Red Seaweed *Eucheuma cottonii* Ethanol Extract Regulates Inflammatory Mediators Production and Prevents Colonic Injury on Acute Colitis Disease in Mice

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Abstract: This study aims to determine the protective effects of red seaweed *Eucheuma cottonii* (EC) ethanol extract on acute colitis disease in mice. Male BALB/c mice used for acute colitis disease model by induced 2.5% (w/v) of dextran sulfate sodium (DSS) for 7 days for all groups, except control group. The DSS-induced mice then treated by three different doses of EC extracts (0.35, 0.70, 1.75 g/kg body weight), curcumin (as a positive control, 0.10 g/kg), and a group was orally only by water. In 8th day, the mice sacrificed and collected the blood, then measured the body weight, colon weight, and colon length. Disease activity index (DAI), pro-inflammatory such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, as well as IL-10 as anti-inflammatory were measured. The results showed that after treatment for 7 days, EC extract protected the weight loss and decreased the colon weight per length ratio. In addition, EC extract also decreased the pro-inflammatory cytokines expression in serum and increased the IL-10. Moreover, EC extract protected the colonic tissue damage. According to this results, the EC ethanol extract might be can used for the treatment of colitis disease.

Keywords: colitis disease; *Eucheuma cottonii*; inflammatory cytokines, red seaweed

1. Introduction

Inflammatory bowel disease (IBD) is known medical burden in developed countries and a significant cause of morbidity [1]. IBD including Crohn’s disease (CD) and ulcerative colitis (UC), are indicated by chronic and relapsed inflammation of gut [2]. UC is associated with intestinal inflammation and often results in diarrhea accompanied by blood and mucus, weight loss, and colon shortening [2,3]. Since the middle of the twentieth century, the incidence of CD and UC have increased in the Western world, which includes North America, Europe, New Zealand, and Australia [4], but IBD was relatively rare in developing country. However, over the past few decades, newly industrialized countries in Asia, the Middle East, and South America have documented the emergence of IBD [5]. Furthermore, the newly industrialized countries such as India and China, with the large populations in conjunction with expanding urbanization and westernization. It might mean that the number of cases of IBD in newly industrialized countries could at some point overtake the number of cases in the Western world. According to this condition, IBD is a global disease [4,5].

Currently, pharmacological and surgical interventions are the two main treatment approaches for IBD [6]. Traditional therapeutic agents are becoming important in steroid-resistant and steroid-dependent patients, such as azathioprine and 6-mercaptopurine, as well as antibiotics [7]. Drugs such as corticosteroids, aminosalicylates, and immune-suppressants, which aimed to decrease the inflammation, show limited effectiveness for long-term remission and are associated with significant side effects [8]. Considering the serious side effects associated with the conventional treatment; natural products, including those from marine origin, have been studied to aid in the improvement of IBD clinical symptoms [9].
Seaweed or marine algae is a potential development as a source of IBD treatment. Some seaweeds used for management or treatment of IBD symptoms such as Caulerpa Mexicana [2], a sulfated polysaccharide from Hypnea musciformis [10], and fucoidan extract from Fucus vesiculosus [6]. These seaweeds have many bioactive compounds, such as polysaccharides, terpenes and flavonoids which have different pharmacological activities with antitumor, antiprot韬al, antiviral, antioxidant, anti-nociceptive, anti-inflammatory, and anticoagulant effects [2,11-13]. A kind of red seaweed, Eucheuma cottonii (EC) reported by some authors possess several functional properties such as antioxidant, anticoagulant, anti-tumor, and anti-inflammation [14-18]. Eucheuma cottonii known in some communities as Kappaphycus alvarezi (KA) or the ‘sea-bird nest’ [19,20]. In addition, extract of this seaweeds reported slow down the growth rate of the tumor cells [21], promoted wound healing [22], and upregulated the cancer cell apoptosis [23]. Moreover, KA extracts improved cardiovascular, liver, and metabolic parameters in obese rat models [24] and showed the anti-diabetic effect on streptozotocin-induced type 2 diabetic mice [25]. However, effects of EC extract on colitis disease on mice haven’t reported.

Various chemical agents have been reported to induction of colitis rodents models such us induced by dextran sodium sulfate (DSS), trinitrobenzene sulfonic acid (TNBS), oxazolone, acetic acid, carrageenan, indomethacin, a non-steroidal anti-inflammatory drugs (NSAIDs), and peptidoglycan-polysaccharide [26]. Acute or chronic inflammation of colon can induced by administration of DSS in drinking water and it effects depending on the administration concentration and time [27]. This study aims to determine the protective effects of Eucheuma cottonii ethanol extract on dextran sulfate sodium (DSS)-induced colitis disease in mice.

