Effects of *Sargassum plagiophyllum* extract pretreatment on tissue histology of constipated mice

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Abstract

**Purpose:** To evaluate the toxicity of the dried seaweed, *Sargassum plagiophyllum*, extract (SPE) pretreatment in constipated mice.

**Methods:** The dried seaweed powder was mixed with distilled water and extracted by autoclave at 121 °C. Antioxidant activity of the extract was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Human normal colon cells were pretreated with SPE at 0 - 100 µg/mL for 24 h before challenging them with 100 µM hydrogen peroxide (H₂O₂). Intracellular reactive oxygen species (ROS) were quantified using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA). Male ICR mice were pretreated for 14 consecutive days with SPE at 100, 500 and 1,000 mg/kg or lactulose at 500 mg/kg. Body weight and food intake were recorded daily. Constipation was induced with loperamide on days 12, 13 and 14 and fecal pellets evacuated over a 4-hr period. The ileum, liver, kidney, and spleen were collected for histopathological examination.

**Results:** The IC₅₀ for the radical scavenging capacity of SPE was 343.90 ± 4.21 µg/mL compared to 14.14 ± 0.71 µg/mL for ascorbic acid. Pretreatment with SPE was significantly reduced ROS production in human normal colon cells. Oral administration of all doses of SPE and lactulose for 14 consecutive days had no effect on food intake or body weight when compared to the normal control group. Defecation was significantly more frequent in mice pretreated with SPE at 100 mg/kg than in the constipation control group. Histopathological examination of the ileum, liver, kidney and spleen of pretreated constipated mice revealed no toxic effect from either SPE or lactulose. On the other hand, the loss of mucus-producing cells in the ileum of constipated mice was significantly lower in mice pretreated with SPE.

**Conclusions:** These findings support the safety of SPE supplementation and may broaden its application in clinical fields as an alternative drug or supplement for constipation management.

**Keywords:** *Sargassum plagiophyllum*, Constipation, Prebiotics, Seaweed, Antioxidant

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INTRODUCTION

Constipation is a common gastrointestinal problem characterized by infrequent stools, difficult stool passage, or both. It causes discomfort and affects quality of life. Common causes of constipation include medications, metabolic abnormalities, and age. The prevalence of constipation increases gradually after the age of 50 and rapidly after the age of 70 [1]. The deleterious effects of constipation can be treated or relieved by different drugs. Among these, linaclotide and lubiprostone are well known, but long-term use commonly produces adverse effects. To enable the management of constipation without the side effects of conventional drugs, attention has gradually turned to the development of medicinal plant extracts with prebiotic properties. The administration of prebiotics has been reported to relieve constipation in patients by increasing stool frequency and improving stool consistency [2]. The possible mode of action may be alteration of gut motility due to stimulation of the colonic microbiota [3]. A known medicinal plant, brown seaweed (e.g. Sargassum polycystum, Sargassum ilicifolium, Sargassum plagiophyllum, etc.) is widely distributed in coastal areas of Thailand and has long been used in the diet and folk medicine of Asian countries [4]. Extracts from Sargassum sp. were reported to exhibit not only prebiotic but also antioxidant activities [5]. Antioxidants promote physiological wellbeing by counteracting oxidative stress and mitigating the pathogenesis of disease conditions. Moreover, extracts of Sargassum sp. have also been reported to possess neuroprotective, hepatoprotective, anti-inflammatory, anti-cancer, and immunomodulatory properties [6].

In order to develop a seaweed extract as an alternative treatment for constipation, it is vital to strengthen its medicinal viability by ruling out any potential toxicity. Although Sargassum plagiophyllum was reported to be abundant on the west coast of Thailand, there is no existing toxicity profile and the antioxidant activity of S. plagiophyllum extract has not been reported. Therefore, the present study aims to evaluate the antioxidant and biochemical activities of S. plagiophyllum extract, and any toxic effect on the ileum, kidney, liver, and spleen of constipated mice.

EXPERIMENTAL

Materials

Unless otherwise indicated, basic chemicals, loperamide and lactulose were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Cell culture reagents were from Gibco (Life Technologies, Carlsbad, CA, USA). Human normal colon cells were kindly provided by Dr. Supansa Kongseng, Prince of Songkla University, Thailand.

