ORIGINAL ARTICLE

The effect of roselle flower petals extract (*Hibiscus sabdariffa* Linn.) on reducing inflammation in dextran sodium sulfate-induced colitis

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ABSTRACT

Aim To determine the effect of roselle (*Hibiscus sabdariffa*) petals in dextran sodium sulfate (DSS)-induced colitis model by measuring pro-inflammatory cytokines expressions (IL-6, and TNF-α), anti-inflammatory cytokine expression (IL-10) and histological colitis inflammation score (HCIS).

Methods This study was conducted in two phases. For the first phase, five DSS-induced colitis mice were sacrificed (group 1) and compared with six healthy mice (group 2) after five-cycle induction (70 days). For the second phase, roselle-treated DSS-induced colitis mice were sacrificed on day 7, 14, 21, and 28 after induction and compared with mesalazine-treated DSS-induced colitis mice. Expressions of IL-6, TNF-α, and IL-10 were determined by immunohistochemistry and HCIS were assessed by two experienced pathologists.

Results The expressions of IL-6, TNF-α, and IL-10, and HCIS in DSS-induced colitis mice were increased compared with control. The expressions of IL-6, TNF-α were significantly higher in roselle-treated group on day 7 and 14, but not on day 21 and 28, whereas, the expression of IL-10 was significantly lower only on day 7 compared with mesalazine-treated group. The inflammation was higher in roselle-treated group assessed by using HCIS. Compared to day 0, the reduction of HCIS was significant in roselle-treated and mesalazine-treated groups.

Conclusion Roselle flower petal can attenuate the inflammation in DSS-induced colitis in mice. The extract of roselle can be given as an adjuvant therapy to the first-line therapy to enhance anti-inflammatory effect by increasing expression of anti-inflammatory cytokines and decreasing pro-inflammatory cytokines.

Key words: anti-inflammatory, cytokines, immunohistochemistry, inflammatory bowel disease, plant extract

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INTRODUCTION

Inflammatory Bowel Disease (IBD) is a chronic or remitting/relapsing inflammation of the intestinal with two major groups: Crohn's Disease (CD), which can affect any region in digestive tract from mouth to anus characterized by transmural granulomatous inflammation, and ulcerative colitis (UC), which continuously affects colonic mucosa proximal from rectum and forms ulcers (1). The etiology of IBD is still unknown; probably it is the result of excessive immune response to gastrointestinal microbiota that was stimulated by increased effector T cell activity and/or decreased regulator T cell activity, changes in the composition of gastrointestinal microorganisms and/or damage of the epithelial barrier (2) including both innate and adaptive immunity. Dysregulation of the homeostasis between intestinal immune systems and microbiota has been shown to be associated with the development of inflammatory bowel diseases (IBD.

The process of immune response in IBD is not uniform. The inflammatory process in CD is mainly mediated by T helper (Th) type 1 or Th type 17 cells, whereas for UC it is generally mediated by Th type 2 cells. However, this model cannot be used generally because of the presence of phases of the disease (acute or chronic), innate immunity pathways involvement, epithelial cells factor, and the use of anti-inflammatory drugs (3). The most important concept of immune response in the pathogenesis of IBD is the involvement of anti-inflammatory cytokines such as interleukin (IL)-10 and pro-inflammatory cytokines, such as IL-6 and tumour necrosis factor (TNF)-α. IL-10, which is known as cytokine synthesis inhibitory factor (CSIF), inhibits the release of pro-inflammatory cytokines, hence reducing inflammatory response in the mucosa (4). Pro-inflammatory cytokines, such as IL-6 and TNF-α, were also known for their roles in the pathogenesis of colitis to induce Th17 to produce other pro-inflammatory cytokines (5)evolution and, ultimately, the resolution of these forms of inflammation. Studies over the last two decades now provide a detailed (but not yet complete.

A study of Barman et al. reported that IL-10 had anti-proliferation effect of T helper cells on human intestinal lamina propria (6). Mice model of IBD with IL-10-deficient has shown a potential role of IL-10 in maintaining intestinal immune regulation, which is very important in pathogenesis of IBD (7). Antigen-presenting cell produces IL-10 that regulates homeostatic T-cell responses to commensal bacteria (8)mediated by T-helper (Th.

