

In vivo study of antiallergenicity of ethanol extracts from *Sargassum tenerrimum*, *Sargassum cervicorne* and *Sargassum graminifolium turn*

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Abstract Food allergy has becoming the serious threat in the world for which the search of an effective anti-allergic drug is the demand of time. Keeping in view of the potentiality of seaweeds, the ethanol extracts from *Sargassum tenerrimum* (ST), *Sargassum cervicorne* (SC), and *Sargassum graminifolium turn* (SG) have been studied in vivo for its antiallergenicity through passive cutaneous anaphylaxis (PCA) and active cutaneous anaphylaxis (ACA) in female BALB/c mice. Intraperitoneal administration of these ethanol extracts inhibit mouse PCA and ACA in a dose-dependent manner using ovalbumin (OVA) and shrimp allergen as triggering agents to induce allergenicity over mice. The extract of ST containing phlorotannin has been found most active over the suppression of PCA triggered by OVA and shrimp with IC₅₀ values of 25.64 and 40.98 mg/kg, respectively and an efficacy comparable to that of an anti-allergic drug disodiumcromoglycate. Similarly, ST inhibits ACA triggered by ova and shrimp allergen in the mouse, with 50% suppression at 25.5 and 43.53 mg/kg, respectively. The results presented here show that these extracts are active on the studied models among which ethanol extract of ST was the most potent, leading toward the promising development of a new class of anti-allergic drugs.

Keywords Passive cutaneous anaphylaxis · Active cutaneous anaphylaxis · Shrimp allergen · Ovalbumin · IgE · Seaweeds

Introduction

Food allergy has emerged as a major health problem which represents the most common disorders of the immune system, affecting about 20% of the world's population [1, 2]. With the rise of allergic threats, treatments of atopic diseases are obligatory to control by suppressing the elements of the allergic inflammatory response. Antihistamines, bronchodilators, anti-allergic drugs and corticosteroids remain by far the most effective therapeutic response to allergic diseases in reducing the inflammatory reactions [3–6]. The widely spread allergic diseases can be treated with a variety of drugs, but without achieving an adequate response. Hence, there remains a need to develop new inhibitors from natural resources for better prevention and treatment of allergic disorders.

Anti-allergenic compounds usually act over mast cells which are known to play principle role in the immediate-type allergic reaction, which is the major mechanism for causing allergic rhinitis and bronchial asthma [7]. The bridging of surface IgE receptors by antigens elevates the degranulation of mast cells, leading to the release of chemical mediators, including histamine, leukotrienes and prostaglandins [8, 9].

Much attention has been focusing on polyphenols for the prevention and treatment of many diseases such as anti-cancer [10], antioxidative [11, 12], and anti-inflammatory [13] agents. Some reports have indicated that polyphenols inhibited the mast cell degranulation which is involved in the IgE-mediated allergic reaction [14, 15]. Marine algal

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polyphenols, which contain phlorotannin that is only present in brown algae and restricted to polymers phloroglucinols (1,3,5-trihydroxybenzene) [16], has been tested so far for its anti-allergic activity [17]. Keeping in mind the biological importance of abundant seaweeds at the coastal area of Pakistan and China, we thus undertaken some of its varieties to evaluate the potentiality of inhibiting mast cell degranulation by measuring the inhibitory effects on atopic reactions in different experimental animal models of allergic disorders, such as PCA and ACA in mice which were proved previously as effective tools to study the antiallergenicity [18, 19].

Materials and methods

Algae

Sargassum tenerrimum (ST) and *Sargassum cervicorne* (SC) were collected from the coastal area of Karachi, Pakistan, and *Sargassum graminifolium* turn (SG), was collected from the coastal area of Qingdao, China, in January 2007. Samples were washed three times with tap water to remove salt, epiphytes, and sand attached to the surface of the samples and then air dried within a shade protecting from direct sunlight. The dried seaweeds were crushed and ground into a powder form to pass through a 40-mesh sieve and stored at room temperature.

