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Original Article

The Antibacterial and Immunomodulatory Effects of Carbohydrate Fractions of the Seaweed *Gracilaria persica*

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Introduction: Red seaweeds are the source of polyanionic polymers that play a critical role in ionic, mechanical, and osmotic functions of the cells. The *Gracilaria* polysaccharides have numerous biological activities. This research aimed to compare the *in vivo* and *in vitro* effects of the various carbohydrate fractions of the seaweed *Gracilariopsis persica*. **Methods:** The crude polysaccharide of the *G. persica* seaweed was extracted using three methods, including soaking in water, hot water extraction, and acid extraction. On the optimal conditions, the seaweed polysaccharides were extracted using HCl 0.1 M 10% (w/v), and the crude carbohydrates were precipitated by ethanol. The extract was fractionated on diethylaminoethyl cellulose (DEAE-C) column using a NaCl gradient. The antimicrobial activity of each fraction was assessed by microdilution broth method against 6 bacteria species, including *Staphylococcus aureus*, *Escherichia coli*, *Methicillin-resistant Staphylococcus aureus* (*MRSA*), *Salmonella typhimurium, Pseudomonas aeruginosa,* and *Aeromonas hydrophila.* Moreover, the obtained fractions were orally administered (100 µg/day) for 7 days to 10 groups of 4 adult NMRY mice. The effects of various fractions were evaluated based on the bactericidal effect of the sera and some immune response indicators, including complement activity and humoral immune response against sheep red blood cells (SRBC). **Results:** Most of the fractions had direct antibacterial effects; however, oral administration of the fractions neither increased the antibacterial effect of sera nor triggered the complement activity. However, the fractions 1, 2, 5, and 6 significantly induced the humoral immune response against SRBC. **Conclusion:** The *G. persica* seaweed has direct antibacterial effects. However, unlike the humoral immune response induction, the carbohydrate fractions have no effects on innate immune responses. *J Med Microbiol Infec Dis, 2018, 6 (2-3): 57-61.*

Keywords: *Gracilaria*, Mice, Anti-bacterial Agents, Immunity.

INTRODUCTION

Algae contain diverse photosynthetic organisms that grow in aquatic environments. According to their morphology and chemical composition, marine macro-algae or seaweeds are classified into green algae (Chlorophyta), brown algae (Phaeophyta), and red algae (Rhodophyta) [1]. They have various bioactive organic and inorganic components with beneficial applications, such as feeding and health protection of humans and animals [2, 3].

Due to the emergence of antibiotic-resistant strains of bacteria, the medicinal plants might play an alternative role in the treatment of infectious diseases. The biological and pharmacological evaluation of seaweeds has led to discovering several natural or semi-synthetic drugs in recent years [4]. In response to harsh environmental conditions, algae produce various secondary metabolites [3, 5] that have bactericidal effects against some of the Gram-positive and Gram-negative bacteria [6]. In addition, brown, red, and green seaweed extracts have been reported to have antioxidant, antiviral [7], antifungal [8], cytotoxic [9], and larvicidal properties [10]. The anti-inflammatory compounds of the seaweeds can be also used for medicinal applications [11].

The *Gracilaria* species grow in the tropical regions throughout the world. These Algae produce sulfated polysaccharides. The polysaccharide backbone of *Gracilaria* composed of 3-linked β-D-galactopyranosyl residues and 4-linked α-L-galactopyranosyl (or 3,6-anhydro-galactopyranosyl) residues; however, various substitutions change the *Gracilaria* structures in different species [7]. The *Gracilaria* polysaccharides have numerous biological activities, such as antitumor [12], antimicrobial [13], anti-inflammatory, and pain reducing [14] effects. The present study evaluated the antibacterial and immunostimulatory effects of the carbohydrate fractions of the seaweed *Gracilariopsis persic*a.

MATERIAL AND METHODS

Collection of seaweed specimens. The *G. persica* seaweed specimens were diagnosed and collected from the shallow seawater of the Persian Gulf beach by our fisheries specialist colleagues (Bushehr province, Iran).

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Bacterial strain. The bacterial strains of *Staphylococcus aureus, Salmonella typhimurium, Aeromona shydrophila, Pseudomonas aeruginosa*, and *Escherichia coli*, were obtained from Department of Microbiology, Faculty of Veterinary; the methicillin-resistant *S. aureus* was a standard strain (PTCC: 1764). Blood agar and Mueller Hinton Broth were used for the culture and maintenance of the bacterial species. Broth cultures were incubated under aerobic conditions at 37°C.