Mesalazine [5-aminosalicylate (5-ASA)] is the first-line therapy for IBD, and it also used for remission maintenance in patients with UC; it is relatively safe and generally well tolerated although its efficacy on CD is still limited (9). Other conventional treatments are corticosteroids, immunomodulatory drugs, biology agents, small molecular therapy as well as immunosuppressant (10). Renewable treatments are still being studied such as mesenchymal stem cell transplantation and faecal microbiota transplantation (11).

Roselle (*Hibiscus sabdariffa* Linn.) is a plant belonging to the family *Malvaceae,* which is ideal for developing countries; it can grow up to 2.4 meters with red or white flower petals, and it is a good nutritious source of minerals and vitamins, especially in the flower petals, which also have numerous health benefits (12,13). A study by Anokwuru et al. stated that roselle flower petals contained several phenolic compounds, with methanol extract giving the highest phenolic contents and antioxidant activity against free radicals (14). Phenolic and flavonoid compounds are compounds that can be used as immunostimulator, as stated by Chiang et al. (15). Research on the immunomodulatory activity of roselle flower petals using the hemagglutination test method was carried out *in vivo* by Fakeye et al.; researches also measured the concentration of interleukin-10 (IL-10) as an anti-inflammatory cytokine (16). The results showed that roselle flower petals were able to increase the immune system. This is due to an increase in IL-10 production so that it can suppress the synthesis of other pro-inflammatory cytokines and affect B lymphocyte cells to produce antibodies (17).

The aim of this study was to determine the effect of roselle (*Hibiscus sabdariffa*) flower petals in dextran sodium sulfate (DSS)-induced colitis model by measuring pro-inflammatory cytokines expressions (IL-6, and TNF-α) and anti-inflammatory cytokine expression (IL-10).

MATERIALS AND METHODS

Animals and study design

This study was conducted in the pharmaceutical laboratory, biological laboratory, and anatomical pathology laboratory of the Universitas Sumatera Utara, Medan, Indonesia during the period between June and September 2019. Fifty five male healthy mice (*Mus musculus*) (Charles River, Inc., Kanagawa, Japan), 6-8 weeks old and weighing 30 grams, were housed at 20 °*C* – 25 °*C with controlled 12 hour light/dark cycle.* Laboratory-standardized cages were used to keep the animals with ad libitum access to food and water.

All animal procedures were based on the Helsinki Declaration.

The experiment was approved by the Institutional Ethics Committee of the Universitas Sumatera Utara, Medan, Indonesia.

This study was conducted in two phases.

In phase one, 11 mice were divided into 2 groups. Group 1 consisted of six mice without DSS induction and group 2 consisted of five DSS-induced colitis mice. Both groups were sacrificed after group 2 completed five-cycle (70 days) induction. Colon of each mice was excised and samples were fixated in phosphate buffered saline (PBS) 10% formalin for the histopathological analysis and for the examination of anti-inflammatory (IL-10) and pro-inflammatory (IL-6, TNF-α) cytokines using immunohistochemistry.

In phase 2, 44 mice were divided into two groups (group 3 and group 4). Group 3 (24 mice) was DSS-induced colitis mice given roselle flower petals extract (300 mg/kg body weight/day) (16) as a therapy after the induction of colitis. One kg of roselle petals were macerated with 4.5 litres of 96% ethanol for 7 × 24 hours, then concentrated with a rotary evaporator until concentrated extract was obtained. Group 4 (20 mice) was DSS-induced colitis mice given mesalazine (7.8 mg/day) (18) as gold standard therapy after the induction of colitis. Six mice from group 3 and five mice from group 4 were sacrificed on day 7, 14, 21, and 28. Histopathological analysis and immunohistochemistry of IL-6, IL-10, and TNF-α were conducted for these mice as described above.

Methods

Chemical induction of colitis. The experimental colitis was chemically induced with dextran sodium sulfate (DSS) (MP Biomedicals LLC). Mice were given 5% DSS in 5 cycles. Each cycle was given for 7 days and then continued by distilled water for 7 days. After 5 cycles (70 days) the mice were sacrificed or treated by roselle flower petals extract or mesalazine as gold standard therapy.