Chemicals

Folin-Ciocalteu's phenol reagent (2 N), disodium cromoglycate (DSCG), dimethyl sulfoxide (DMSO), Phloroglucinol and aluminum hydroxide gel (alum) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Evan's blue was obtained from Fluka, Germany. All other chemicals were ACS and spectrophotometric grade. Water was purified by milli-Q system.

Extraction

Crude extracts of brown seaweeds were prepared using the modified method described elsewhere [20]. 10 g of dried samples of various brown seaweeds were immersed in 100 mL optimized 85% ethanol and stirred for 24 h at 25 °C, followed by centrifuging the extract to collect the supernatant. The residues were transferred to a conical flask and washed with 85% ethanol. The procedure was repeated twice to obtain the supernatants. The combined supernatant was filtered and then concentrated to about 50 mL under vacuum using a rotary evaporator (Buchi R-200V, Brinkmann Instruments, Mississauga, ON), at 40 °C, as described elsewhere [21]. The concentrated

sample was then washed three times with the equal volume of chloroform to remove fats and pigments in a separatory funnel. The upper layer, corresponding to the non-lipid fraction was extracted with ethyl acetate (3×50 mL). The ethyl acetate fraction was dried under reduced pressure. The residues (crude extract) so obtained (part of it) were then dissolved in PBS to produce required concentration of the doses set for the mice. While the remaining part of the dried crude extracts were dissolved in 10 mL of DMSO to make a known concentration for the determination of phlorotannin.

Total phlorotannin content

Total phlorotannin content in the ethanol extracts was determined according to a modified version of Folin-Ciocalteu method [22] using phloroglucinol as the standard. The crude extracts dissolved in DMSO were diluted taking into account the measurable range of the spectrophotometer (e.g., a 0.025–0.1 mL aliquot of extracts of soluble phenolics was mixed with 0.475–0.4 mL water). A 0.1-mL aliquot of the diluted crude extracts was mixed in a test tube with 0.5 mL of 2 N Folin-Ciocalteu reagent and 0.5 mL of water. The mixture was allowed to stand for 3 min following addition of 2.0 mL of 20% Na_2CO_3 . Samples were incubated in the dark at room temperature for 45 min and centrifuged at $1,600 \times g$ for 8 min. Absorbance of the supernatant was measured at 730 nm using spectrophotometer. Total phlorotannin content was calculated using the standard graph plotted and expressed as a percentage.

Animals

Six weeks old female BALB/c mice (22–25 g) were purchased from Vital River Co., Beijing. They were fed a standard diet and kept in controlled climatic conditions. Food and drinking water were available ad libitum.

Antigens

Ovalbumin (OVA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) while shrimp allergen (tropomyosin, isolated protein from shrimp, 36 kDa) was produced by food safety laboratory, Ocean University of China.

Antibodies production

Anti-OVA IgE and anti-shrimp IgE antibodies were produced by the modified method described elsewhere [18].

Female BALB/c mice were immunized three times with 200 µg of OVA and shrimp allergen to produce their

respective antibodies with 1 mg of Alum contained in 100 μ L of PBS, intraperitoneally at intervals of 7 days. Serum samples for the PCA tests were obtained 7 days after the last immunization. The PCA reaction conditions were optimized to find the minimum dose required to elicit a positive response.

Mouse belly passive cutaneous anaphylaxis

Mouse belly PCA was conducted following the modified procedure described elsewhere [23].

Experimental design

Female BALB/c mice were divided into several groups marked separately for both shrimp allergen and OVA respectively, based on the specific treatments given at the stage of sensitization. Each group contains three mice. Group 1 belongs to sensitized animals for blank (positive control) treated with 50 μ L diluted sera (30 μ L sera + 20 μ L PBS, optimized). Group 2 belongs to non-sensitized animals for blank (negative control) treated with 50 μ L PBS only. Groups 3a–3c, 4a–4c, 5a–5c, and 6a–6c belong to the sensitized mice for samples ST, SC, SG, and DSCG, respectively. These groups were treated same as group 1. In addition, groups for the preliminary experiments were under taken to optimize the sera content

in 100 μ L PBS, were given intraperitoneally to groups 3a–3c, 4a–4c, 5a–5c, and 6a–6c of mice sensitized by injection of diluted sera, 30 min prior to eliciting PCA by antigenic challenge. While group 1 (positive control) and group 2 (negative control), received an intraperitoneal injection of 100 μ L of PBS. PCA reaction was elicited by an intravenous injection of 0.25 mg of OVA or shrimp allergen dissolved in 100 μ L of PBS containing 1.8% (w/v) Evan's blue to all groups. Thirty minutes after the antigenic challenge, blue spot wheal appeared on the skin, the mice were killed and their skins removed for the measurement of extravasated dye spectrophotometrically.