Preparation of the Sargassum plagiophyllum extract (SPE)

Adult stage S. plagiophyllum was harvested from Lanta Island, Krabi, Thailand. S. plagiophyllum was washed with distilled water, dried at 60 °C for 48 h, and pulverised. To prepare the SPE, 10 g of dried S. plagiophyllum powder were mixed with 1 L of distilled water and the mixture was autoclaved at 121 °C for 20 min. The autoclaved mixture was filtered through cheesecloth. The filtrate was centrifuged at 2,220 g for 10 min. The supernatant was collected and freeze dried (CHRIST, DELTA 2-24 LSCplus, Germany).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

Free radical scavenging capacity was determined by using DPPH radicals. Briefly, 100 µL of 400 µM DPPH solution dissolved in methanol were mixed with 100 µL of SPE or ascorbic acid at the desired concentration. The mixture was incubated for 30 min at room temperature and then absorbance was measured at 517 nm with a microplate reader (Synergy HT; BioTek, USA). The percentage radical scavenging capacity (RSC) was calculated as in Eq 1.

\[ \text{RSC} \% = \left\{100 - \frac{(\text{Aed} - \text{Ae})}{\text{Ac}}\right\} \times 100 \]  

where Aed is the absorbance of the extract with DPPH; Ae is the absorbance of the extract alone; and Ac is the absorbance of DPPH alone. RSC was expressed as 50 % inhibition concentration (IC50).

Cell culture and reactive oxygen species (ROS) production assay

Human normal colon cells were cultured in growth medium (GM) composed of Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified CO2 incubator at 37 °C. For the ROS production assay, human normal colon cells were seeded into 96-well plates at 5000 cells/well and allowed to grow for 24 h. The cells were pretreated with 0-100 µM SPE in GM for 24 h. H2O2 was directly added to each well for a final concentration of 100 µM. After incubation for 6 h, the medium was discarded. The cells were then incubated at 37 °C for 30 min with 10 µM
H2DCFDA in phosphate buffer saline (PBS). After washing the cells several times with PBS, fluorescence intensity was measured at 485 nm excitation and 535 nm emission wavelengths using a fluorescence microplate reader (Synergy HT; BioTek, USA).

Animals and treatment groups

Male ICR mice (Mus musculus; 6 weeks old, weighing 30 - 35 g) were obtained from the National Laboratory Animal Center and housed at the Southern Laboratory Animal Facility, Thailand. All mice were acclimatized for 7 days before the experiment started. They were placed in stainless steel cages, kept under standard environmental conditions at 23 - 27 °C with 50 - 55% humidity under a 12-hour light/12-hour dark cycle. They were given standard commercial food pellets and reverse osmosis purified water ad libitum.

The mice were randomly divided into 6 groups: a normal control, a constipation control, a positive control constipation group that received 500 mg/kg lactulose, and three constipation groups that received different doses of SPE (100, 500, and 1000 mg/kg). The treatments were administered once a day for 14 days. On days 12, 13 and 14, constipation was induced in all groups, except the normal control, by oral administration of loperamide at a dose of 5 mg/kg body weight. Body weight and food intake were recorded daily. Fecal pellets evacuated over a four-hour period were counted and hourly averages calculated. This study was approved by the Animal Ethics Committee of Prince of Songkla University, Thailand (ethical clearance no. MOE 0521.11/1555, Ref.68/2018) and followed international guidelines for animal studies.

Histopathological examination

After treatment with SPE once a day for 14 days and with loperamide on days 12, 13, and 14, the mice were sacrificed by sodium thiopental injection. The ileum, kidney, liver, and spleen were immediately excised, cut into small pieces, and fixed with 10% formalin for 24 h. The fixed tissues were dehydrated with a graded ethanol series, embedded in paraffin blocks, and sectioned at 5 µm thickness. The sections were stained with hematoxylin and eosin (H & E), periodic acid-Schiff (PAS) and Masson's trichrome following the standard protocols. The change in epithelial and cellular morphology, number of goblet cells, and accumulation of collagen fiber in stained tissues were examined under a light microscope (Olympus DP73).

Statistical analysis

The data from at least 4 independent experiments were presented as means ± standard error of the mean (SEM). Statistical differences were analyzed by one-way analysis of variance (ANOVA), followed by the Bonferroni test using GraphPrism (version 5). The data were considered statistically significant when \( p < 0.05 \).

