In vivo Anti-inflammatory, Antipyretic, and Analgesic Activities of the Aquaculturable Green Seaweed Codium fragile Extracts in Mice

Ji-Young Kang¹, Quoc-Hai Luyen¹, Mohammed Nurul Absar Khan¹,², Jae-Suk Cho³, In Soon Choi⁴ and Yong-Ki Hong¹*

¹Department of Biotechnology, Pukyong National University, Namku, Busan 608-737, Korea
²Department of Fisheries Technology, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
³IES Center, IACE, Silla University, Saseong-gu, Busan 617-736, Korea
⁴Department of Biological Science, Silla University, Saseong-gu, Busan 617-736, Korea

Received March 12, 2012 / Revised May 17, 2012 / Accepted May 23, 2012

Dichloromethane, ethanol, and boiling water extracts of the green seaweed Codium fragile; used as an herbal medicine and known as an invasive species over the world, were examined for anti-inflammatory, antipyretic, and analgesic activities in mice. The dichloromethane and ethanol extracts inhibited inflammatory symptoms of mouse ear edema and erythema by 74% or higher. The extracts also demonstrated inhibition of pyrexia, similar to that of acetyl salicylic acid. Eicosapentaenoic acid was isolated from the seaweed as the main active anti-inflammatory compound. These findings are consistent with various claims that the seaweed can be used as remedies for inflammation-related symptoms.

Key words: Codium fragile, anti-inflammation, eicosapentaenoic acid, indigenous medicine, green seaweed

Introduction

Many studies have concentrated on the contribution of marine organisms, including seaweeds, to the search for new drugs from natural products. For drug development, selection of samples for biological activity is often based on assaying for active ingredients of organisms used in folk remedies [17]. A number of seaweed species are used as traditional medicines, foods and health-care in various regions of the world. A green seaweed Codium fragile (Suringar) Harriot as known as Chungkag, Nogkaghe, or Chungkagche is used as a food additive, an anti-helminthic [10], and to treat fever, especially in children, with fever accompanying pain, cough, and cold sweat, with no side effects [3]. It is also used as an herbal medicine in China to treat many urinary diseases, dropsy, and helminthiases [19]. As material sources of herbal medicine in oriental countries, most of those medicinal effects of C. fragile are directly or indirectly related to anti-inflammatory action of the seaweed. Recently, Kang et al. [9] found that methanol extract of C. fragile regulated the expression and secretion of LPS-induced inflammatory mediators by inhibiting NF-κB activity. Annual production of C. fragile in 2009 was estimated at 553 tons (wet weight) by natural collection and 1,796 tons (wet weight) by aquaculture in Korea [14]. Annual growths of C. fragile as an invasive species, are now found in temperate regions all over the world [20]. To evaluate the in vivo medicinal activity of C. fragile, abundant species with immense aquaculture potential, we measured anti-inflammatory activity of each extract of dichloromethane, ethanol, and boiling water against edema, erythema, local blood flow, hyperpyrexia, and algesthesia in mice. In an attempt to identify the main active anti-inflammatory compound from the seaweed, we found that eicosapentaenoic acid (EPA) showed inhibitory effects against inflammatory symptoms. By identifying large amounts of the anti-inflammatory EPA from C. fragile, we support claims that the seaweed has been used in health care and indigenous medicine as a remedy for inflammation-related symptoms.

Materials and Methods

Tested materials and animals

Thalli of the green seaweed Codium fragile (Suringar) Harriot were collected from an aquaculture farm of Wando, Korea, in June 2010 and 2011. A voucher specimen was deposited in our laboratory (Y. K. Hong). Extracts of dichloro-
methane (yield: 2.8%) and ethanol (yield: 7.1%) were obtained from 20 g of C. fragile powder in 1 liter of each solvent by 1-h shaking at room temperature. The water extract (yield: 10.6%) was obtained by boiling in water for 1 h. BALB/c mice (8-10 weeks old, 20-25 g body weight) were used for assaying various activities and anti-inflammatory effects. They were maintained in standard environmental conditions, with free access to food and water. Animal experiments were performed in accordance with the U.S. NIH Guidelines for the Care and Use of Laboratory Animals.