***In vivo* assays**. The albino mice, weighing 20±2 g, were obtained from the laboratory animals section of the Jundishapur University of Medical Sciences. All animals were housed in a temperature-controlled room and received water and food ad libitum. All experiments were performed according to the guidelines of the Ethics Committee of the Faculty of Veterinary Medicine of the Shahid Chamran University of Ahvaz, Iran (Ethics Advisory Committee 2016).

Extraction of the carbohydrates from seaweed. The seaweed specimens were washed with limpid seawater and air dried in an oven at 40ºC for 24 h and then grounded to powder. Different extraction methods were tested to obtain the optimal condition for preparation of crude polysaccharide of the seaweed. Two extracts were prepared as described by others [13]. In brief, 2 g of the seaweeds were subjected to mechanical stirring in 100 ml of distilled water for 12 h at room temperature (25°C). The residue was removed by filtration and centrifugation at 6000 g for 10 min. The compounds in the supernatant were precipitated with absolute EtOH (1:2, v/v), centrifuged at 2500 g for 10 min, dissolved in distilled water, and labeled as E1. The remaining residue was re-extracted in water at 80°C for 12 h, and the procedure was followed as described above and the precipitate was labeled as E2. Moreover, the acid extraction method was used as described elsewhere [15]. In brief, 2 g of algae powder was mixed with 100 ml of HCl 0.1 M, followed by stirring at room temperature for 6 h. The suspension was filtered, and pH of the solution was adjusted to 7 using 0.5 M NaOH. The solution was then centrifuged at 6000 g for 20 min, and two volumes of ethanol were added to the supernatant. Each method was repeated three times, and then the resulting extracts were collected in a single tube. Then the suspensions were dialyzed in PBS. The polysaccharide level in each extract was determined using a phenol-sulfuric acid method as mentioned by others [16] with d-glucose as the standard. Briefly, 15 µL of phenol 5% was added to 25 µL of the sample solution followed by the addition of 75 µL concentrated H_2SO_4 and reading the optical absorbance after 10 min at 490 nm. The carbohydrate fractions were purified from the sample, which contained the highest amount of the carbohydrate.

Purification of the carbohydrate fractions of *Gracilaria*. The crude polysaccharides were fractionated using anion exchange chromatography on a diethylaminoethyl cellulose (DEAE-C) (Sigma, cat No: D6418) column as described by others [17] with minor modifications. In brief, 20 ml of the extract was filtered through a Whatman filter paper (Sigma, WHA10347509) and loaded onto the DEAE-C column, previously equilibrated with 10 Mm tris–HCl buffer (pH 8.5). After washing away the unbound components (F1), the bound components (F2-F9), were eluted using a linear gradient of sodium chloride (0.1 M, 0.25 M, 0.5 M, 0.75 M, 1 M, 1.25 M, 1.5 M, 2 M, and 4 M). The total carbohydrate content was determined as described above. The obtained fractions were concentrated and dialyzed for 48 h against distilled water.

Evaluation of the direct bactericidal effects. Antibacterial effects of the obtained carbohydrate fractions of *G. persica* were evaluated using microdilution broth method in 96-well microplates. The antibacterial activity of each fraction was evaluated against 6 bacteria, including *Staphylococcus aureus, Escherichia coli, Methicillin-resistant Staphylococcus aureus* (MRSA), *Salmonella typhimurium, Pseudomonas aeruginosa*, and *Aeromonas hydrophila.* The bacterial strains were cultured on blood agar medium and incubated overnight at 37°C. One colony from each strain was introduced into Mueller Hinton Broth at 37°C and monitored until it reached the turbidity equal to 0.5 McFarland standard. The serial two-fold dilutions (1/2, 1/4, 1/8, and 1/16) of each fraction, were prepared in 65 µl of brain heart infusion (BHI) medium. The prepared bacteria in a volume of 25 µl, were added to the specified wells. After reading the optical densities (ODs) at 600 nm using a spectrophotometer (AccuReader, Taiwan), the plates were incubated at 37°C for 24 h, and the ODs were reread in the same conditions as before. The results were interpreted by calculation of the bacterial growth in test and control wells [18].

