Original Article
The effect of lunasin from Indonesian soybean extract on histopatologic examination and cox-2 expression in dextran sodium sulfate-induced mice colon

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Abstract: Inflammatory bowel disease (IBD) is a condition describing chronic gastrointestinal inflammation. Chronic inflammation in colon can develop into colon cancer. Lunasin has been known to inhibit inflammatory reactions induced by lipopolysaccharide *in vitro*. The effect of lunasin to inhibit inflammation *in vivo* is not widely known. In this study, we analyzed the effect of lunasin from soybean to decrease the risk of inflammation by analyzing histopathologic feature and the expression of COX-2. 30 mice are divided into 6 groups. Normal group was not induced by dextran sodium sulfate (DSS). The other groups were induced by 2% DSS through drinking water for 9 days. After 9 days, negative control group did not receive any treatment. The other groups received treatment given lunasin dose 20 mg/kg body weight (BW) and 40 mg/kg BW, commercial lunasin and positive control given aspirin. Treatment was performed for 5 weeks. Inflammatory colon histopathologic examination and immunohistochemical score of COX-2 proteins were analyzed using statistical tests. Lunasin dose 20 mg/kg BW and 40 mg/kg BW were able to significantly reduce inflammation ($P<0.05$) performed by histopathologic feature with an average score of 2.52 and 2.16 COX-2 expression decreased significantly ($P<0.05$) with an average score of 43.674 and 33.349. Lunasin dose 20 mg/kg BW and 40 mg/kg BW were able to inhibit inflammation and decrease the expression of COX-2 in colon induced by DSS.

Keywords: Inflammation, lunasin, histopathology, COX-2

Introduction

Inflammatory bowel diseases (IBDs) are idiopathic inflammatory disorders of the gastrointestinal tract that can be subdivided into two major disorders: ulcerative colitis and Crohn's disease. In ulcerative colitis the disease extends proximally from the anal verge to involve all or part of the colon. Crohn's disease is typically a patchy disease that can affect the gastrointestinal tract anywhere from the mouth to the anus. Ulcerative colitis and Crohn's disease are characterized by episodes of remission and exacerbations in which the patient experiences abdominal pain, diarrhea, blood in the stool, and systemic symptoms. The peak incidence of IBD occurs in patients between the ages of 15 and 30 years; a second peak occurs between the ages of 50 and 80 years [1].

The prevalence of IBD is higher in the westernized world compared to developing countries, with approximately 1.4 million Americans affected with ulcerative colitis or Crohn's disease [2]. Since Crohn and Rosenberg first described IBD-associated colorectal cancer (IBD-CRC) in 1925 [3] the colon remains the primary site of neoplasms in IBD patients today, and colorectal cancer accounts for approximately 10-15% of all deaths in IBD patients [4]. Although IBD-CRC accounts for only 1-2% of all cases of colorectal cancer, IBD with colon involvement is among the top three high-risk conditions for colorectal cancer. Patients with IBD colitis are 6 times more likely to develop colorectal cancer than the general population and have a higher frequency of multiple synchronous colorectal cancers [5]. Because IBD incidence is highest among young people, the mean age for developing IBD-CRC is lower than

for sporadic colorectal cancer (40-50 years of age vs. 60 years of age) [6]. Ulcerative colitis-associated colorectal cancer is most common in the rectum and sigmoid colon, whereas Crohn's disease-associated colorectal cancer is evenly distributed between the different colon segments [7].

IBD may lead to colon cancer known as colitis-associated cancer (CAC). This cancer is usually difficult to treat, thus increasing the mortality rate [8]. It is reported that more than 20% of IBD patients develop cancer and 50% of these will die of colon cancer [9]. These patients are reported to have increased inflammatory infiltration and increased expression of inflammatory genes [10]. Patients with a potentially higher UC develop into colon cancer compared to CD patients. Higher risk for colon cancer is observed in IBD patients who have a family history of CRC [11].