Anti-inflammatory activities

C. fragile extracts in ethanol (0.4 mg per 10 μl per ear) or various concentrations of the purified compound were prepared in 10 μl of 100% ethanol and applied topically to the whole inner side of the mouse ear. Phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO, USA; 0.2 μg in 10 μl acetone) was applied topically to the same side of the ear 30 min later to allow absorption of anti-inflammatory compounds. Ear edema (swelling) was measured 10 hr after the PMA application, using a spring-loaded micrometer (Mitutoyo Corp., Tokyo, Japan) [6]. Ear erythema was determined at 10 hr using digital photography, adjusted to balance white, and Photoshop 7.0 (Adobe, San Jose, CA, USA) to measure the magenta value [12]. To confirm the anti-inflammatory activity of the seaweed, local blood flow in the mouse ear was measured at 10 hr, using laser speckle flowgraphy (Inflameter LFG-4; SoftCare, Fukuoka, Japan) [15]. Edema (AU), erythema (AU), and blood flow values (AU) were calculated as (I0 – I)/I0, where I0 is the measurement at 10 hr after PMA application, and I is the measurement at 0 hr. Relative inhibition rate (%) was expressed as [(value of the control - value of the extract)/value of the control]×100. Indomethacin (0.3 mg per 10 μl ethanol per ear) was used as a standard.

Antipyretic and analgesic activities

Mice were divided into five groups of seven mice each. The brewer’s yeast-induced pyrexia model in female mice was used to test the antipyretic activity of seaweed extracts and fractions [18]. When the rectal temperature peaked after 24 hr, either 4 g of extracts in 10 ml of 5% Tween-80 or 10 ml of 5% Tween-80 (control) per kg body weight were administered orally, and the rectal temperature (°C) was recorded after an additional 45 min, using an electric thermometer and probe, inserted 2 cm into the rectum. Relative antipyrexia (%) was expressed as [(value of the control - value of the extract)/value of the control]×100. Acetyl salicylic acid (150 mg/kg) was used as a standard. For analgesic activity, either extracts (1.5 g in 10 ml of 5% Tween-80 per kg) or control was administered intraperitoneally to mice by the tail-flick test [5], and the tail-flick latency time (sec) was measured using the tail-flick unit (Ugo Basile, Varese, Italy) 1 hr later. Relative latency (%) was expressed as [(value of the extract - value of the control)/value of the control]×100. Acetyl salicylic acid (150 mg/kg) was used as a standard.

Acute toxicity test

Mice were fasted for 6 hr, with water provided ad libitum. Extracts (5 g in 10 ml of 5% Tween-80 per kg) were administered orally to mice (n=5, each). The animals were observed for any abnormal behavior for 3 hr, and mortality was noted up to 2 weeks. A group of animals treated with the Tween-80 served as the control.

Isolation of anti-inflammatory compound

To isolate the anti-inflammatory compound from C. fragile thalli, the algal powder (200 g) was extracted three times with 2 liters dichloromethane, and the crude extract was evaporated under vacuum to give a dark brown residue (22 g). The dichloromethane extract was chromatographed on a silica gel column (70-230 mesh, 22 g, 4.5 cm i.d. × 40 cm) and successively eluted with 90 ml each of n-hexane, dichloromethane, acetonitrile, and methanol. The active dichloromethane eluent (1.3 g) was dried and dissolved in 4 ml of methanol for reverse-phase high performance liquid chromatography (RP-HPLC). Each 300-μl (37.5 mg) aliquot was separated on a C18 column (10 mm i.d. × 25 cm) (Waters 5-μm 300A; Beckman Coulter, Fullerton, CA, USA). The analysis was performed on a Waters 600 gradient liquid chromatograph (Waters, Milford, MA, USA) monitored at 213 nm. The mobile phase consisted of two solvent systems: acetonitrile with 0.1% TFA and distilled water with 0.1% TFA. Elution was performed with a linear gradient of 0 to 100% v/v acetonitrile over 30 min and with isocratic 100% v/v acetonitrile over 10 min more for the active compound, at a flow rate of 2 ml/min. Each eluted compound was dried under a stream of nitrogen gas. For analytical method, the purified compound was analyzed on a GC-MS-QP5050A (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector, and compared to the spectral data from the
database. Electron Impact Mass Spectrometric (EIMS) and High-Resolution Fast Atom Bombardment Mass Spectra (HR-FABMS) data were obtained from a JMS-700 spectrometer (JEOL, Tokyo, Japan) and a JMS HX 110 Tandem Mass Spectrometer (JEOL), respectively. Infrared spectrum was recorded on a Fourier Transform IR spectrophotometer (IFS-88; Brucker, Karlsruhe, Germany). The 1-D Nuclear Magnetic Resonance (NMR; $^1$H, $^{13}$C, and DEPT) and 2-D NMR (HMQC, HMBC, and COSY) spectra were taken on a JNM-ECX 400 NMR Spectrometer (JEOL), using methanol-d$_2$ (CD$_2$OD). The structure of the purified compound was identified and confirmed to be identical to the spectral data in Fu et al. [4]. For quantification of the compound in *C. fragile* the thalli were completely dried in shade at room temperature for a week, and then ground for 5 min to powder. The powder (0.4 g) was extracted with 8 ml dichloromethane on a rotator for 1 hr at 30 rpm. After centrifugation at 2,000× g for 5 min, 4 ml of the clear supernatant was evaporated to 5 mg/ml for RP-HPLC. Each 100-μl aliquot was separated on an Ultrasphere C18 column, using the same isolation procedure. The amount was assessed by measuring the dimensions of HPLC peaks, using the standard curve of the pure compound.