RESULTS

Antioxidant activity of SPE

The yield of SPE extracted by autoclave was 23.46 ± 0.26 % of dried weight. An in vitro assay revealed that the antioxidant activity of SPE was concentration-dependent (Figure 1). The IC50 for the RSC of SPE was 343.90 ± 4.21 µg/mL whereas the IC50 for the RSC of the standard ascorbic acid was 14.14 ± 0.71 µg/mL (Figure 1 A). Moreover, ROS production in human normal colon cells after stimulation with 100 µM H2O2 was significantly lower after pretreatment with SPE at 10 - 100 µg/mL for 24 h (Figure 1 B).

Figure 1: Antioxidant activity of Sargassum plagiophyllum extract (SPE) (A) and effect of SPE pretreatment on reactive oxygen species (ROS) production in human normal colon cells (B). * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) compared to H2O2-treated control

Effect of SPE pretreatment on food intake, body weight and constipation

Changes in body weight and feeding activity were used as an indicator of the toxic effects of SPE. Up to the fourteenth day, there was no significant difference between the body weights of mice in all six groups (Table 1). Body weight was consistent with food intake, which also did not significantly change throughout the experiment (Table 2). However, the frequency of defecation did vary among groups. The
Table 1: Effect of SPE pretreatment on body weight in constipated mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1 (g)</th>
<th>Day 5 (g)</th>
<th>Day 9 (g)</th>
<th>Day 13 (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>37.05 ± 1.37</td>
<td>36.90 ± 1.01</td>
<td>36.86 ± 1.02</td>
<td>37.04 ± 1.00</td>
</tr>
<tr>
<td>Constipation control</td>
<td>38.01 ± 0.42</td>
<td>38.18 ± 0.47</td>
<td>38.20 ± 0.57</td>
<td>37.75 ± 0.78</td>
</tr>
<tr>
<td>Lactulose (500 mg/kg) + constipation</td>
<td>35.20 ± 0.77</td>
<td>36.31 ± 0.88</td>
<td>36.73 ± 0.62</td>
<td>36.84 ± 0.60</td>
</tr>
<tr>
<td>SPE (100 mg/kg) + constipation</td>
<td>39.09 ± 1.46</td>
<td>39.18 ± 1.49</td>
<td>39.46 ± 1.43</td>
<td>39.95 ± 1.44</td>
</tr>
<tr>
<td>SPE (500 mg/kg) + constipation</td>
<td>38.00 ± 0.80</td>
<td>37.84 ± 1.08</td>
<td>38.55 ± 1.21</td>
<td>39.43 ± 1.20</td>
</tr>
<tr>
<td>SPE (1000 mg/kg) + constipation</td>
<td>39.70 ± 1.33</td>
<td>39.80 ± 1.48</td>
<td>39.79 ± 1.32</td>
<td>40.36 ± 1.28</td>
</tr>
</tbody>
</table>

Values are expressed as means ± S.E.M.

Table 2: Effect of SPE pretreatment on food intake in constipated mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1 (g)</th>
<th>Day 5 (g)</th>
<th>Day 9 (g)</th>
<th>Day 13 (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>4.56 ± 0.18</td>
<td>4.76 ± 0.10</td>
<td>4.60 ± 0.09</td>
<td>4.66 ± 0.11</td>
</tr>
<tr>
<td>Constipation control</td>
<td>4.46 ± 0.07</td>
<td>4.29 ± 0.13</td>
<td>4.18 ± 0.13</td>
<td>4.02 ± 0.03</td>
</tr>
<tr>
<td>Lactulose (500 mg/kg) + constipation</td>
<td>4.76 ± 0.28</td>
<td>4.84 ± 0.10</td>
<td>4.44 ± 0.10</td>
<td>4.13 ± 0.06</td>
</tr>
<tr>
<td>SPE (100 mg/kg) + constipation</td>
<td>5.14 ± 0.08</td>
<td>4.55 ± 0.17</td>
<td>4.56 ± 0.14</td>
<td>4.04 ± 0.19</td>
</tr>
<tr>
<td>SPE (500 mg/kg) + constipation</td>
<td>4.06 ± 0.19</td>
<td>4.85 ± 0.31</td>
<td>4.61 ± 0.24</td>
<td>4.63 ± 0.25</td>
</tr>
<tr>
<td>SPE (1000 mg/kg) + constipation</td>
<td>4.83 ± 0.24</td>
<td>4.72 ± 0.21</td>
<td>4.62 ± 0.14</td>
<td>4.64 ± 0.16</td>
</tr>
</tbody>
</table>

Values are expressed as means ± S.E.M.