The increased risk of IBD development into colitis-associated cancer (CAC) depending on the duration and extent of inflammation [1]. Area of inflammation can be driven by the accumulation of immune cells and inflammatory mediators, such as cytokines, chemokines, growth factors, lipid molecules, reactive oxygen species, and nitrogen species. The interactions between immune cells, inflammation and cytokines lead to signal stimulation that helps the development, growth, and metastases of tumor cells. A clear link exists between inflammation (IBD) and colon cancer [8].

COX-2 is one of the most important molecule that has main role in inflammation. COX-2 is an enzyme involved in the conversion of arachidonic acid to prostaglandins, is induced by inflammatory and mitogenic stimuli. Thus, the synthesis of prostaglandins is increased in inflamed tissues. Prostaglandin (PGE2) is the most important mediator in inflammation process. There is ample evidence suggesting that COX-2 expression is important in carcinogenesis. Selective COX-2 inhibitors can interfere with tumorigenesis in experimental systems including colorectal cancer. 10 Accumulating evidence indicates that non-steroidal anti-inflammatory drugs (NSAIDs) can reduce the mortality rate by 40% to 50%, decrease cell proliferation to give smaller tumors, and reduce the number of intestinal polyps. NSAIDs reduce the number and size of polyps in patients with familial adenomatous polyposis [12].

Soybean has been known as one of the utilization of natural ingredients that have anti-inflammatory effect. Lunasin is a polypeptide that had been identified in soybean [13-15]. Lunasin peptide also can be found in wheat and dairy products such as soy milk, tofu, tempeh, whole wheat bread, and others [14, 15].

In vitro studies have demonstrated the effect of lunasin to suppress inflammatory reactions induced by lipopolysaccharide. The ability of lunasin in suppressing the inflammatory activity thought to be used as a deterrent to the development of colon cancer-related inflammation. Some studies indicate lunasin has the potential to reduce the production of proinflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) as well as proinflammatory mediators, such as prostaglandin E2 (PGE2), through modulation of cyclooxygenase-2 (COX-2)/PGE2 and inducible nitric oxide synthase (iNOS)/nitric oxide pathway by inhibiting NF-κB pathway in RAW 264 macrophage cells induced by lipopolysaccharide [16-18].

The role of lunasin as anti-inflammatory *in vivo* is not widely known. Research on the expression of COX-2 in colorectal epithelial cells of mice is expected to provide some information regarding to the effect of lunasin on the expression of three proteins to inhibit inflammation and potentially reduce the risk of colon cancer, particularly those initiated by inflammation (IBD).

Methods

Plant material and extraction

Soybean was obtained from Indonesian Legumes and Tuber Crops Research Institute Malang of East Java, Indonesia. Soybean variety used in this study was Grobogan because it contains the highest protein than other varieties (to reach 43.9%). Soybean seed was pressed to obtain free oil defatted soybean. The defatted soybean grobogan was macerated with aquadest (1:5, 30 minutes) and filtrated three times. Filtrate was dried with rotary vapor resulted soybean extract. The result was tested by high performance liquid chromatog-

raphy to determine the content of lunasin according to the previous study [19].

High-performance liquid chromatographic analysis of lunasin

Analysis of lunasin in extract was tested by HPLC. 100 mg extract is dissolved in 8 mL of aquadest, then the solution is dismonicated for 30 min and then added aquadest up to 10 mL (10.000 ppm solution). The solution was then centrifuged at 12,000 rpm for 30 min and the filtrate was filtered with a 0.22 μm millipore filter to obtain a clear and colorless solution. The lunasin level in the solution was determined by HPLC using C18 column and UV-Vis detector, with acetonitrile water phase: water at 5:95 ratio, retention time 35 minutes, wavelength 295 nm, injection volume 20 μL and motion phase velocity 2 mL/minute [19].

Commercial lunasin

Commercial lunasin was produced by Reliv International Inc. USA and imported by PT. Reliv Indonesia. Commercial lunasin used in this research is the most pure, concentrated form of lunasin ever produced. It contains 125 grams of high quality soy protein.

