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Original Article

EFFECT OF LUNASIN-RICH SOYBEAN EXTRACT UPON TNF-α EXPRESSION ON COLONIC EPITHELIAL CELLS OF MICE INDUCED BY AZOXYMETHANE/DEXTRAN SODIUM SULFATE

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ABSTRACT

Objective: Colorectal cancer (CRC) contributes to 9.7% of all cancer, and its pathogenesis is related to chronic inflammation. However, current cancer therapy options are lacking, and peptide in food has become popular among researchers because it is cheap, easy to get, has a low toxicity, and is a promising cancer-preventing agent. This research aimed to investigate whether lunasin from soybeans can reduce the expression of pro-inflammatory cytokine TNF-α in colonic epithelial cells.

Methods: Thirty Swiss Webster mice were randomly allocated to six groups. One group was normal, and in five groups, carcinogenesis was induced using azoxymethane (AOM) and dextran sodium sulfate (DSS). The mice were then given nothing (negative control), aspirin (positive control), and lunasin-rich soybean extract (LSE) in three different doses (250, 300, and 350 mg/kgBW) for four weeks. Distal colon tissue was immunohistochemically stained and then observed under a light microscope with 400X magnification to count epithelial cells, based on their color. The index was calculated using optical density scores.

Results: LSE was shown to decrease the expression of tumor necrosis factor (TNF)-α. This decrease was statistically significant between the negative control and a dose of 300 mg/kgBW (p=0.016) and 350 mg/kgBW (p=0.009), yet it was not significant with a dose of 250 mg/kgBW (p=0.754).

Conclusion: a dose of 300 mg/kgBW or higher of LSE can reduce the expression of TNF-α.

Keywords: Azoxymethane, Colonic epithelial cell, Dextran sodium sulfate, Lunasin, Tumor Necrosis Factor-alpha

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INTRODUCTION

Colorectal cancer (CRC) is the third most common type of cancer in men and the second most common type of cancer in women, contributing to 9.7% of all cancer worldwide [1, 2] In 2012, 746,000 men and 614,000 women were diagnosed CRC, and 694.000 people died from CRC [2]. Its incidence is correlated with genetic factors and lifestyle, such as an increased intake of red and processed meat, a high-fat diet, a decreased intake of high-fiber food (e. g., vegetables and fruits), high alcohol intake, and smoking. This can lead to chronic inflammation, which plays major role in CRC pathogenesis [3, 4].

The high prevalence of cancer and cost of its treatment have amplified the importance of CRC screening and the promotion of a healthy lifestyle. Although the incidence of CRC decreased by 34% from 2000 to 2014 in the>50 age group, there was an increase of 13% in the<50 age group during the same period [5]. In addition, current cancer therapies, such as surgery, radiation, chemotherapy, and immunotherapy are far from flawless; radiation and chemotherapy have a low therapeutic index and high toxicity, and sometimes, tumor cells can grow back. Meanwhile, immunotherapy shows toxicity and autoimmunity, and its effects quickly disappear. Therefore, innovative therapeutic strategies are needed to reduce the risk of cancer and modify tumors by utilizing knowledge in tumor biology and tumor-host interactions [6].

In recent years, peptides and proteins in foods are gaining attention as a cancer adjuvant therapy. Unlike drug molecules, peptides have a higher affinity and specificity to a target organ, a lower toxicity, and better penetration in tissue [7]. Therefore, there is a possibility of using proteins and peptides as cancer prevention methods in the phases of initiation, promotion, and progression [8]. One interesting peptides for this purpose is lunasin, a peptide consisting of 43 amino acids isolated from soybeans. One of its anticancer mechanisms is its anti-inflammatory activity, which it does by suppressing nuclear factor-kappa beta (NF-κB) [9] NF-κB activation increases inflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α, which are proven in cancer pathogenesis. In an *in vitro* study, the administration of lunasin for 24 h on inflammatory-induced macrophage using lipopolysaccharide (LPS) showed that lunasin significantly reduced IL-6 and TNF-α, as well as reactive oxygen species (ROS) [10].

Until now, there has been no *in vivo* research focused on the immune system in cancer carcinogenesis that observes TNF-α expression after the administration of lunasin from soybean extract. Therefore, in this paper, we use animal models that were induced with colorectal carcinogenesis using dextran sodium sulfate (DSS) and azoxymethane (AOM). Lunasin was administered orally to measure the expression of TNF-α on colonic epithelial cells.

MATERIALS DAN METHODS

Lunasin extraction

Grobogan soybean variety, obtained from Indonesia Legumes and Tuber Crops Research Institute in Malang, East Java, Indonesia, was used in this study because it is one of the superior varieties and has the highest protein content. The soybean was pressed to separate its oil and obtain the residue, which is in the form of a powder. The residue was macerated with phosphate buffer saline (1:5) for 60 min, then filtrated thrice. The procedure of maceration was based on that of previous studies [11, 12]. The filtrate was dried in an evaporator at 50 °C. The concentration of lunasin in soybean extract was determined using high-performance liquid chromatography (HPLC).

Animals

Thirty male Swiss Webster mice (12 w old and±25 g body weight) were supplied by the Agency for Health Research and Development, Ministry of Health, of the Republic of Indonesia. The mice were acclimatized for one week in the Pathological Anatomy Laboratorium of the Faculty of Medicine, University of Indonesia,

under controlled temperature (25 °C), humidity (55%), and light (12 h/12 h light/dark cycle), and with free access to food and drink. Their physical condition was examined, and only the healthy ones were chosen for use in the experiment.