Histopathological analysis. The colon specimens were paraffin-embedded, subsequently cut, and they were stained with hematoxylin and eosin (H&E) using standard technique. A histological score was assessed by two experienced pathologists from the Anatomy Pathology Department of the School of Medicine, Universitas Sumatera Utara. Severity of inflammation was scored as follows: 0 - rare inflammatory cells in the lamina propria, 1 - increased number of granulocytes in the lamina propria, 2 - confluence of inflammatory cells extending into the submucosa, 3 - transmural extension of the inflammatory infiltrate. Crypt damage was scored as follows: 0 - intact crypts, 1 - loss of one-third basal, 2 - loss of two-thirds basal, 3 - entire crypt loss, 4 - change of epithelial surface with erosion, 5 - confluent erosion. Ulceration was scored as follows: 0 - absence of ulcer, 1 - 1 or 2 foci of ulcerations, 2 - 3 or 4 foci of ulcerations, 3 - confluent or extensive ulceration. Values were added to give a maximal histological score of 11 (19).

Immunohistochemical analysis of IL-6, IL-10, and TNF-α. The chosen paraffin-embedded slides were deparaffinized, rehydrated, and heated on microwave with 0.01 M citrate buffer (pH 6.0) for 30 minutes. Endogen peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes, and then washed with sulfate salt buffer. The specimens were incubated overnight in 4 °*C*, then immune stained with primary antibody (rabbit polyclonal IgG to bind the mice IL-6, IL-10, and TNF-α cytokines) (Wuhan Fine Biotech Co., Ltd., China) in concentration of 1 mg/mL diluted by 1:600. Primary antibody was detected by avidin-biotin peroxidase solution (ScyTek Laboratories, Inc., USA), and a signal was visualized by using diaminobenzidine (ScyTek Laboratories, Inc., USA). The slides were then counterstained with hematoxylin and assessed by two blinded experienced pathologists from the Anatomy Pathology Department of the School of Medicine,

Universitas Sumatera Utara. The slides were categorized as (0) for 0-15%, (1) for 15-25%, (2) for 26-50% and (3) for 51-100% stained cells for TNF-α, IL-6 and IL-10. Scale 0 and 1 was categorized as (-), 2 and 3 as (+) (20).

Statistical analysis

The results of IL-10, IL-6, and TNF-α on phase 1 (group 1 and 2) were analysed by using Fisher's exact probability test and HCIS on phase 1 were analysed by using Mann-Whitney U test. The results of IL-10, IL-6, and TNF-α on phase 2 (group 3 and 4) were analysed by using Fisher's exact probability test, and HCIS on phase 2 were analysed by using independent t-test if the distribution was normal, or Mann-Whitney U test if the distribution was not normal to compare the groups in each week, and also using one-way ANOVA test or Kruskal-Wallis test. Bonferroni post-hoc test was conducted if there were significant differences using one-way ANOVA test. Mann-Whitney U test comparing day 0 with each day 7, 14, 21, and 28 was conducted if there were significant differences using Kruskal-Wallis test. Differences were considered statistically significant when $p<0.05$.

RESULTS

Phase 1: Effect of DSS-induced colitis on inflammatory cytokines and histopathology of mice colon

The expression of pro-inflammatory (IL-6 and TNF-α) and anti-inflammatory (IL-10) cytokines by immunohistochemistry and the degree of inflammation by HCIS in five healthy mice were compared with six DSS-induced colitis mice (Table 1). Pro-inflammatory cytokines were expressed in all six mice of Group 2 (DSS-induced colitis group) compared to Group 1 (control) (none) (p=0.002). The expression of anti-inflammatory cytokines in Group 2 was also found in all mice compared to Group 1 (p=0.015). The degree of inflammation was also significantly higher in Group 2 comparing to Group 1.