Dye extraction and measurement were performed as described elsewhere [19]. Each of removed skin was dissolved with 0.5 mL of 1 M KOH solution at 37 °C overnight, and 2 mL of a mixture of 0.6 M phosphoric acid and acetone (5:13) were added. After vigorous shaking, precipitates were filtered off and the amount of dye was determined spectrophotometrically at 620 nm.

Calculation

The decrease value of dye content (absorbance) in comparison to the positive control refers to the suppression or inhibition of antigens induced PCA reaction by the intraperitoneal administration of crude extracts and the DSCG. The percent inhibition was then calculated as follows:

$$\text{Inhibition percent} : 1 - \left\{ \frac{[\text{mean of experimental group} - \text{mean of negative control group}]}{[\text{mean of positive control group} - \text{mean of negative control group}]} \right\} \times 100.$$

required to produce bluing reaction. Three groups for three different concentrations (3 mice for each concentration) of sera (20, 30, 40 μ L, diluted with PBS to make up 50 μ L) were sensitized same as group 1.

Procedure

In brief, the whole procedure is commenced with treatments mentioned above in “[Experimental design](#)” in which female BALB/c mice were anesthetized by ether and 50 μ L of a diluted sera containing anti-shrimp IgE or anti-ovalbumin IgE antibodies, at optimized concentration (30 μ L), were intradermally injected into the belly skin for all the groups except the non-sensitized group 2.

Forty-eight hours after sensitization, samples ST, SC, SG and DSCG at doses of 5, 50 and 100 mg/kg dissolved

The 50% inhibitory concentration (IC₅₀) was calculated using the observations of three different concentrations containing three mice of each group [24].

Preliminary experiments

Sera containing IgE was injected intradermally inside the skins at different dilutions (mentioned in “[Experimental design](#)”) in order to determine the optimal concentrations to produce bluing reactions confined within skin. The PCA procedure was followed as mentioned above.

Mouse belly active cutaneous anaphylaxis

Mouse belly ACA was conducted following the modified procedure originally described elsewhere [25].

Experimental design

Female BALB/c mice were grouped same as mentioned in PCA section with the marking of different group numbers. Group 7 belongs to immunized animals for blank (positive control) treated with 100 μ L PBS, 25 μ g OVA and 1 mg aluminum hydroxide gel (alum). Group 8 belongs to non-immunized animals for blank (negative control) treated with 100 μ L PBS and 1 mg alum. Groups 9a–9c, 10a–10c, and 11a–11c belong to the immunized mice for samples ST, SC, SG, respectively. These groups were treated same as group 1. In addition, preliminary groups were under taken to optimize the antigens content required to produce bluing reaction. Three groups for three different concentrations of antigens (0.001, 0.01, 0.1 μ g/ μ L) were immunized same as group 1.

Procedure

The whole procedure is commenced with treatments mentioned above in “Experimental design” in which female BALB/c mice were immunized by a 100 μ L intraperitoneal injection of PBS containing 25 μ g of ovalbumin or shrimp allergen adsorbed on 1 mg of aluminum hydroxide gel (alum) as adjuvant for all groups of mice mentioned in “Experimental design” except group 8 which received an intraperitoneal injection of 1 mg of alum without the antigen (negative control).