**Statistical analysis**

All animal experiments except the toxicity test were performed with at least seven mice for each group, and the highest and lowest values were discarded. Data are reported as means±SE. The significance of the results was calculated using Student's *t*-test and was deemed statistically significant when *p*<0.01.

**Results**

In preparing traditional medicines and health care foods, it is common to boil the materials in water or to soak them in beverage alcohol. To understand more detailed investigations of the active substances, we prepared boiling water, alcohol, and dichloromethane-soluble extracts of the seaweed, and determined their anti-inflammatory, antipyretic, and analgesic activities in mice. The dichloromethane extract (0.4 mg/ear) demonstrated inhibition of inflammatory symptoms: mouse ear edema, erythema, and blood flow by 89.5%, 74.1%, and 42.0%, respectively (Table 1). The ethanol extract also showed inhibitions by 74.4%, 79.3%, and 173%, respectively. Thus, the dichloromethane and ethanol extracts (i.e., likely nonpolar compounds) from the *C. fragile* showed potent (*p*<0.01) anti-inflammatory activities, especially anti-edema and anti-erythema actions, compared to indomethacin of the standard anti-inflammatory drug. The seaweed extract's antipyretic activity was evaluated by measuring changes in rectal temperature. The mice were injected with brewer's yeast, and the rectal temperature peaked at 39.19±0.07°C after 24 hr. Oral administration of dichloromethane and ethanol extracts (4 g/kg body weight) potently lowered rectal temperature in hyperthermic mice to 35.46±0.46°C and 35.73±0.21°C, respectively (Table 1). Acetyl salicylic acid (150 mg/kg body weight) as

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Edema (Inhibition %)</th>
<th>Erythema (Inhibition %)</th>
<th>Blood flow (Inhibition %)</th>
<th>Temperature (Antipyresia %)</th>
<th>Tail flick (Latency %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.86±0.03</td>
<td>0.58±0.01</td>
<td>0.081±0.005</td>
<td>36.92±0.10</td>
<td>3.08±0.04</td>
</tr>
<tr>
<td>CHCl$_3$ extract</td>
<td>0.9±0.05</td>
<td>0.15±0.05</td>
<td>0.047±0.007</td>
<td>35.46±0.46</td>
<td>3.56±0.17</td>
</tr>
<tr>
<td>EtOH extract</td>
<td>0.22±0.04</td>
<td>0.12±0.03</td>
<td>0.067±0.015</td>
<td>35.73±0.21</td>
<td>3.47±0.21</td>
</tr>
<tr>
<td>Boiling water</td>
<td>0.84±0.06</td>
<td>0.54±0.07</td>
<td>0.079±0.014</td>
<td>36.73±0.16</td>
<td>3.24±0.06</td>
</tr>
<tr>
<td>Acetyl salicylic acid</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>35.38±0.58</td>
<td>4.4±0.30</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.14±0.01</td>
<td>0.22±0.08</td>
<td>0.024±0.016</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

1Results are represented as means±SE (n≥5).
2Statistical significance is "*p*<0.01, "*p*<0.001 as compared to the untreated controls. ND, not determined.
Fig. 1. Chemical structure of 5,8,11,14,17-eicosapentaenoic acid (20:5 ω3) isolated from the green seaweed Codium fragile.

a standard antipyretic drug was 35.38±0.58°C when orally applied in the hyperthermic mice. The seaweed revealed potent suppression of body temperature similar to the standard drug. Tail-flick behavior in mice was used to evaluate the analgesic activity of the seaweed extracts. As controls, mice injected with 5% Tween-80 responded by tail flicking in 3.08±0.04 sec on average. The intraperitoneal injection of dichloromethane extract into the abdominal cavity showed marginal activity against the algesia with latency increase by 3.56±0.17 sec. We evaluated any acute toxicity that the extracts might pose in mice, even though the seaweed is using as food additives in diets of Koreans. Over the 2 weeks of observation, no death or obvious symptoms occurred in any group of five mice administered a dose of 5 g/kg body weight.

