Note

The Anticoagulant Activity and Hypocholesterolemic Effect of a Hot Water Extract from the Red Alga Ibaranori (*Hypnea charoides*)

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Received March 16, 2012; Accepted May 6, 2012

Ibaranori (*Hypnea charoides*), a red alga, grows along the coast of the Japanese islands and is commonly eaten in southwestern areas such as Okinawa Prefecture. While *H. charoides* is thought to be a health-promoting food, the underlying mechanism of this benefit is not well understood. In this study, in order to examine the health benefits of *H. charoides*, a hot water extract was evaluated for its anticoagulant activity by using thromboelastography and measuring the hypocholesterolemic effect on hypercholesterolemic rats administered the hot water extract orally. The extract significantly exerted anticoagulant activity, as indicated by changes in thromboelastogram parameters and suppression of the increase in rat liver cholesterol ester and rat serum cholesterol. Based on these results, the health benefits of *H. charoides* as a useful food resource and the potential for effective use of the alga are suggested.

Keywords: *Hypnea charoides*, anticoagulant activity, hypocholesterolemic effect, carrageenan

Introduction

Ibaranori (*Hypnea charoides*), a red alga, grows along the coast of the middle and south Japanese islands (Yoshida, 1998). This alga is a common part of the diet in Okinawa Prefecture, and although the total yield of the alga in Okinawa Prefecture is not obvious due to a lack of public statistical data in recent years, it is known that the yield of the alga from a fisheries trader in the prefecture is 31.6 kg/year (wet weight) (Ikeguchi, 2005). In particular, the alga itself and dishes that use the alga are called “Mooi”; “Mooi-Tofu” is well-known as a traditional Okinawan food. Regarding the health benefits of this alga, it has been reported to have an anticancer effect (Nakazawa et al., 1974), an immunomodulating effect (Mizukoshi et al., 1993) and an effect on the lipid profiles of hypercholesterolemic rats (Wong et al., 1999).

While *H. charoides* grows throughout Japan, its dietary applications and health benefits beyond those previously mentioned remain largely unknown. An increased knowledge of the health benefits could lead to an expansion of the effective use of *H. charoides* in both food and pharmaceutical products. In order to demonstrate the health benefits of the alga, we focused on the blood and liver, which are often the targets of various lifestyle-related diseases. Because the bioactivity of seaweed polysaccharides such as alginic acid and fucoidan are well known (Brownlee et al., 2005; Li et al., 2008), hot water extraction of polysaccharides contained in the alga was performed. Based on reports that some seaweed might possess anticoagulant activity and hypocholesterolemic effects (Amano et al., 2005; Nishino and Nagumo, 1987; Ren et al., 1994; Wijestinghe et al., 2011), the hot water extract was examined for anticoagulant activity evaluated by thromboelastography and its effect on lipid levels (serum and liver) in hypercholesterolemic rats.

Materials and Methods

Materials and animals

Commercially-dried *Hypnea charoides* was purchased from the Kaneshiro Store in the Makishi public market (Naha, Okinawa, Japan). It was harvested from the coast of the northern main island of Okinawa in spring. The seaweed was washed with tap water and air-dried. To improve hot water extraction, the dried sample was cut with scissors. Male Wistar rats (seven weeks old) were...
purchased from Kyudo Co., Ltd. (Tosu, Saga, Japan).

**Hot water extraction** Chopped *H. charoides* (0.75 g) was mixed with 50 mL of distilled water. The mixture was boiled for 30 min, and 33 mL of the supernatant was centrifuged at 1400 × g for 5 min. The supernatant was retained as the hot water extract of the alga. Air-drying the supernatant for 7 d resulted in 300.3 mg of residue. Therefore, the concentration of solids in the original extract was calculated to be 9.1 mg/mL; and this value was used for subsequent experiments.

**Evaluation of the anticoagulant activity**

(i) **Animals and phlebotomy** Wistar rats were housed in individual cages kept between 23 – 26°C under a 12 h light/dark cycle until testing. Food and water were freely available. Rats from 8 to 12 weeks old were used. Blood samples were taken from the carotid artery under anesthesia with diethyl ether and collected in a test tube including 1/9 volume of saline supplemented with 3.8 % sodium citrate as an anticoagulant.