Table 3: Effect of SPE pretreatment on the frequency of defecation in constipated mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Frequency of defecation (pellet/hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>1.95 ± 0.09</td>
</tr>
<tr>
<td>Constipation control</td>
<td>0.60 ± 0.20</td>
</tr>
<tr>
<td>Lactulose (500 mg/kg) + constipation</td>
<td>2.30 ± 0.09#</td>
</tr>
<tr>
<td>SPE (100 mg/kg) + constipation</td>
<td>1.85 ± 0.13#</td>
</tr>
<tr>
<td>SPE (500 mg/kg) + constipation</td>
<td>1.15 ± 0.17</td>
</tr>
<tr>
<td>SPE (1000 mg/kg) + constipation</td>
<td>1.71 ± 0.53</td>
</tr>
</tbody>
</table>

Values are expressed as means ± S.E.M., *p < 0.05 compared with the normal control, #p < 0.05 compared with the constipation control.

The frequency of defecation was significantly lower in the constipation control group than the normal control group; but between the normal control group and all three SPE pretreatment groups, there was no significant difference. Moreover, the frequency of defecation was significantly higher in mice pretreated with 500 mg/kg lactulose and 100 mg/kg SPE than in the constipation control group (Table 3). These results indicated that SPE did not detrimentally affect the growth of the experimental animals and that it could restore the frequency of defecation in constipated mice.

Effect of SPE pretreatment on the number of mucus-producing cells in the ileum

Periodic acid Schiff (PAS) staining was used to aid the investigation of the protective effect of SPE pretreatment on the number of mucus-producing cells, or goblet cells, in constipated mice. The goblet cells located in the epithelium of villi and crypts were stained dark pink (Figure 3 A). The numbers of goblet cells per villus (Figure 3 C), but not per crypt (Figure 3 B), were significantly reduced in the constipation control group compared to the normal control group. All three SPE pretreatment groups showed significantly higher numbers of goblet cells per villus compared to the constipation control group (Figure 3C).

Effect of SPE pretreatment on the kidney histology of constipated mice

The kidneys are responsible for the elimination of toxic agents. In the application of herbal extracts, the development of renal toxicity is a major limiting factor. This study showed that supplementation with SPE at 100, 500, and 1000 mg/kg and lactulose at 500 mg/kg for 14 consecutive days did not produce any signs of renal toxicity (Figure 4). There was no glomerulus atrophy and urinary space dilation in all treatment groups. Also, the epithelial thickness of proximal and distal convoluted...
Effect of SPE pretreatment on the architecture of the ileal mucosa in constipated mice

Figure 2: Effect of *Sargassum plagiophyllum* extract (SPE) pretreatment on the architecture of the ileal mucosa in constipated mice (A). The length of villi (B) and depth of crypts (C) were measured. The ratios of villus length-crypt depth were calculated (D). Scale bar = 20 µm.

Effect of SPE pretreatment on the ileal histology of constipated mice

The ileal histology showed changes in the crypt-villus architecture and goblet cell numbers. SPE pretreatment appeared to have a protective effect on the ileal mucosa compared to the constipation control group.

Figure 3: Effect of *Sargassum plagiophyllum* extract (SPE) pretreatment on numbers of goblet cells in the ileum of constipated mice (A). Numbers of goblet cells were determined per crypt (B) and per villus (C). *p < 0.05* compared to normal control, *#p < 0.05* compared to constipation control, scale bar = 20 µm.

Effect of SPE pretreatment on the liver histology of constipated mice

Histological changes in the liver, the organ essential for metabolism and glycogen storage, were investigated. After histopathological assessment, comparison with the control groups revealed that no significant morphological changes had occurred in the liver tissue of all three SPE pretreatment groups and the lactulose pretreatment group (Figure 5). Liver tissue from all groups showed regular hepatic lobules with central veins and peripheral portal triads. Normal hepatocytes and hepatic cords extended radially from the central veins. The sinusoidal spaces in each group were the same. PAS staining showed abundant distributions of glycogen granules within the cytoplasm of hepatocytes in all groups. Masson's trichrome staining revealed small collagen fiber deposits supporting structures in the portal triad; branch of portal vein, hepatic artery, and bile ducts. Increases in collagen fiber deposits have been used as an index to evaluate liver injury and drug and chemical hepatotoxicity. However, after 14-days of treatment with SPE and lactulose, the amount of collagen fiber in the portal triad of mice in those groups showed no change compared to mice in the normal control and constipation control groups.