2. Results

2.1. Weight Loss and Disease Index

The body weight loss significantly (p<0.05) increased in DSS-induced colitis disease mice and treatment with EC extract or Curcumin maintenance the body weight loss (Figure 1a). Untreated DSS-induced colitis mice higher of disease index in the end of experiment compared than treated mice with EC extract or Curcumin (Figure 1b).

Figure 1. Effect of EC extract on (a) weight loss in DSS-induced colonic mice during experiment and (b) disease index after treatment for 6 weeks. The values represent the mean ± S.E.M. (n = 8).

2.2. Colon Weight and Length

DSS-induced colitis mice decrease the colon length and after treatment for 7 days with a high dose of EC extract or Curcumin significantly (p<0.05) protected the shortening of the colon (Figure 2a). In addition, both of treatment also decreased the colon weight per length ratio (Figure 2b) and Figure 2c showed the representative colon of each group.
Figure 2. Effects of EC extract on colon DSS-induced after treatment for 7 days: (a) Colon length; (b) Colon weight/length ratio; (c) Representative colons of each group. The values represent the mean ± S.E.M. (n = 8).

2.3. Inflammatory Cytokines

In mice induced by DSS, the pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 significantly (p<0.05) increased compared than the control group (Figure 3a and 3b). DSS-induced mice company with treatment EC extract or Curcumin reduced the TNF-α, IL-1β, and IL-6 expression in mice serum. In addition, EC extracts also significantly (p<0.05) reduced the expression of IL-1β in colon tissue (Figure 4a). On the others hand, IL-10 expression high in healthy mice and decreased in DSS-induced colitis mice and treatment with both of EC extract or Curcumin regulated the expression IL-10 in colon tissue (Figure 4b).
Figure 3. Effects of EC extract on inflammatory cytokines expression in serum after treatment for 7 days: (a) TNF-α; (b) IL-1β; (c) IL-6. The values represent the mean ± S.E.M. (n = 8).

Figure 4. Effects of EC extract on inflammatory cytokines expression in colon tissue after treatment for 7 days: (a) IL-1β; (b) IL-10. The values represent the mean ± S.E.M. (n = 8).

2.4. Colonic Histopathology

Under hematoxylin and eosin (H&E) observation, normal colon shows the good architecture of all the layer and no loss of the crypts cell (Score 0), however in DSS-induced mice shows the thickness of mucosal layer accompanied by erosion and the score is 4 for the microscopic damage (Figure 5). After treated with EC extract (especially in high dose) and Curcumin showed the amelioration effects of the treatment by prevented the loss of crypts cell (Score 1).
3. Discussion

Crohn’s disease (UC) and ulcerative colitis (UC) are two major of inflammatory bowel diseases (IBD), which recognized by acute and chronic inflammation of the intestinal [28]. Various animal models have been studied to characterize the complexity of IBD pathogenesis such as induced the animal models by dextran sodium sulfate (DSS), trinitrobenzene sulfonic acid (TNBS), oxazolone, acetic acid, carrageenan, indomethacin, a non-steroidal anti-inflammatory drugs (NSAID), and peptidoglycan-polysaccharide [26]. DSS-induced in drinking water is the most common used for mouse models of colitis disease due to simplicity, rapidly, reproducibility, and controllability. Administration range of doses between 2.5%-5.0% of DSS in BALB/c mice [28].

DSS-induced in mice decrease the body weight with high level of weight loss and after treated with EC extract or Curcumin showed that both of treatment help to maintenance the body weight loss (Figure 1a). In addition, disease index also increases in DSS-induced mice (Figure 1a). Previous study reported that weight loss occurred in mice which administration of DSS in drinking water [28] and increased the disease index [29,30]. Oral administration of EC extract or Curcumin have alleviated the disease index of DSS-induced mice. In colitis induced by DSS associated with acute histological changes such weight loss, diarrhea, and rectal bloody, which the results increased the disease index. Colitis disease also characterized by shortening the colon (Figure 2a and 2c) and increase the colon weight/length ratio (Figure 2b). Previous study reported that colitis disease recognized by increase of colon weight. EC extract or Curcumin treatment have prevented effect for the colon weight. Overall, after treated with EC extract or Curcumin reduced the clinical signs and symptoms of colitis disease in mice induced by DSS.