Analysis of the effects of fractions on immune responses. Ten groups of adults NMRY mice (4 mice in each group), were intraperitoneally injected with 100 µl of 2% sheep red blood cells (SRBC). The obtained aqueous fractions of the extracts were orally administered to the mice (100µg/day in a volume of 250 µl) for one week. The effect of administered fractions on bactericidal activity against *E. coli* and *S. aureus* was assessed as described above (evaluation of the direct bactericidal effects). The complement activity of the sera of the treated mice was evaluated by their hemolytic activity against rabbit red blood cells [19]. The direct haemagglutination test in microplate was used to determine the SRBC antibody titer. For this, 1% SRBC was added to serial dilutions of the sera in a microplate. The plates were incubated at 37ºC for 45 min and then examined for haemagglutination. The test was repeated three times for each sample, and the antibody titer was determined to equal to the highest dilution exhibiting hemagglutination [20].

Statistical analysis. In this study, the differences between groups were assessed using one-way analysis of variance ANOVA. In all statistical analyses, a p-value less than 0.05 was considered significant.

RESULTS

Crude polysaccharide extraction. The ethanol precipitated approximately half of the total carbohydrates in

each extract. The highest amount of the purified polysaccharide was isolated using acidic condition (69 mg/ml) in comparison with E_1 (14.4 mg/ml) and E_2 (43.65 mg/ml) extraction methods.

Purification and Fractionation of crude polysaccharides. Ten carbohydrate fractions were purified using DEAE-C column (Table 1). Most of the carbohydrates were eluted using 0.1, 1.5, and 1.25 M NaCl. The other fractions released by lower and higher NaCl concentrations had a lower amount of carbohydrates.

Direct bactericidal effects. Most of the fractions exhibited antibacterial effects (Table 2). Out of 10 carbohydrate fractions, the fractions 10, 9, and 3 had the highest bactericidal effects. These fractions, in comparison with other fractions, prevented the growth of most of the examined bacteria in higher dilutions. The most resistant bacteria to the bactericidal effects of the carbohydrate fractions were *S. typhimurium*, *E.coli*, and *A. hydrophila*.

The effects on immune responses. The fractions 1, 2, 5, and 6 significantly induced the humoral immune response against SRBCs (Table 3), while showed no significant effect on the complement activity.

In comparison with the sera of the control group treated with PBS, the serum of the mice in the groups 1, 2, and 3 prevented the *S. aureus* growth in the higher dilutions. However, the sera of treated animals had no antibacterial effect on *E. coli* (Table 4).

Table 1. The level of carbohydrate in the isolated fractions (1-10) of *G. Persica*

Fraction	1	2	3	4	5	6	7	8	9	10
Carbohydrate (mg/ml)	**0.2**	**5.4**	**0.225**	**1.1**	**1.2**	**1.1**	**4.2**	**4.3**	**0.9**	**0.312**

Table 2. The *in vitro* antibacterial effects of the *G. persica* carbohydrate fractions

Tested bacterial strains	**MIC of the carbohydrate fractions**									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
S. aureus	2	8	16	2	-	16	2	2	16	8
MRSA	2	4	8	2	2	4	4	8	8	16
E. coli	2	2	2	2	2	2	2	2	4	4
S. typhimurium	2	2	2	-	2	2	2	2	2	4
P. aeruginosa	8	8	16	8	16	16	16	8	8	16
A. hydrophila	-	2	2	2	2	2	2	4	8	8

The minimum inhibitory concentrations (mean) were shown as the last effective dilution.

Table 3. The hemagglutination titers against SRBCs and complement activity (mean±sd) in the mice orally treated with *G. persica* carbohydrate fractions

Fraction	PBS	SRBC	1	2	3	4	5	6	7	8	9	10
Anti-SRBC titer	**0**	**70±20^a**	**200±46.5^b**	**240±93.1^b**	**60±28.2^a**	**80±5^a**	**280±56.5^b**	**200±61^b**	**160±25^c**	**120±46.5^c**	**80±10^a**	**120±65^c**
Complement activity	**5.5±42^a**	**5.47±0.3^a**	**5.6±0.5^a**	**5.55±0.3^a**	**5.65±0.1^a**	**5.75±0.3^a**	**5.75±0.2^a**	**5.7±0.2^a**	**5.65±0.2^a**	**5.40±0.1^a**	**5.45±0.3^a**	**5.5±0.1^a**

The different superscript letter in each row shows a significant difference at $p<0.05$.