Animal

Male Swiss-Webster mice (8-10 weeks) with an average weight of 20 g were supplied by the Health Research and Development Agency of the Ministry of Health of the Republic of Indonesia. The mice were kept in independent ventilation cages (48 cm×35 cm×20 cm) with free access to food and water under controlled humidity (55%±5%), light/dark cycle (12 hrs/12 hrs) and temperature (23±1°C). The mice were carefully examined to ensure that they are in healthy conditions and acclimatized for 1 week before any experimental procedure was performed. All protocols and surgical procedures were approved by the Animal Care and Use Committee of the Faculty of Medicine of the University of Indonesia.

Induction of inflammation

Mice were given a dextran sodium sulfate (DSS) 2% in drinking water for 9 days. This procedure was adopted from Perse *et al* [20].

Experimental groups

Mice were randomized into the following 6 groups: normal group, which received no DSS and no other treatment; negative group, which only received induction 2% DSS for 9 days; positive group, which received daily oral aspirin suspension at a dose of 42.25 mg/kg BW after 9 days induction; soybean extract treatment groups (SE1 and SE2), which received daily oral soybean extract contained lunasin at a dose of 20 and 40 mg/kg BW after 9 days induction; respectively; commercial lunasin treatment groups (CL), which received daily oral commercial lunasin at a dose of 16.25 mg/kg BW, after 9 days induction. After 5 weeks, the mice were sacrificed and colon tissues of mice were taken.

Tissue sample preparation

Colon tissues of mice were cleaned with water. Tissues were fixed with 10% of buffered neutral formalin and embedded into paraffin blocks.

Hematoxylin-eosin staining

Tissue samples were cut with a thickness of 3-5 μm for HE staining. The specimens were deparaffinized with xylol I, II and III for 5 minutes each, then rehydrated with absolute alcohol, 90% alcohol and 70% alcohol for 5 minutes each. After that, the slides were placed in hematoxylin for 5-10 minutes, washed in running water for 5-10 minutes, placed in a saturated lithium carbonate solution and washed again in running water for 5-10 minutes. The slides were then placed in eosin for 1-3 minutes. After being stained, the slides were dehydrated with 70% alcohol, 90% alcohol and absolute alcohol for 3-4 minutes each, then cleared with xylol I, II and III before being mounted with entellan.

Immunohistochemistry staining

Tissue samples were cut with a thickness of 3-5 μm for immunohistochemistry staining. The specimens were deparaffinized and rehydrated following HE staining procedure previously. Specimens were spilled by 0.5% of H_2O_2 to eliminate endogenous peroxide for 30 minutes at room temperature. Then, specimens were incubated for 10 minutes in tris-EDTA at 96°C temperature to block non-specific antigen sites. Specimens were incubated with rabbit poly-
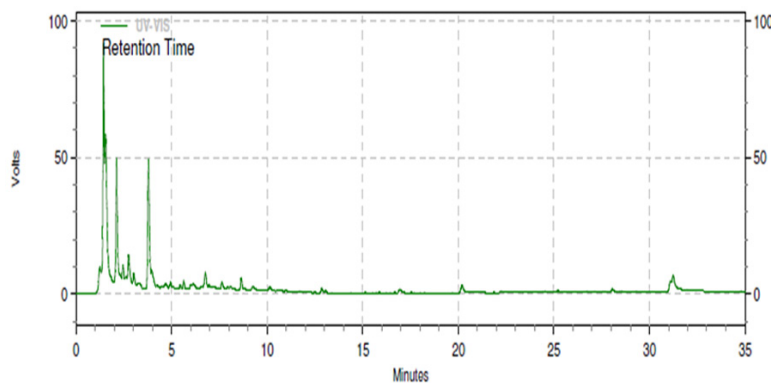
Figure 1. HPLC profile of lunasin from grobogan soybean.

Table 1. Lunasin content of the grobogan soybean extract

Sample	n	Lunasin content (mg/g)
Grobogan soybean extract	1	0.808
	2	0.838

clonal antibodies of inducible nitric oxide synthase (1:100 dilution) or rabbit polyclonal β-catenin (1:300 dilution) for one hour at room temperature. After that, specimens were incubated with the appropriate secondary antibody for 15 minutes, followed by incubation with HRP-conjugated streptavidin for 15 minutes. Proteins visualized using 3,3' diaminobenzidine (DAB) for 2 minutes. Then, specimens were counterstained with hematoxyllin, dehydrated, cleared and mounted.