The results from molecular observation supported the amelioration effects of EC extract or Curcumin on the phenotype changes in mice induced by DSS. The EC extract or Curcumin treatment can reduce the pro-inflammatory cytokines level such as TNF-α, IL-1β, and IL-6 in serum of DSS-induced mice, whereas untreated DSS-induced mice showed the increased expression of these cytokines (Figure 3). TNF-α, IL-6, and IFN-γ are the inflammation mediator in colitis mice [30,31]. TNF signaling induced the pleiotropic pro-inflammatory effects in colitis disease including activation effector T cell and macrophages and the direct damage of intestine epithelial cells (ICEs) via myosin light chain kinase (MLCK) activation [32,33]. Increasing production of IL-6 have been reported in experimental colitis, which produced by lamina propria and CD4+ T cells [34]. Recent study reported that anti-TNF-α is a strategy management the colitis disease [35]. On the other hand, regulate the
expression of IL-10 may can use for prevented the colitis disease [32,36]. Present study showed that in no treated DSS-induced mice, the IL-10 expression in colon tissue lower than treated with EC extract or Curcumin (Figure 4b). Previous study also reported that untreated IL-10 gene-deficiency mice showed high histopathologic injury score and high colon weight/length ratio as well as high expression of IFN-γ and IL-17 [37]. Colitis disease shows the severity colonic damage. The colon of untreated mice shows the thickness of mucosal layer accompany by erosion. After treatment with EC extract or Curcumin reduced the microscopic score and characterized by high number of crypts cell in mucosal layer (Figure 5). Previous study reported that colonic mice induced by 5% of DSS showed the mucosal thickness and epithelial injury as well as increased the microscopic score damage [29].

EC extract shows had some function properties such as antioxidant, anti-inflammation, and anti-cancer [20,21,38,39]. EC extract reported slow down the growth rate of the tumor cells [21], promoted wound healing [22], and upregulated the cancer cell apoptosis [23]. Moreover, extract of this seaweed improved cardiovascular, liver, and metabolic parameters in obese rat models [24] and showed the anti-diabetic effect on streptozotocin-induced type 2 diabetic mice [25]. As positive control, Curcumin reported can prevent the colitis disease by suppress the NF-kB [40] and inhibit the STAT3 signaling [41]. In addition, Curcumin also modulation of some inflammatory mediator such as TNF-α and NO [42]. Future research for this study may be can observe the microbiota variation in gut intestine of the mice. Previous studies reported that the gut microbiota is altered in inflammatory bowel diseases [43,44].

4. Materials and Methods

4.1. Materials

Red seaweed Eucheuma cottonii was harvested in Sabah, North Borneo, Malaysia. Curcumin was purchased from Nakari Tesk (Kyoto, Japan). Dextran sulfate sodium (DSS, MW ~40,000 Da) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Tumor necrosis factor (TNF)-α (Cat. No. ARG80206), interleukin (IL)-6 (Cat. No. ARG80199), IL-1β (Cat. No. ARG80196), and IL-10 (Cat. No. ARG80200) ELISA kits were purchased from Arigo Biolaboratories Corporation (Hsinchu, Taiwan). Formalin was purchased from Macron Fine Chemicals (Pennsylvania, USA). Ethanol and phosphate buffered saline (PBS) powder were purchased from Sigma-Aldrich (St. Louis, Missouri, U.S.A.).

4.2. Seaweed Extraction

Seaweed extraction processes based on the previous method [45] with a modification. Briefly, 40 g dried seaweed Eucheuma cottonii powder and put into Erlenmeyer flask and added 200 ml of 70% ethanol. The extraction was performed at 50°C for 3 h with stirring by a magnetic stirrer. Then, filtration used a filter paper as the results were filtrate (liquid) and residues (waste). Take the residues and replay the extraction with fresh solvent until the filtrate colorless. Use vacuum evaporator at 40°C of the water bath to evaporate the solvent. After evaporation completed, remove the evaporated extract to the new bottles for drying used freeze dry machine and the result was the E. cottonii (EC) extract with extraction yield was 17.79%.

4.3. Animal Model

All procedures have followed the standard of Institutional Animal Care and Use Committee (IACUC Approval No. 107003) of National Taiwan Ocean University. Six-week-old of male BALB/c mice were purchased from National Laboratory Animal Center (Taipei, Taiwan). The mice were acclimatized for 1 week before the experiment began and housed 4 mice per cage in a room maintained at 25°C, under a 12 h day/night cycle throughout the experiments. The acute colitis mice were established by the previous method [30] with a modification. Briefly, the mice were induced by administrating 2.5% (w/v) of DSS for 7 days [28]. Forty-eight mice were weight before the experiment and were divided to six groups (8 mice per group) that included the control, either 2.5% DSS-induced colitis, or 2.5% DSS-induced colitis and EC extracts were administrated (EC1, 0.35; EC2, 0.70; or EC5, 1.75 g/kg/day). The positive control group was treated by curcumin (Cur, 0.10 g/kg/day). Mice were
checked daily for body weight, stool consistency, and the presence of fecal blood. EC extract and curcumin orally administered once a day of DSS treatment for 7 days. The mice were sacrificed at 8th day and fasted for 12 h before sacrifice. The colon, spleen, kidney, and liver were measured on the day of sacrifice. The blood and colon tissue were kept in -20°C for use in the future analysis.