Two weeks later, samples ST, SC, and SG at doses of 5, 50 and 100 mg/kg dissolved in 100 μ L PBS, were given intraperitoneally to groups 9a–9c, 10a–10c, and 11a–11c, of mice immunized by antigens, 30 min prior to eliciting ACA by injection of ovalbumin or shrimp allergen. While group 7 (positive control) and group 8 (negative control), received an intraperitoneal injection of 100 μ L of PBS. ACA was elicited in mice belly by intradermally injecting the 0.5 μ g antigens in 50 μ L of PBS (optimized), followed by an intravenous injection of 100 μ L of PBS containing 1.8% (w/v) Evan’s blue to all groups of mice including the negative control group.

Thirty minutes after the antigenic challenge, blue spot wheals appeared on the belly skins and the remaining procedure for the extraction and measurement of extravasated Evan’s blue dye were performed as described in PCA section.

Calculation

Inhibition (%) and IC_{50} values were calculated as described previously in this report.

Preliminary experiments

In preliminary experiments, 50 μ L of antigen was injected into the shaved belly of immunized mice at different concentrations (mentioned in “Experimental design”) to determine the optimal concentrations of reagents needed to produce blueing reactions limited within skins. The ACA procedure was followed as mentioned above.

Statistic

The data were analyzed using SPSS 11.0 software. One-way analysis of variance was performed and was followed by Duncan’s Multiple Range test. A value of $P < 0.05$ was used to indicate significant differences.

Results

The anti-allergic activity in vivo was evaluated for the ethanol extracts of *Sargassum tenerrimum*, *Sargassum cervicorne*, and *Sargassum graminifolium turn* using different animal models of allergenic disorders, such as passive cutaneous anaphylaxis and active cutaneous anaphylaxis in the mouse. The ethanol extraction was designed in a manner to obtain the crude phlorotannin to be used as anti-allergenic agents. The total phlorotannin content of the crude extracts of ST, SC, and SG were resulted as 5.06 ± 0.24 , 3.30 ± 0.20 , and

Table 1 Suppression of PCA over mice induced by ova and shrimp allergens along with corresponding values of the total phlorotannin content

Materials	Suppression of PCA induced by shrimp allergen IC_{50} values ^a (mg/kg)	Suppression of PCA induced by OVA allergen IC_{50} values ^a (mg/kg)	Total phlorotannin ^b (%)
DSCG ^c	50.27 ± 2.77	27.33 ± 3.91	–
<i>Sargassum tenerrimum</i>	40.98 ± 8.12	25.64 ± 7.09	5.06 ± 0.24
<i>Sargassum cervicorne</i>	124.23 ± 2.61	107.78 ± 5.44	3.30 ± 0.20
<i>Sargassum graminifolium turn</i>	258.13 ± 5.19	256.99 ± 8.21	1.71 ± 0.16

^a The mean value of (IC_{50}) was calculated using the observations of three different concentrations containing three mice of each for all the samples (algae extracts) including the control DSCG

^b Total phlorotannin content of the residual extract on dry weight basis, was determined by Folin-Ceocalteu’s method using phloroglucinol as standard

^c Disodiumcromoglycate, used as control

Table 2 Suppression of ACA over mice induced by OVA and shrimp allergens

Materials	Suppression of ACA induced by shrimp allergen IC ₅₀ values ^a (mg/kg)	Suppression of ACA induced by OVA allergen IC ₅₀ values ^a (mg/kg)
<i>Sargassum tenerrimum</i>	43.53 ± 10.07	25.50 ± 5.02
<i>Sargassum cervicorne</i>	125.23 ± 6.87	116.24 ± 5.74
<i>Sargassum graminifolium turn</i>	367.32 ± 6.80	345 ± 4.27

^a The mean value of (IC₅₀) was calculated using the observations of three different concentrations containing three mice of each for all the samples (algae extracts)

Table 3 Optimization of bluing reaction for shrimp allergen

Sera content ^a (μL)	Size of PCA absorbance ^b (nm)	Antigen content ^c (μg/μL)	Size of ACA absorbance ^b (nm)
20	0.115 ± 0.009	0.001	0.132 ± 0.006
30	0.392 ± 0.006	0.01	0.424 ± 0.005
40	0.398 ± 0.013	0.1	0.429 ± 0.008

All data are the mean values at different concentrations using three mice of each