(ii) **A measurement by thromboelastogram** Two hundred seventy microliters of the collected blood samples and 30 μL of the hot water extract of *H. charoides* (9.1 mg/mL) or distilled water (control) were mixed and incubated for 2 min at 37°C. Calcium chloride (final concentration = 13.3 mM) was added to the mixtures to start a clotting reaction, and the thromboelastogram was immediately obtained using a ROTEG thromboelastogram measuring device (Pentapharm Ltd., München, GmbH). The anticoagulant activity was evaluated with four parameters: clotting time (CT), clot formation time (CFT), maximum clot firmness (MCF) and clot formation rate (CFR) (Fig. 1). When the values of CT and CFT increased, and MCF and CFR decreased more than those in the controls, respectively, it was concluded that the extract possessed anticoagulant activity.

**The effect of *H. charoides* on hypercholesterolemic rat**

(i) **Animals and experimental design** For an acclimation period of about one week, the Wistar rats were housed as described above. The rats were divided into four groups (I (n = 3), II (n = 3), III (n = 5) and IV (n = 5)). Group I was given 2 mL of a sucrose solution (9.1 mg/mL) and MF (KBT Oriental Co. Ltd., Tosu, Saga, Japan) as a basal diet. Group II was given 2 mL of the *H. charoides* hot water extract (9.1 mg/mL) and the MF diet. Group III was given 2 mL of the sucrose solution and a cholesterol diet, comprising MF supplemented with 2 % cholesterol and 0.5 % cholic acid. Group IV was given 2 mL of the hot water extract and the cholesterol diet. Because not only the maximum but also a smooth flowing volume of the hot water extract in an oral feeding needle was 2 mL in our preliminary check, we decided on 2 mL for the administration volume. Food and water were freely available. The upper limit of the diet consumption was 25g/day/rat.

The sucrose solution and hot water extract were administered orally to the rats in the mornings and evenings for 7 d using an animal feeding needle. On the final day of administration, a blood sample was taken from a heart puncture and the liver was excised under anesthesia with diethyl ether. To obtain the serum, the collected blood sample was allowed to settle for 30 min in a test tube and then centrifuged at 1900 × g for 20 min. The excised liver was washed with saline and stored at −30°C until use.

All of the animal experiments were performed with permission of the “Committee for Use and Care of Laboratory Animals” of the National Fisheries University, and in compliance with the “Guideline for Animal Experiments in Research Institutes under the jurisdiction of the Ministry of Agriculture, Forestry and Fisheries” (Approval number, 10-7; March 31, 2010).

(ii) **Determination of lipid levels in liver** Lipids from 5 g of liver were extracted by the method of Folch et al. (1959). The extract was diluted to 10 mL with a chloroform/methanol (2/1 v/v) solution and retained as the sample. The total amount (w/w) of hepatic lipids was measured by drying the solvent of the sample solution and weighing the residue.

Lipid class (cholesterol ester and triglyceride) analysis was carried out according to the method of Peyrou et al. (1996) with some modifications. Three milligrams of the hepatic lipids were dissolved in 600 μL of chloroform/methanol (2/1 v/v). Two microliters of the solution was spotted onto preheated S-III chromarods with a 5 μL micro-dispenser (Drummond Scientific Co., Broomall, PA, USA). After spotting, the rods were placed in the developing tank until the developing solvent reached 10 cm. The composition of the developing solvent was hexane-ether-formic acid (60: 5: 0.15, v/v). The rods were then dried with Roddryer (TK-8, Iatron Laboratories, Inc., Tokyo, Japan) for 1 min. The spots on the rods were analyzed by a hydrogen flame-ionization detection (HFID) scan using an IATROSCAN MK-5 analyzer (Iatron Laboratories). The hydrogen and air flow rates of the HFID were 160 and 2000 mL/min, respectively. The scanning speed was set at 30 cm/min.

(iii) **Determination of lipid levels in serum** The total cholesterol level was assayed by the cholesterol oxidase-DAOS method using a Cholesterol E-test kit (Wako Pure Chemical Industries Ltd., Osaka, Japan) according to the manufacturer’s protocol. The triglyceride level was assayed by the GDP-DAOS method using the Triglyceride E-test kit (Wako) according to the manufacturer’s protocol.

**Statistical analysis** Statistical analyses of the thromboelastogram were performed using the Student’s t-test. Statis-
tical analyses of the multiple comparisons of hypocholesterolemic effects were done by the Dunnett’s test using “Excel Statistics” software (Esumi Co. Ltd., Tokyo, Japan). Data were expressed as the mean ± SD. \( P < 0.05 \) was considered statistically significant.