4.4. Disease Activity Index

Disease activity index (DAI) measured by previous methods [30,46,47]. Disease index was calculated based on presence of fecal blood, stool consistency, and percentage of weight loss. DAI values were calculated as [(weight loss score) + (stool consistency) + (rectal bleeding score)]/4 and scored from 0-4 (Table 1).

Table 1. Scoring of disease activity index (DAI) in DSS-induced colitis disease*

<table>
<thead>
<tr>
<th>Score</th>
<th>Occult/gross bleeding</th>
<th>Weight loss (% of initial weight)</th>
<th>Stool consistency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>&lt; 1</td>
<td>Normal stools</td>
</tr>
<tr>
<td>1</td>
<td>Small spots of blood stool; dry anal region</td>
<td>1-5</td>
<td>Soft pellets not adhering to the anus</td>
</tr>
<tr>
<td>2</td>
<td>Large spots of blood stool; blood appears through anal orifice</td>
<td>5-10</td>
<td>Very soft pellets adhering to the anus</td>
</tr>
<tr>
<td>3</td>
<td>Deep red stool; blood spreads largely around the anus</td>
<td>10-15</td>
<td>Liquid stool on long streams; wet anus</td>
</tr>
<tr>
<td>4</td>
<td>Gross bleeding</td>
<td>&gt; 15</td>
<td>Diarrheal</td>
</tr>
</tbody>
</table>

*Table adapted from previous studies [30,46,47].

4.5. Inflammatory Cytokines Analysis

The blood serum collected using syringe and centrifuge for 15 min at 3000 rpm and stored at -20°C. The extract of colon tissue was prepared by weighted 100 mg of colon tissue and diluted in 900 µl of cold PBS and keep in -20°C and thawed at room temperature. Then centrifuge for 15 min at 5000 rpm and collected the supernatant [48,49]. Pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and anti-inflammatory (IL-10) were detected by enzyme-linked immunosorbent assay (ELISA) kit as described by Arigo Biolaboratories. Briefly, added 100 µl of sample or cytokines standard into antibody-coated microplate and incubated for 1.5 h at 37°C. Washed with Wash buffer and added 100 µl of Antibody conjugated to each well and incubated for 1 h at 37°C. Washed and added 100 µl of Streptavidin conjugated to Horseradish peroxidase (HRP) and incubated for 30 min at 37°C. Washed and added 100 µl of substrate solution (3,3',5,5'-Tetramethylbenzidine, TMB) and incubated for 15 min at 37°C. Protected the plate from light. Added 100 µl of Stop solution and read the optical density (OD) at 450 nm immediately.

4.5. Histopathological analysis

At the day of sacrifice, the mice colons collected and the distal colon fixed in 10% neutral-buffered formalin. These specimens were embedded in paraffin, and sections were cut at a thickness of 5 µm and stained with hematoxylin and eosin (H&E) as described by previous methods [50] and was done by Lie Pei Co., Ltd. The colonic histology was scoring followed by previous methods [29,51]: Score 0, normal colonic mucosa, Score 1, loss of one-third of the crypts; Score 2, loss of two-third of the crypts, Score 3, lamina propria covered with a single layer of epithelial cells with mild inflammatory cell infiltration; and Score 4, erosions and marked inflammatory cell infiltration.

4.6. Statistical Analysis

Data are reported as means of at least three replicates. Statistical analyses with Duncan’s multiple test (p<0.05) were conducted using SPSS 22.0 software to analyze the experimental data. All data were expressed as mean ± standard error of measurement (S.E.M.).
5. Conclusions

The Eucheuma cottonii ethanol (EC) extract has protected effects on the colonic disease induced by dextran sulfate sodium in mice. Treatment with EC extract reduces the weight loss and disease index. In addition, EC extract also regulates the pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-6, and interleukin-1β. EC extract also reduces colon injury in DSS-induced mice. Based on the results, the E. cottonii ethanol extract might be used for the treatment of colitis disease.

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