Evaluation of hematoxyllin-eosin (HE) staining

Histopathological features were scored by using Roger's criteria at 400× magnification. The severity and extent of colitis were scored according to the following criteria [21]:

Score	Criteria
0	Healthy colon
1	Minimal inflammation with minimal to no separation of crypts (generally focal affecting <10% of mucosa)
2	Mild inflammation with mild separation of crypts (generally affecting 11%-25% of mucosa or mild, diffuse inflammatory infiltrates with minimal separation of crypts)
3	Moderate inflammation with separation of crypts, with or without focal effacement of crypts (generally affecting 26%-50% of mucosa or moderate, diffuse separation of crypts)
4	Extensive inflammation with marked separation and effacement of crypts (generally affecting 51%-75% of mucosa)
5	Diffuse inflammation with marked separation and effacement of crypts (generally affecting >75% of mucosa)

Evaluation of immunohisto-chemistry staining

The total number of cells stained at each intensity and the number of cells in each field were counted. COX-2 expression in the cytoplasm of colon crypt epithelial cells was scored semi-quantitatively by using H-score assessment at 400× magnification. The percentage of positive cells was calculated using the following formula [22]:

$$S = (SSS \times SP) + (ISS \times IP) + (WSS \times WP) + (NSS \times NP)$$

S: total score
SSS: strong staining score (3)
SP: strong percentage (0-100%)
ISS: intermediate staining score (2)
IP: intermediate percentage (0-100%)
WSS: weak staining score (1)
WP: weak percentage (0-100%)
NSS: negative staining score (0)
NP: negative percentage (0-100%)

Statistical analysis

The average total scores are reported as the mean rank. Since histopathological feature score is quantitative data, the score was analyzed using the Mann-Whitney test or Kruskal-Wallis one-way analysis of variance by ranks. Immunohistochemical score was analyzed using the Tukey test or ANOVA one-way analysis of variance. A p value of less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 20.0 statistical package.

Results

HPLC analysis of lunasin

HPLC analysis of lunasin from Grobogan soybean was shown in **Figure 1** [19]. Using HPLC, it was shown that lunasin had retention at 0-5 minutes. Through two further measurements, the levels of lunasin were obtained at 0.808 and 0.838 mg/g lunasin respectively in soybean extract.

Lunasin content of the Grobogan soybean extract was measured in duplo, shown in **Table 1**, following previous studies [19].
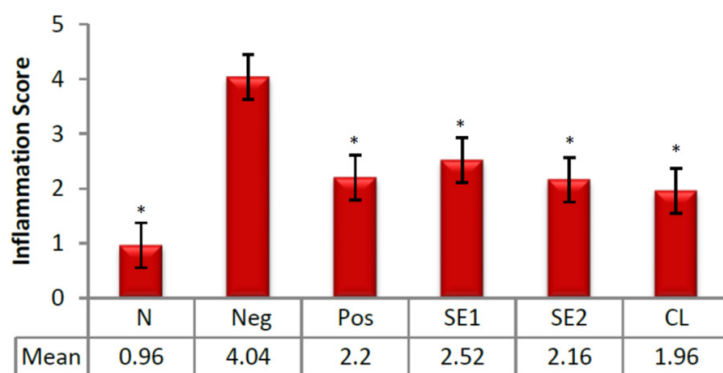
Figure 2. Mean inflammation score of mice crypt epithelial cells after induction of DSS. *P<0.05 = significantly different compared to the negative control group (n = 5 per group).

Histopathological features

Administration of lunasin at a dose of 20 mg/kg BW and 40 mg/kg BW, aspirin, and commercial lunasin can reduce the inflammatory score caused by DSS. Mann Whitney test statistical analysis proved that there were significant differences between the positive group, SE1 group, SE2 group and CL group compared with the negative control (P<0.05). However, the Mann Whitney test statistical analysis did not show a significant difference among positive group, SE1 group, SE2 group and CL group. The results of the observation of histopathological features in each group are presented in **Figure 2** below.