^a Sera contents were diluted with PBS solution to make up to 50 μL, injected intradermally into the belly skin of mice for PCA reaction

^b Size of PCA and ACA was evaluated as the measurements of dye content in terms of absorbance taken at 620 nm spectrophotometrically

^c Contents of shrimp allergen dissolved in PBS (50 μL), injected intradermally to the immunized mice for ACA reaction

1.71 ± 0.16% of the dried extract, respectively (Table 1). Mast cell activations were elicited by using two different antigens OVA and shrimp allergens react with their respective IgE antibodies. Anti-ovalbumin IgE and anti-shrimp IgE were produced by the intraperitoneal injections of their respective antigens and the preliminary tests were conducted through PCA to confirm the productivity and to optimize the concentrations for the bluing reaction. While testing through PCA, antibodies showed the positive bluing reactions as allergenic response and optimized at a concentration of 30 μL sera. While optimizing the antigen contents through ACA, 0.01 μg/μL of both the antigens was found enough for the bluing reaction (Tables 3, 4). The antiallergicity of ethanol extracts was observed by eliciting PCA in mice. The intra-peritoneal administration of the crude extracts including the control DSCG showed their respective inhibition of PCA reaction against the antigenic challenge with ova and shrimp allergen separately (Table 1); the IC₅₀ values as per dose of the mice were elucidated as 40.98 ± 8.12, 124.23 ± 2.61,

Table 4 Optimization of bluing reaction for OVA

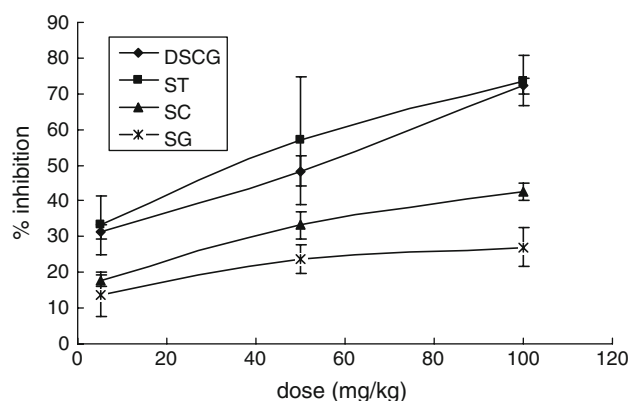
Sera content ^a (μL)	Size of PCA absorbance ^b (nm)	Antigen content ^c (μg/μL)	Size of ACA absorbance ^b (nm)
20	0.091 ± 0.007	0.001	0.114 ± 0.005
30	0.365 ± 0.007	0.01	0.41 ± 0.008
40	0.385 ± 0.006	0.1	0.399 ± 0.01

All data are the mean values at different concentrations using three mice of each

^a Sera contents were diluted with PBS solution to make up to 50 μL, were intradermally injected into the belly skin of mice for PCA reaction

^b Size of PCA and ACA was evaluated as the measurements of dye content in terms of absorbance taken at 620 nm spectrophotometrically

^c Contents of OVA dissolved in PBS (50 μL), injected intradermally to the immunized mice for ACA reaction

**Fig. 1** Inhibition (%) of PCA over mice induced by shrimp allergen. DSCG, Disodiumcromoglycate; ST, *Sargassum tenerrimum*; SC, *Sargassum cervicorne*; SG, *Sargassum graminifolium turn*

and 258.13 ± 5.19 mg/kg for ST, SC, and SG, respectively in the case of shrimp allergen-induced immunization while 25.64 ± 7.09, 107.78 ± 5.44, and 256.99 ± 8.21 mg/kg for ST, SC, and SG, respectively in case of OVA-induced immunization. Figures 1 and 2 show that suppression was dose-dependent. To verify the effectiveness of these results, the anti-allergic property of DSCG, a prophylactic anti-allergic drug which acts by stabilizing mast cell membranes, decreasing the release of histamine [26] was also comparatively evaluated in PCA mouse belly experiments and its IC₅₀ values were recorded as 50.27 ± 2.77 and 27.33 ± 3.91 mg/kg for shrimp and ova allergen-induced immunization, respectively. Table 1 shows that ST was found as the most potent anti-allergic agent comparable to that of DSCG which has greater IC₅₀ value than ST but lesser than that of SC and SG.