Effect of SPE pretreatment on the spleen histology of constipated mice

Histopathological examination of spleen tissue revealed normal histology in all groups (Figure 6). There was no evidence of splenic hyperplasia or splenic volume loss in any SPE pretreatment group nor in the lactulose pretreatment group. Furthermore, lymphoid infiltration of white pulp and red pulp was not significantly different when compared to the normal controls.
DISCUSSION

The antioxidant activity of SPE in this study was higher than previously reported for another Sargassum species [7], the difference may be due to species variance and the extraction method used [7]. Other studies have provided evidence that Sargassum extracts from several species are good sources of natural antioxidants [5]. Several lines of evidence indicate that the antioxidant activity of Sargassum extract is exerted through the presence of polyphenols and Fe$^{2+}$-chelating ability [4]. Accordingly, SPE can possibly be recommended as an antioxidant that prevents oxidative stress by attenuating oxidative damage.

The administration of SPE changed neither body weight nor food intake and did not have detrimental effects on the growth of the experimental animals. These results are consistent with previous reports of the effects of seaweed extract on body weight [8] and food intake [9] in animal models. In a study of Wistar rats [10], the oral administration of Sargassum extract for 28 consecutive days showed no toxic effect on the epithelium of stomach and intestine. However, the numbers of goblet cells were significantly reduced in the mucosa of loperamide-induced constipation rats. The benefits of SPE pretreatment may be seen in the frequency of defecation. In a study of constipation in rats, pretreatment with SPE shortened gastrointestinal transit time, which in turn alleviated reductions in the numbers of goblet cells [11]. In ICR mice, the consumption of prebiotic extracts of dragon fruit stimulated gut motility [3]. These results suggested that SPE pretreatment increased the frequency of defecation and prevented the loss of goblet cells in constipated rats, which in turn protected the intestinal epithelium.

With regard to the effect of SPE on internal organs, the results of this study indicated that oral administration of SPE was safe. This conclusion is supported by the findings of previous reports. The administration of seaweed extract has been reported safe for the kidneys, since it did not interfere with renal functioning [8] and did not cause any alteration of renal histology [12]. It has also been reported that the administration of seaweed extract did not alter liver tissue histology or levels of serum ALT and ALS, which reflect normal liver functioning [12]. Amount of collagen fiber deposition at the portal triad also normal as this index has been used to evaluate liver injury and hepatotoxicity [13]. The consumption of seaweed extract also showed no effect on the spleen histology of Swiss mice [14]. In previous research, the major constituents of seaweed, fucoidan [15], fucoxanthin [16] and laminarin [17], showed no toxic effect on the kidney, liver, or spleen after treatment in vivo. These reports support the results of this study.

Figure 5: Effect of Sargassum plagiophyllum extract (SPE) pretreatment on the liver histology of constipated mice. The liver tissues were stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS) and Masson’s trichrome. C: central vein; P: portal triad; scale bar = 20 µm

Figure 6: Effect of Sargassum plagiophyllum extract (SPE) pretreatment on the spleen histology of constipated mice. The spleen tissue was stained with hematoxylin and eosin (H&E). R: red pulp; W: white pulp; scale bar = 20 µm
that SPE presents no risk to the kidney, liver, and spleen.

CONCLUSION

The findings of this study reveal that Sargassum plagiophyllum extract exhibits antioxidant activity, and has no toxic effect on the histology of the ileum, kidney, liver, and spleen of constipated mice. Furthermore, pretreatment with Sargassum plagiophyllum extract protects against the reduction of goblet cells in constipation mice. Therefore, these results strongly suggest that Sargassum plagiophyllum extract is a promising supplement for constipation management.

DECLARATIONS

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Conflicts of interest

There were no conflicts of interest to declare.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Chittipong Tipbunjong, Saranya Peerakietkhajorn, Pissared Khuituan - conceived and designed the experiment and wrote the manuscript; Chittipong Tipbunjong, Saranya Peerakietkhajorn, Rattanaporn Sengkhim, Nilobon Jeanmard, Supattra Pongparadon, Thanvarin Thitiphatphuvanon, Piyaporn Surinlert - collected and analyzed the data; Chittipong Tipbunjong - approved final version of the manuscript. All authors read and approved the manuscript for publication.

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