Effect of soybean extract administration on the COX-2 expression

Induction of DSS 2% can increase expression of COX-2 in negative group which is significantly different from normal group (P<0.05). Mann Whitney test shows statistically significant difference between the negative control group and SE1 group (P<0.05), SE2 group (P = 0.05), CL group (P = 0.05). This shows that expression of COX-2 caused by DSS 2% can be decreased by lunasin in soybean extract. Mann Whitney test does not show statistically significant difference in COX-2 expression score among the treatment groups. The effect of soybean extract administration on the COX-2 expression is shown in **Figures 4** and **5**.

Discussion

Lunasin is one of the peptides found in soybeans. Previous research has proven that luna-sin from soybeans has various clinical effects including anti-inflammatory, antioxidant, anti-cancer and so on [23]. Based on this, a study was conducted which aimed to determine the effect and activity of lunasin active compounds in soybeans to prevent the onset of colon cancer by chronic inflammatory diseases of the colon, commonly called inflammatory bowel disease (IBD). Colon cancer which is initiated by an inflammatory disease is often called colitis-associated colon cancer (CAC).

The dose of lunasin used in this study was 20 mg/kg BW and 40 mg/kg BW. According to Dia et al, [24] lunasin at a dose of 20 mg/kg BW given orally in an *in vivo* model can reduce markers in colon cancer metastasis.