Furthermore, the suppression of ACA was also conducted to verify the effectiveness of the polyphenol based seaweeds extracts ST, SC, and SG. Figures 3 and 4 show

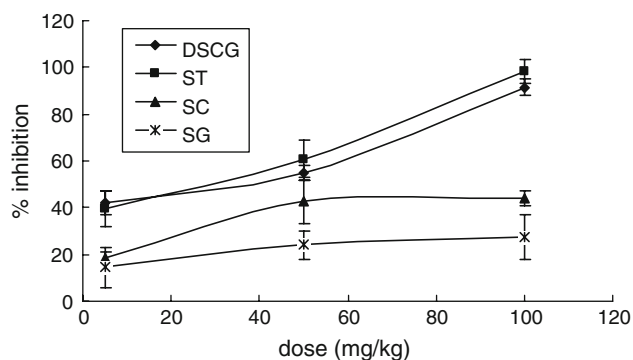


Fig. 2 Inhibition (%) of PCA over mice induced by OVA allergen. DSCG, Disodiumcromoglycate; ST, *Sargassum tenerrimum*; SC, *Sargassum cervicorne*; SG, *Sargassum graminifolium turn*

that administration of extracts of ST, SC, and SG injected intraperitoneally 30 min prior to the antigenic challenge, inhibit ACA response in a dose-dependent manner. On measuring the IC_{50} values (Table 2), it was confirmed again that the suppression caused by ST was the highest among all the extracts with IC_{50} values of 43.53 ± 10.07 and 25.5 ± 5.02 mg/kg in cases of both shrimp allergen and OVA-induced immunization, respectively. While the IC_{50} values of SC, and SG were resulted as 125.23 ± 6.87 and 116.24 ± 5.74 mg/kg for shrimp allergen induced immunization; 367.32 ± 6.80 and 345 ± 4.27 mg/kg for ova allergen-induced immunization, respectively.

Discussion

The inhibitory effect of extracts from the ST, SC, and SG over IgE-dependent cellular deregulation is clearly of therapeutic interest, as confirmed by several in vivo experiments using different animal models of allergic disorders. To evaluate the effects of anti-allergic drugs on allergenic rhinitis, PCA reaction is preferable [27].

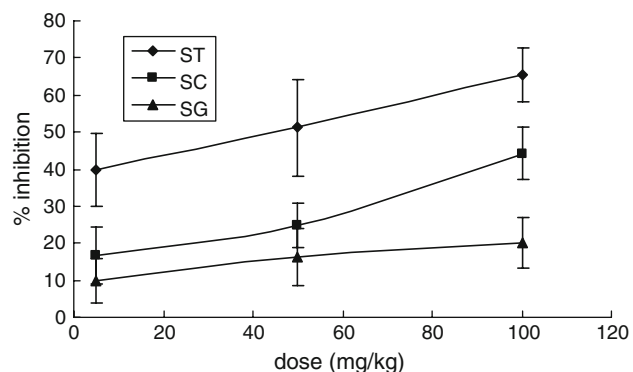


Fig. 3 Inhibition (%) of ACA over mice induced by shrimp allergen. ST, *Sargassum tenerrimum*; SC, *Sargassum cervicorne*; SG, *Sargassum graminifolium turn*

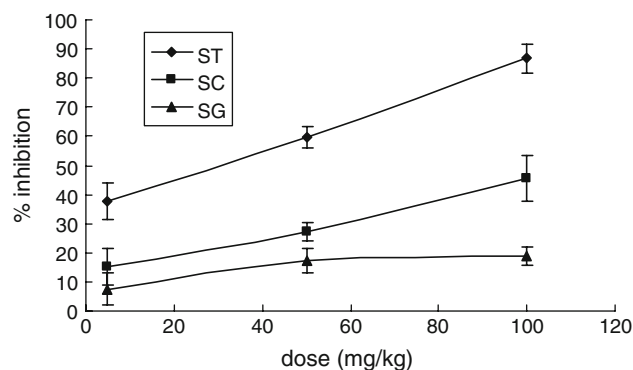


Fig. 4 Inhibition of ACA over mice induced by OVA allergen. ST, *Sargassum tenerrimum*; SC, *Sargassum cervicorne*; SG, *Sargassum graminifolium turn*