Table 1. Comparison of inflammatory cytokines expressions on immunohistochemistry and histological colitis inflammatory score (HCIS) between healthy (group 1) and colitis mice (group 2) groups

Variable	No (%) of mice		p
	Group 1	Group 2	
Immunohistochemical categorization of slides*			
	IL-6		0.002†
-	5 (100)	0 (0)	
+	0 (0)	6 (100)	
	TNF-α		0.002†
-	5 (100)	0 (0)	
+	0 (0)	6 (100)	
	IL-10		0.015†
-	4 (80)	0 (0)	
+	1 (20)	6 (100)	
HCIS	0 (0 – 1)	7.5 (6 – 9)	0.004‡

*(-): (0) for 0-15% and (1) for 15-25%; (+): (2) for 26-50% and (3) for 51-100% stained cells for TNF-α, IL-6 and IL-10; †Fisher's exact probability test; ‡Mann-Whitney U test

Phase 2: Effect of roselle compared to mesalazine on inflammatory cytokines and histopathology of colon in DSS-induced mice colitis

The expression of pro-inflammatory (IL-6 and TNF-α) and anti-inflammatory (IL-10) cytokines by immunohistochemistry and the degree of inflammation by HCIS from six roselle-treated DSS-induced colitis mice were compared with five mesalazine-treated DSS-induced colitis mice on day 7, 14, 21, and 28 (Table 2). There were significant differences in the number of mice in which pro-inflammatory cytokines (IL-6 and TNF-α) were expressed in roselle-treated group comparing to mesalazine-treated group on day 7 (p=0.015 and p=0.015, respectively) and 14 (p=0.015 and p=0.015, respectively), but not on day 21 (p=1.000 and p=0.182, respectively) and 28 (p=1.000 and p=1.000, respectively). Anti-inflammatory cytokine expression was significantly more frequent in the mesalazine-treated group comparing to roselle-treated group on 7 (p=0.048), but not on day 14, 21 and 28 (p=0.455, p=1.000, and p=1.000, respectively) (Table 2).

Table 2. Comparison of inflammatory cytokine expression on immunohistochemistry between roselle group and mesalazine group*

Day	No (%) of mice														
	IL-6					TNF-α					IL-10				
	Roselle group (n=6)		Mesalazine group (n=5)		p	Roselle group (n=6)		Mesalazine group (n=5)		p	Roselle group (n=6)		Mesalazine group (n=5)		p
	+	-	+	-		+	-	+	-		+	-	+	-	
7	5	1	0	5	0.015	6	0	1	4	0.015	1	5	4	1	0.048
14	5	1	0	5	0.015	5	1	0	5	0.015	4	2	5	0	0.455
21	1	5	0	5	1.000	3	3	0	5	0.182	5	1	5	0	1.000
28	1	5	0	5	1.000	1	5	0	5	1.000	5	1	5	0	1.000

*(-): (0) for 0-15% and (1) for 15-25%; (+): (2) for 26-50% and (3) for 51-100% stained cells for TNF-α, IL-6 and IL-10;

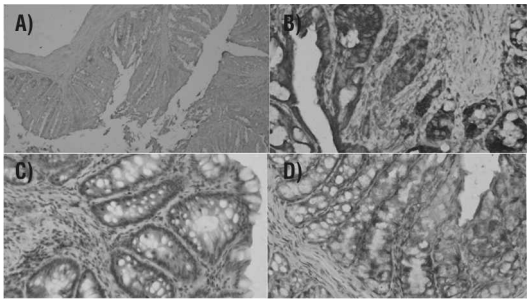

Figure 1. Histopathology of large intestine of mice. A) no IL-6 expression in control group or group without DSS induction; B) IL-6 (+) expression in large intestinal of mice that had not been given intervention; C) IL-10 (–) in large intestine of mice that had been given roselle flower petals extract on day 7; D) expression of IL-10 (+) in large intestines of mice that had been given roselle flower petals extract on day 28

Histopathology slides were examined and HCIS were assessed for each slide (Figure 1). The HCIS in roselle-treated group were also significantly higher than in mesalazine-treated group on day 7, 14, 21, and 28 ($p<0.001$, $p<0.001$, $p=0.005$, and $p=0.009$, respectively). Compared to day 0, significant decrease of HCIS was found on either roselle-treated and mesalazine-treated group ($p<0.001$ and $p<0.001$, respectively). From post-hoc analysis, HCIS from roselle-treated was significantly decreased on day 21 and 28 ($p<0.001$ and $p<0.001$, respectively), and HCIS from mesalazine-treated was significantly decreased on day 7, 14, 21, and 28 (all $p<0.001$) (Figure 2).