In this study, the Swiss Webster mice were induced by DSS to induce inflammation in the colon. Trivedi et al's study [25] showed that DSS can significantly increase various inflammatory markers such as NF-κB, COX-2 iNOS, IL-6, TNF-α and PGE2 in plasma.

Histopathological observation with Hematoxilin-Eosin staining was done to see the level of damage to the colon tissue caused by inflammation showed in **Figure 3**. Tissue damage was assessed acco-rding to Rogers's scoring criteria [21]. Observations showed that the 2% DSS-induced group with MW 60,000 (60 kDa) produced significantly different inflammatory features. These results are based on research conducted by Perse et al, [20] who reported that the molecular weight of DSS was a very important factor in inducing colitis. The highest level of inflammation in colitis was experienced by Balb/c mice given DSS with BM 40,000 (40 kDa), mice given DSS with molecular weight (MW) 5000 (5 kDa) experienced moderate inflammation, while mice given DSS MW 500,000 (500 kDa) have mild inflammatory lesions in mice colon tissue. Histopathological features of DSS-induced colitis describe the incidence of IBD in humans. Colitis can be induced by giving 2-5% DSS for 4-9 days or with a lower concentration given 4 cycles periodically [26].
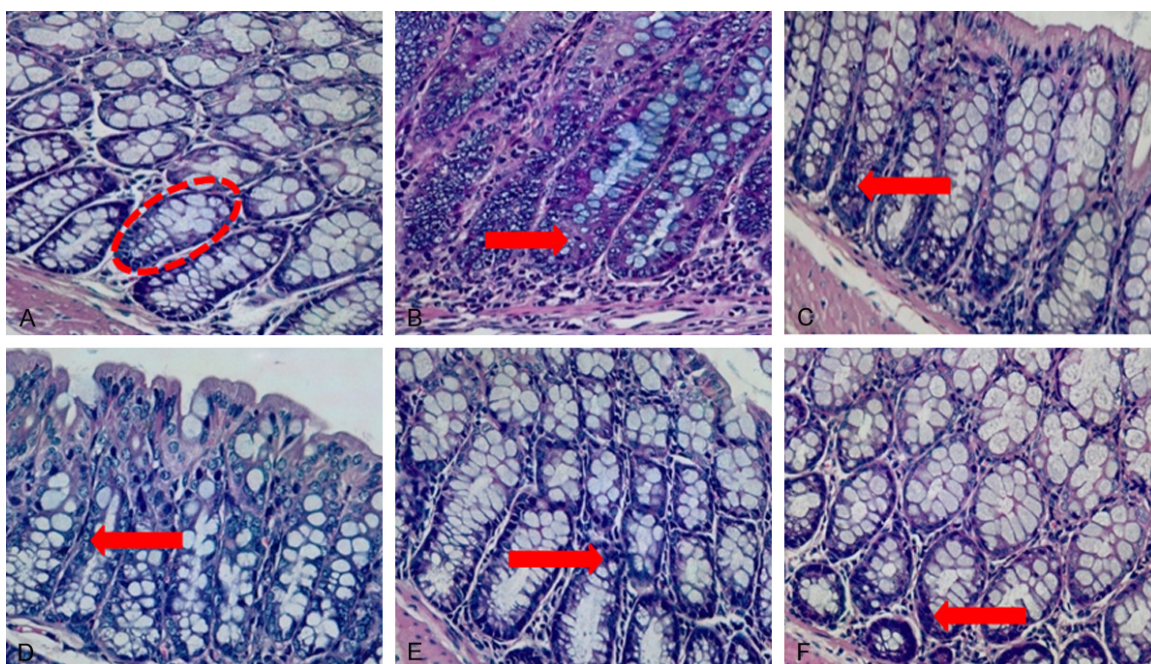
Figure 3. Histopathological features in colorectal epithelial cell of mice (magnification ×400). (A) Normal group, (B) Negative group, (C) Positive group (Aspirin), (D) SE1 (lunasin 20 mg/kg BW), (E) SE2 (lunasin 40 mg/kg BW), (F) CL (Commercial Lunasin). The red circle shows crypta and red arrows show inflammation.
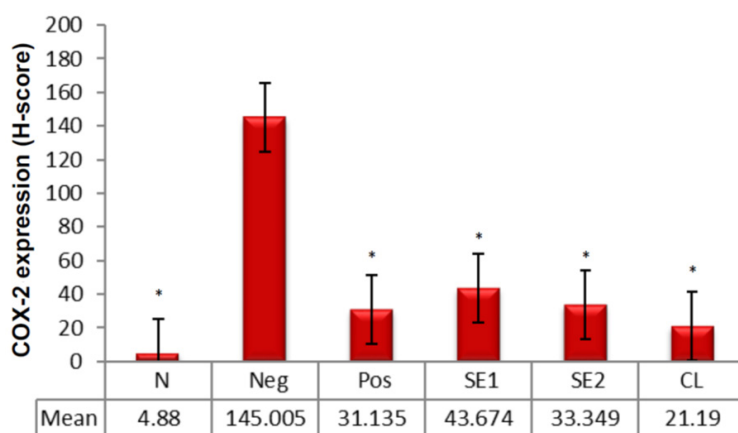