Although passive sensitization provides a simple and useful experimental model for studying drugs for anti-allergic activity, active sensitization has its own significance to investigate both allergic reactions and drug action, since allergic disorders are caused by active sensitization [19]. Therefore, the PCA technique was extended in the mouse belly to the ACA elicited in female BALB/c mice. Animals were immunized with the respective antigens in the presence of alum, and, after 2 weeks, ACA was elicited in the mouse belly by intradermal injection of the antigen followed by an intravenous injection of Evan's blue solution. The ACA reaction induced by intradermal injection of antigen was applicable to both high- and low-molecular weight antigens even at a lower concentration [18]. Intradermal delivery of antigen and dermal test is likely to be a good model for food allergy to quantify the suppression of mast cell degranulation. Though oral administrations of drug and algae extracts are preferred and more physiological, IP delivery is systematic and recommended for preclinical studies. Both PCA and ACA are less time consumable process. However, further study is required to introduce oral administration of drugs and algae extracts to PCA and ACA model in order to make it more physiological.

Before commencing the PCA and ACA reactions, preliminary experiments were conducted for the optimization of sera and antigens concentration for both ova and shrimp allergens through PCA and ACA, respectively (Tables 1, 2). As the difference of PCA ranges between 30 and 40 μ L injected sera was very little, lower concentration was considered enough for bluing reaction. Similarly, the concentration of 0.01 μ g/mL for both the antigens was found enough to produce bluing reaction in the case of ACA. Intraperitoneal administration of ST extracts resulted in remarkable suppression of PCA in the mice, with an IC_{50} produced by a dose of 40.98 ± 8.12 and 25.64 ± 7.09 mg/kg for both shrimp and OVA allergens-induced immunization, respectively with an efficacy comparable to that of

DSCG, a potent anti-allergic drug [28], and a topically applied anti-allergic agent, has been shown to inhibit mast cell activation [29] with IC_{50} values of 50.27 ± 2.77 and 27.33 ± 3.91 mg/kg for shrimp allergen and ova-induced immunization, respectively. Similarly, ST inhibited ACA in the mouse as well, with 50% suppression produced by a dose of about 43.53 ± 10.07 and 25.5 ± 5.02 mg/kg for shrimp and OVA allergen-induced immunization, respectively. The IC_{50} values of SG, and SC extracts were more than ST and DSCG indicating that these are the less potent inhibitors of allergic response among extracts tested in our study. The suppression of PCA and ACA by all the extracts including the control was more for the case of OVA than shrimp allergen. This is due to the fact that each allergen has specific potential of allergenicity [30]. So the inhibitors can suppress the bluing reactions more for the case of lower potential allergens induced with the IgE causing allergenicity. The ethanol extracts suppressing the PCA and ACA contains phlorotannin contents (Table 1) which were found in an increasing order with the decreasing of IC_{50} values of the dose given to the mouse, suggesting that phlorotannin might be among the compounds responsible for the anti-allergic activities. But the relation between the IC_{50} values and the total phlorotannin content does not reflect that phlorotannin is the only compound for the anti-allergenicity. As the percentage of phlorotannin in the crude extract was very low, some other bioactive components might play the role for the suppression of PCA and ACA, like porphyran of red algae, *Porphyra tenera* and *P. yezoensis*, which inhibited the allergenic activity [31]. However, this cannot be ignored as in a report [17], it was found that anti-allergic effect was observed even at a lower yield of crude extract containing phlorotannin from brown algae *Eisenia arborea*. Further study is still to be needed like isolation and characterization of the active compound to explore the facts.

From the result above, we concluded that the ethanol extracts of ST containing active compound appears very promising and it could be a valid candidate for development of a new therapeutic agent in the treatment of allergic diseases, with markedly high potency, compared to the anti-allergic compounds which are being used recently.

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