**Results and Discussion**

As shown in Fig. 1 and Table 1, the MCF and CFR values of the *H. charoides* hot water extract were significantly lower than those of controls. The CT and CFT values of the extract were not significantly different from those of the controls. Because *H. charoides* includes abundant \( \kappa \)-carrageenan, a seaweed sulfated galactan (Shimahara and Sugiyama, 1974; Qi *et al.*., 1997), it is thought that the hot water extract would include substantial amounts of \( \kappa \)-carrageenan. Güven *et al.* (1991) reported that \( \kappa \)-carrageenan barely affected activated partial thromboplastin time (APTT), thrombin time (TT) and prothrombin time (PT), which are biochemical markers of anticoagulant activity. In this study, because the extract more significantly influenced MCF and CFR values attributed to physicochemical factors than CT and CFT values attributed to biochemical factors, the components of the hot water extract may influence the rigidity of the clot but not the biochemical reactions of clotting. Therefore, it is thought that the results obtained in this study are consistent with prior knowledge (Güven *et al.*, 1991). Concerning other algal polysaccharides, it has been reported that sulfated fucan from *Ecklonia cava* exhibited anticoagulant activity by elongating APTT, TT and PT and accelerating antithrombin III activity (Jung *et al.*, 2007; Wijesinghe *et al.*, 2011). Therefore, the obtained results attributed to physicochemical factors in this study may represent a novel mechanism of the anticoagulant activity by algal polysaccharides.

As shown in Fig. 2, although the hepatic cholesterol, total lipid and triglyceride level of the rats fed with the cholesterol diet (group III and IV) were significantly higher than those of the rats fed with MF (group I and II), a significant difference in total lipid and triglyceride levels between groups III (sucrose) and IV (extract) was not observed (Fig. 2B, C). However, the extract significantly (\( P < 0.01 \) versus group III) suppressed the rise in the hepatic cholesterol ester level of the group IV rats (Fig. 2A). Furthermore, in a complementary study, no significant difference in the hepatic fatty acid composition among the test groups was observed in an analysis by gas chromatography (data not shown). Additionally, as shown in Fig. 3, the extract significantly (\( P < 0.05 \) versus group III) suppressed the rise in the total serum cholesterol level in the group IV rats (Fig. 3A), while the serum triglyceride level was not influenced (Fig. 3B). These results implied that the active components included in the

![Fig. 1. Thromboelastograms for the *H. charoides* hot water extract assay.](image)

A, control; B, extract (0.91 mg/mL). CT, Clotting Time; CFT, Clot Formation Time; MCF, Maximum Clot Firmness; CFR, Clot Formation Rate.

<table>
<thead>
<tr>
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<th>CT</th>
<th>CFT</th>
<th>MCF</th>
<th>CFR</th>
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<tr>
<td>Control</td>
<td>261.0 ± 37.3</td>
<td>136.3 ± 39.2</td>
<td>62.3 ± 4.9</td>
<td>66.7 ± 4.7</td>
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<tr>
<td>Extract (0.91 mg/mL)</td>
<td>236.3 ± 24.8</td>
<td>198.3 ± 31.1</td>
<td>46.7 ± 4.0*</td>
<td>56.0 ± 3.0*</td>
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CT, Clotting Time (Sec); CFT, Clot Formation Time (Sec); MCF, Maximum Clot Firmness (mm); CFR, Clot Formation Rate (degree). Values were calculated from the results of triplicate experiments. Data are expressed as means ± SD (\( n = 3 \)). Asterisks indicate that the values of the extract are significantly lower than those of the control. *, \( P < 0.05 \).
Fig. 2. The hypocholesterolemic effect of the *H. charoides* hot water extract on hypocholesterolemic rats for hepatic cholesterol ester (A), total lipid content (B) and triglyceride (C) levels. Rats in groups II and IV were administered 2 mL of the hot water extract (9.1 mg/mL) orally for 7 d. Cholesterol ester and triglyceride levels were detected by HPLC scan using an IATROSCAN MK-5 analyzer, and the lipid content was obtained by the weight method as described in “Materials and Methods”. Data are expressed as means ± SD (group I and II, n = 3; group III and IV, n = 5). *P* < 0.01 and *P* < 0.05 represent significance between the compared groups. NS represents no significance between the compared groups.

Rats in groups II and IV were administered 2 mL of the hot water extract (9.1 mg/mL) orally for 7 d. Cholesterol ester and triglyceride levels were detected by HPLC scan using an IATROSCAN MK-5 analyzer, and the lipid content was obtained by the weight method as described in “Materials and Methods”. Data are expressed as means ± SD (group I and II, n = 3; group III and IV, n = 5). *P* < 0.01 and *P* < 0.05 represent significance between the compared groups. NS represents no significance between the compared groups.