	N	Neg	Pos	SE1	SE2	CL
Mean	4.88	145.005	31.135	43.674	33.349	21.19

Figure 4. Mean COX-2 expressions score of mice crypt epithelial cells after induction of DSS. *$P < 0.05$ = significantly different compared to the negative control group (n = 5 per group).

which causes inflammation in the colon [28].

The treatment group which was given commercial lunasin at a dose of 16.25 mg/kg BW was better at reducing the inflammatory score than the lunasin of soybean extract at a dose of 20 mg/kg BW and a dose of 40 mg/kg BW. This can be caused by the purity of the commercial lunasin which is better and has been tested compared to lunasin in the Grobogan variety soybean extract which still needs further isolation and purification processes.

The results of histopathological observation showed that the normal group that was not treated also experienced inflammation with a score of 0.98. This can be caused by environmental factors such as food and drinking water that are thought to trigger inflammation in the colon [27, 28]. A study has shown that high-fat foods can increase the risk of IBD through modulation of toll-like receptors in macrophages [27]. In addition, water with high iron levels can also trigger IBD by stimulating bacterial growth

Lunasin from soybean extract at a dose of 20 mg/kg body weight and a dose of 40 mg/kg BW can reduce the inflammatory score of the DSS-induced mice group. This is consistent with observations of de Mejia et al [29] who reported that lunacin from soy can inhibit inflammation through the NF-κB pathway thereby reducing regulation of gene expression associated with inflammation such as IL-6, TNF-α, COX-2, iNOS, PGE2 and NO. Lunasin can inhibit the translocation of p50 and p65 subunits in
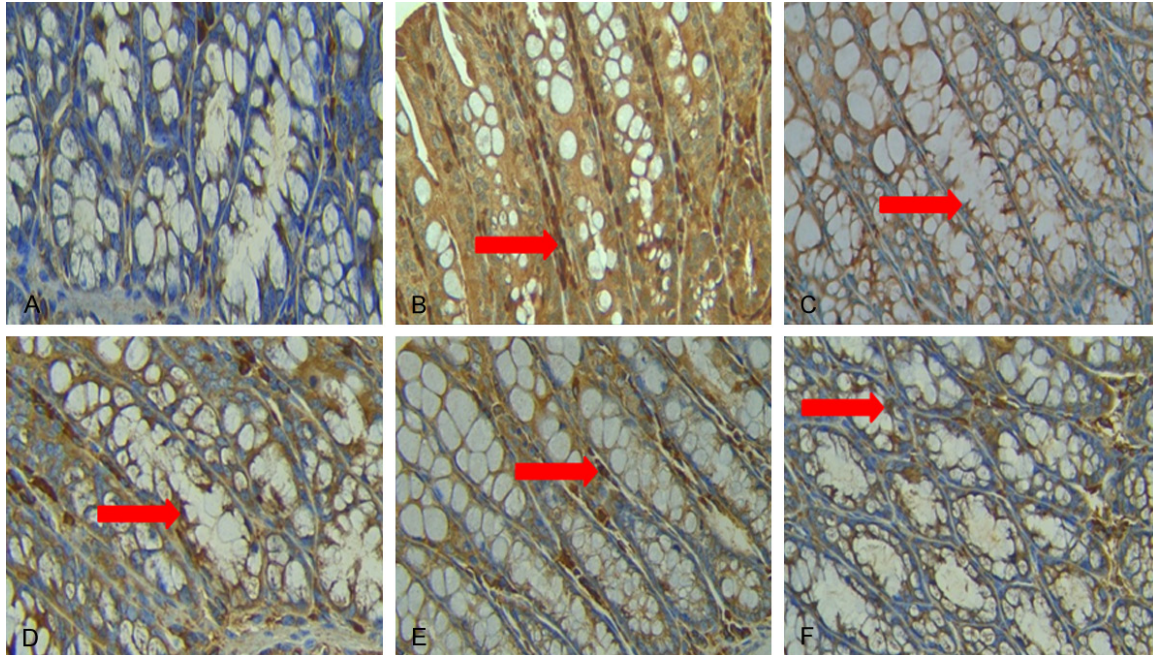
Figure 5. Expressions of COX-2 in colorectal epithelial cell of mice (magnification ×400). (A) Normal group, (B) Negative group, (C) Positive group (Aspirin), (D) SE1 (lunasin 20 mg/kg BW), (E) SE2 (lunasin 40 mg/kg BW), (F) CL (Commercial Lunasin). The red arrows show COX-2 expression in cytoplasm of colorectal epithelial cell.

NF-κB in the cell nucleus thereby reducing the binding of NF-κB to its DNA target and inhibiting gene transcription of proinflammatory molecules [29].

Immunohistochemical observations showed that COX-2 expression was highest in the negative control group. Various studies, [31, 32] have shown that induction of colon cancer in animal models trying to use DSS can increase COX-2 expression. DSS can cause damage to the epithelial mucosal barrier function, which allows the entry of bacteria and antigens into the colonic mucosa, and causes a severe inflammatory response [32]. Inflammatory cells can induce the COX-2 enzyme which plays a role in converting arachidonic acid to prostaglandin E2 (PGE2) which is a mediator most important in the inflammatory response.

Lunasin in soybean extract at a dose of 20 mg/kg BW and 40 mg/kg BW was proven to significantly reduce the level of COX-2 expression induced by DSS, but not as good as commercial saliva and aspirin. The decrease in COX-2 expression is probably caused by lunasin in soybean extract which acts as an anti-inflammatory agent. In accordance with research conducted by de Mejia et al, [29] reported that lunasin contained in soy can reduce COX-2 and vascular expression and stimulate prostaglandin production.

Conclusion

In conclusion, we have demonstrated that the administration of lunasin in the form of soybean extract in mice induced dextran sodium sulfate can inhibit inflammation by reducing expression of COX-2.

Acknowledgements

Disclosure of conflict of interest

None.

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