are the effectiveness, toxicity, cost, and practicability. The effect of algidical octadeca-6,9,12,15-tetraenoic (C18:4) acids of *N. decipiens* appeared rather immediately that this compound killed *H. circularisquama* cells than one hour at 5 µg/ml. Thus, this method has an advantage over bacterial methods, which need at least several days to kill or inhibit the growth of HAB species (Imai et al., 1995). For these purposes, further information on the interaction between octadeca-6,9,12,15-tetraenoic (C18:4) acids from *N. decipiens* and other HAB species should be accumulated. The distribution of *N. decipiens* in the world will also give an opportunity to use algidical substances of *N. decipiens* may useful mitigation agents to remove *H. circularisquama* as HAB species without causing detrimental effects on surrounding marine living organisms.

**References**