Table 4. The antibacterial effect of the mice sera (mean) following oral administration of the carbohydrate fractions of *G. persica*

Fraction	**PBS**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
E. coli	4	4	4	4	4	4	4	4	4	4	4
S. aureus	4	6	5	5	4	4	4	4	4	4	4

The minimum inhibitory concentrations are shown as dilution factor.

DISCUSSION

The various fractions may contain carbohydrates with different level of negative ions due to their sulfate groups. Most of the fractions showed bactericidal effects, which might be due to the presence of sulfated molecules, such as carrageenans [21], fucans [22], and laminarin [23] in different fractions.

Some reports have previously indicated the bactericidal effects of different seaweeds against Gram-positive and Gram-negative bacteria. The methanolic water extracts of the *Heliamphora elongata* had bactericidal effects on *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Listeria monocytogenes*, and *Salmonella abony* [24]. The ethyl acetate crude extract of *Caulerpa also* showed antibacterial activity against *E. coli*, *S. aureus*, *Streptococcus* sp., and *Salmonella* sp. [25] strains. In contrast to the results of the current study, another investigation has shown that the extract of *G. corticata* was more effective against Gram-negative than Gram-positive bacteria due to the lipophilic

nature of the active components in the crude extract [6]. The more potent antibacterial effects of *Gracilaria edulis* were observed in the methanolic extracts [26]. However, the crude sulfated polysaccharide of *Gracilaria ornate* was only effective against *E. coli* [13]. Some reports have indicated that antibacterial effects of the seaweed extracts are affected by various factors, such as habitat and the season of algae collection, growth stage of the plant, species of the algae, carbohydrate extraction, and evaluation methods [27]. The species of bacteria also play a significant role in the effectiveness of the extracts. In this study, the Gram-positive species had more sensitivity to the carbohydrate fractions compared to Gram-negative bacteria. This might be due to the carbohydrate fractions effect on the formation or degradation of the peptidoglycan cell wall of the Gram-positive bacteria [28].

Previous reports have demonstrated the phenolic compounds as the source of antimicrobial activities [29, 30]. However, the current study showed the direct bactericidal property of the carbohydrate fractions. The antibacterial activity of the treated mice sera showed no correlation with the *in vitro* bactericidal effects of the fractions. The main reason might be the presence of lower amounts of the polysaccharides in the mice sera due to their degradation in the digestive tracts. Previous studies reported degradation of seaweeds oligosaccharides in the digestive tract of rats [2, 31].

The carbohydrate fractions have variable effects on the humoral immune response. However, most of them stimulated the thymus-dependent humoral immune response to SRBC. In agreement with the current study, the crude extracts of the *Gracilaria verrucosa*, which contained carbohydrates or glycoproteins component, enhanced T cell immune responses [32]. However, in contrast to the results of the current study, other studies [33, 34] reported the stimulatory effects of the dietary administration of *Gracilaria* seaweed on innate immune responses of different fish species. Furthermore, the hot-water extract of various *Gracilaria* species increased immune responses and resistance of the *Macrobrachium rosenbergii* and white shrimp to *Vibrio* infection [35, 36]. The seaweed components also have probiotic like effects on intestinal microbiota through enhancing the growth of beneficial bacteria in chicken and piglets [37, 38]. Also, other effects, such as protection of intestinal epithelial cell, macrophage and lymphocyte proliferation and differentiation, and modulation of the immune responses, were previously observed [39]. Treatment of cells or animals with crude or protein extracts of the seaweed limits the heme-oxygenase-1 pathway [11], protects the cells against oxidative stress [5], induces IL-10 production [40], and reduces pro-inflammatory cytokine production. However, like the stimulated humoral immune response in the current experiment, most of the seaweed's sulfated saccharide, such as porphyran induced the macrophage activities [9, 41].

The results of this study demonstrated the *in vitro* antibacterial effects of the carbohydrate fractions of the *G. persica* seaweed, while this effect was not found in the sera of the treated mice. Also, most of the fractions stimulated the thymus-dependent humoral immune response. More researches should be conducted for purification of the specific carbohydrate of the *G. persica* which have antibacterial and immunostimulatory effects.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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