Fig. 3. Hypocholesterolemic effects of the *H. charoides* hot water extract on hypocholesterolemic rats in the serum cholesterol (A) and triglyceride (B) levels. Rats in groups II and IV were administered 2 mL of the hot water extract (9.1 mg/mL) orally for 7 d. Cholesterol and triglyceride levels were detected using the Cholesterol E-test and Triglyceride E-test kits, respectively. Data are expressed as means ± SD (group I and II, n = 3; group III and IV, n = 5). *P* < 0.01 and *P* < 0.05 represent significance between the compared groups. NS represents no significance between the compared groups.

The extract might exert a suppressing effect on the intestinal absorption of cholesterol, because the hepatic total lipid level, the hepatic triglyceride level, the hepatic fatty acid composition and the serum triglyceride level that are attributed to cholesterol metabolism were not significantly influenced by the extract. Wong *et al.* (1999) reported an effect of dried *H. charoides* powder on hypercholesterolemic Sprague Dawley rats. However, in that report, the total serum cholesterol level of Sprague Dawley rats fed with the alga only tended to decrease and the effects of the alga on lipid metabolism were
unclear. In our preliminary study, the effect of the dried powder of the alga on hypercholesterolemic Wistar rats was also unclear (data not shown). Therefore, in the present study, the hot water extract of \textit{H. charoides} was examined. On the other hand, there are reports that fucoidan and brown algal polysaccharides lower the serum triglyceride level, but not the total serum cholesterol level in Wister rat (Ren et al., 1994), and that low viscosity alginic acid affects the cholesterol biosynthetic hormone system and leads to a reducing of rat serum cholesterol level (Wong et al., 2003). Their mechanisms are different from the suppression of the intestinal absorption of cholesterol by the \textit{H. charoides} hot water extract. Generalizing the above descriptions, this study is a novel report that the \textit{H. charoides} extract may possess a hypcholesterolemic effect. In the experimental rats, although a lesion in the gut due to the acidity of the extract that includes many sulfate groups was suspected, no abnormal symptoms such as diarrhea and bloody feces were observed in either experimental group. Among the experimental group, there were no significant differences in diet consumption, weight gain and liver weight.

It is well-known that \textit{Hypnea charoides} includes abundant quantities of κ-carrageenan, a seaweed sulfated galactan (Shimahara and Sugiyama, 1974; Qi et al., 1997), and thus preliminary studies were carried out to examine whether carrageenan is the main active component. When elemental analyses of the \textit{H. charoides} hot water extract and \textit{Gelidium elegans} hot water extract (agar) were carried out by inductively coupled plasma mass spectrometry (ICP-MS), the amount of sulfur atoms in the \textit{H. charoides} hot water extract was about 34 times that in the same volume of agar. When the amount of crude carrageenan included in the \textit{H. charoides} hot water extract was measured according to the method of Qi et al. (1997), the resulting 228.3 mg of the crude carrageenan residue represented 76.0 % (w/w) of the initial solids (300.3 mg). From these preliminary experimental data, the main active component included in the extract was postulated to be carrageenan. Qi et al. (1997) reported that the amount of κ-carrageenan was about 42.1 % (w/w) of dried \textit{H. charoides} (30.4 % as crude carrageenan in this study). Based on report that \textit{Eucheuma cottonii}, a well-known resource of κ-carrageenan, contains 34.3 % (w/w) of the polysaccharide (Matsushashi and Itoh, 1986), \textit{H. charoides} may also be regarded as a useful resource of κ-carrageenan. In fact, commercial carrageenan powder has been known to exert hypcholesterolemic effects on human volunteers (Panlasigui et al., 2003). Also, because lowering the human serum cholesterol level leads to reduction of thrombogenic risk (Lacoste et al., 1995) and suppresses the intestinal absorption of cholesterol by the \textit{H. charoides} hot water extract, as implied in this study, carrageenan included in the alga may exert multiple hypcholesterolemic effects and anticoagulant activity in the human body. Therefore, \textit{H. charoides} and polysaccharides (κ-carrageenan) from the alga exhibit great potential as health-promoting food resources.

In this study, because a health-promoting effect of a \textit{H. charoides} hot water extract was demonstrated for the first time, the usefulness of this alga might be expanded in the future. However, further studies are needed to determine the active compound(s) and the detailed mechanism(s) of anticoagulant activity and hypcholesterolemic effects.

Acknowledgements We thank Dr. N. Murase and Dr. M. Abe (National Fisheries University) for contributing the knowledge of \textit{Hypnea charoides}, Mr. K. Yonezawa and Mr. K. Fujimoto (National Fisheries University) for the technical assistance, as well as Dr. D. A. Coury (Phycal Inc., Highland Heights, OH, USA) for reviewing the English.

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