To identify the main anti-inflammatory compound from C. fragile, 200 g of seaweed powder was extracted with dichloromethane and chromatographed on a silica gel column. The active dichloromethane fraction was then separated by RP-HPLC, using a gradient of acetonitrile in water and isocratic 100% acetonitrile. The active peak was eluted at 100% (on 31.7 min) acetonitrile. It appeared as an oily compound, weighing 2.4 mg, and yielding 1.2×10^-5% from the seaweed powder. The GC-MS analysis of the compound led to the tentative identification of eicosapentaenoic acid using library of the GC-MS. The molecular composition is C_{20}H_{32}O_{2} based on HR-FABMS data (negative mode, [M-H]^- at m/z 301.2168), which indicated that the compound contained six double-bond equivalents, comprising five carbon – carbon double bonds and one carbonyl carbon. IR (dry film) absorptions for OH (3,000-2,500 cm^-1) and carbonyl function (1,704 cm^-1) were observed. The 1H NMR spectrum revealed the presence of a methyl proton at δ 0.90 (H-20), eight methylene protons, and ten methine protons. The 13C NMR spectrum revealed one carbonyl carbon at δc 174.9 (C-1), one methyl carbon at δc 150 (H-20), eight methylene carbons, and ten methine carbons. From these spectral data, it was identified as eicos-5,8,11,14,17-pentaenoic acid (EPA) (C20:5 ω3), or timnodonic acid (Fig. 1), and confirmed to be identical to data for authentic EPA.

Discussion

The green seaweed C. fragile is used as a food additive or herbal medicine in Korea and China. According to WHO [21], herbal medicine is said to be toxic if the LD₅₀ is lower than 5 g/kg body weight. From this assertion, it can be said that the C. fragile extracts are not toxic and can be safely used by humans at moderate doses, since no mortality at 5 g/kg body weight was recorded. In present study, dichloromethane and ethanol extracts of C. fragile demonstrated potent anti-inflammatory activities in PMA-induced mouse ear, and the main active constituent in the C. fragile extract was the polyunsaturated fatty acid EPA. In a previous work [11], the EPA concentrations producing 50% inhibition (IC₅₀) by topical application were 230, 462, and 236 μg per ear for edema, erythema, and blood flow, respectively. It showed almost half the anti-inflammatory activity of indomethacin. We also examined the amount of EPA in C. fragile thalli. Mature thalli of commercial product contained EPA with approximately 136 μg/g-dry powder. Known for its anti-inflammatory effect, the compound in the seaweed may play as a competitive inhibitor of cyclooxygenase and/or lipoxigenase in an inflammation reaction, resulting in decreased production of prostaglandins and leukotrienes [8]. Ear inflammations induced by arachidonic acid and ultraviolet-B irradiation was also significantly suppressed in mice at a dose of 300 mg EPA per kg body weight per day for 2 weeks [2]. EPA inhibited UV-induced dermal fibroblasts [13], leukocyte – endothelial interactions [16], and inflammatory mediator release in blood and splenocytes of mice [7]. Supplementing with 50-100 μg/ml ω-3 PUFAs reduces the expression and activity of aggreganases and inflammation-inducible cytokines and cyclooxygenase-2 [1]. However, they had no effect on constitutively expressed cyclooxygenase-1. Thus, these findings for EPA from C. fragile reinforce the claims of the health-care industry and indigenus medicine that the seaweed can be used as a remedy for inflammation-related symptoms. In addition, the amount of EPA in C. fragile can be used as criteria for quality assessment of the seaweed products and for strain improvement.

Acknowledgements

This research was supported by a grant from the Busan Sea mustard Sea tangle Regional Strategic Food Industry
Promotion Agency funded by MIFAFF, Korea. We thank the Brain Busan 21 program for graduate support (JYK, QHL).

References