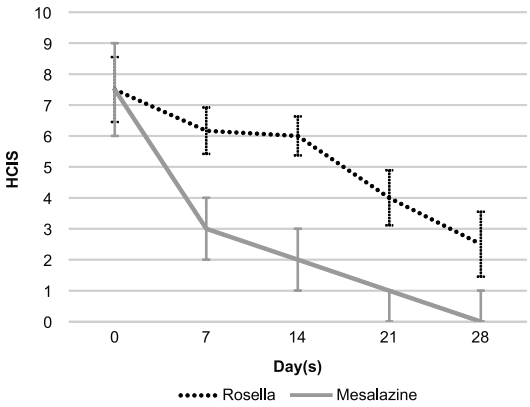

Figure 2. Histological colitis inflammatory score (HCIS) after intervention. In mice treated with roselle compared to mesalazine HCIS was significantly higher (day 7, 14, 21 and 28 ; $p< 0.05$). Compared to day 0, HCIS decreased significantly in mice treated with roselle ($p < 0.001$) and mesalazine ($p<0.001$). In post-hoc analysis, compared to day 0, HCIS decreased significantly by day 21 and day 28 in roselle group , and by day 7, 14, 21, and 28 in mesalazine group

DISCUSSION

Inflammatory bowel disease is caused by disturbance in the balance of pro-inflammatory and anti-inflammatory cytokines in colonic mucosa that leads to disease states (4). Some of pro-inflammatory cytokines that contribute to the pathogenesis of IBD are IL-6 and TNF-α (21). The anti-inflammatory cytokine that can help maintain the balance of inflammatory cytokines in colonic mucosa is IL-10 that can reduce the synthesis of pro-inflammatory cytokines by suppressing effector Th1/Th17 cells, NK cells, and also macrophages (7). Results from this study have shown that there is a significant increase in either pro-inflammatory or anti-inflammatory cytokines on DSS-induced colitis. A conversion from acute to chronic colitis in this model has not been completed yet. Chronic colitis is characterized by the increased IL-6, TNF-α, but decreased expression of IL-10 (19). It is known that the mechanism through which DSS induces inflammation of colonic mucosa is still not completely understood, some studies state that DSS disrupts intestinal barrier function (20,21)mannan-binding lectin (MBL, and other state that DSS is associated with medium-chain-length fatty acids (MCFAs) in the lumen of colon prior induction of colitis (17).

The results of this study have shown that roselle flower petals are able to increase the immune system, by increasing anti-inflammatory cytokines production and also by reducing the synthesis of pro-inflammatory cytokines. Increased synthesis of IL-10 causes suppression of TNF-α production and induces B lymphocyte to produce antibodies (17). The result of our study correlates with study by Fakeye et al. which stated that the phenolic extract from roselle flower petals was not toxic at the doses of immunostimulatory activities, which increased production of anti-inflammatory cytokine (IL-10) (22). The roselle flower petals were also known to have anthocyanin (flavonoids) compound, which has antioxidant activity (14).

Roselle flower petal is still inferior compared with mesalazine. Mesalazine has increased levels of IL-10 and decreased levels of IL-6 and TNF-α since the first week of the treatment, results that are far different from the roselle flower petal extract, which only resulted on day 21 and 28 for IL 10 and IL 6, on day 28 for TNF-α. This proves that mesalazine is indeed recognized as the first

choice for colitis therapy because mesalazine has excellent efficacy and ability to maintain colitis remission (23).

Roselle flower petal is able to reduce the inflammation by increasing expression of anti-inflammatory cytokines (IL-10) and therefore decreasing expressions of pro-inflammatory cytokines (IL-6 and TNF-α). These could be attributed to the immunomodulation properties of phenolic compound and also antioxidative nature of anthocyanin within roselle flower petals (12). Whether active phenolic compounds or anthocyanin that contribute the most to the immunomodulation is within our study limitations. In-depth investigation using those active compounds is needed in the future for the treatment of colitis.

In conclusion, roselle flower petal can attenuate the inflammation in DSS-induced colitis model. Roselle flower petal extract can be given as an adjuvant to the first-line therapy to enhance the anti-inflammatory by immunomodulation of cytokines.

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CONFLICTS OF INTEREST

Conflicts of interest: None to declare.

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