Colorectal carcinogenesis induction

The method for the induction of colorectal carcinogenesis was adopted from Kusmardi *et al.* [13]. Mice were given a single intraperitoneal (i. p.) injection of 10 mg/kgBW azoxymethane (AOM), diluted in 0.9% NaCl, early in week one. Throughout the second week, the mice were given food and drink containing dextran sodium sulfate (DSS) at 2%, daily.

Experimental group

Mice were randomly allocated to six experimental groups, as follows: the normal group, in which mice did not receive AOM/DSS but only received oral administration of physiological saline for 4 w; the negative control group, in which mice were given AOM/DSS followed by oral administration of physiological saline for 4 w; the positive control group, in which mice were given AOM/DSS, followed by oral administration of 150 mg/kgBW aspirin for 4 w; and the lunasin-rich soybean extract (LSE) treatment group, in which mice received AOM/DSS, followed by the oral administration of LSE (250, 300, or 230 mg/kg BW) for 4 w (fig. 1). At the end of the week, all the mice were sacrificed.

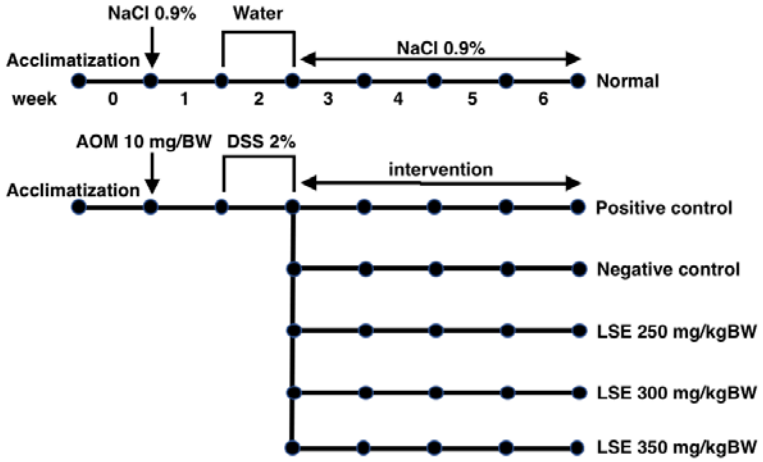

Fig. 1: Experimental groups for the colorectal carcinogenesis model

Immunohistochemistry

Distal colorectal tissue obtained from the mice was washed with water and fixed in buffered formalin 10%. The tissue was embedded in paraffin block, then cut into 4 µm-thick sections. Sections were incubated with primary (anti-TNF-α) antibodies (1:100 v/v) at room temperature for one hour. After a 10-minute wash, the samples were incubated with secondary antibodies at room temperature for 15 min. TrekAvidin-HRP was added to the samples and incubated at room temperature. After two 15-minute washes, chromogen (diamino benzidine) was added to the samples, and the reaction took 30 seconds. The samples were washed with water, soaked in lithium carbonate solutions for one minute, and washed with water again. They were then dehydrated with ethanol.

Quantification of TNF-α expression

The samples were observed under light microscope (400X magnification), and five fields of view were randomly taken of each of the samples. Epithelial cells that did not express TNF-α in their cytoplasm appeared blue (negative), while cells that expressed TNF-α appeared brown (positive). The intensity of this brown color was differentiated into three classes: high, moderate, and low positive (fig. 2). The number of cells in each group was transformed into a percentage (%). The index of TNF-α expression was calculated using optical density score, as follows: (4*%high positive)+(3*%moderate positive)+(2*%low positive)+(1*%negative).

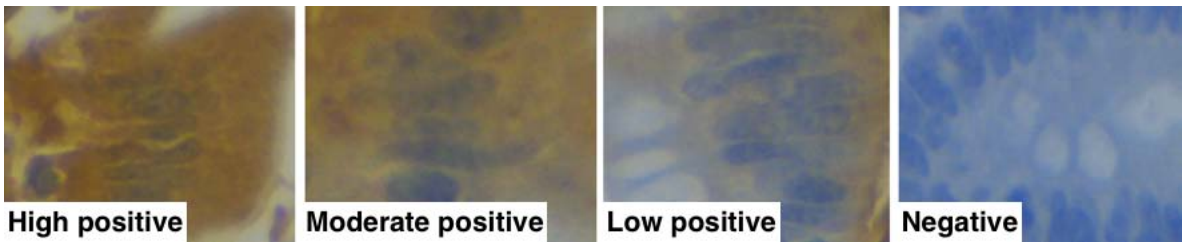

Fig. 2: Intensity difference of cells that expressed TNF-α under light microscope (400X magnification)

Statistical analysis

Data were analyzed using SPSS version 23.0 for Mac. Data is presented as mean±standard deviation. Because the distribution of data was normal, yet the variance was not homogenous, differences in the index expression of TNF-α among the groups were assessed using Kruskal–Wallis and Mann–Whitney U tests. A p value of<0.05 was considered statistically significant.

RESULTS

The more TNF-α was expressed in the cytoplasm of colonic epithelial cells, the greater intensity of the brown color that appeared after immunohistochemical staining was. It was seen that the greatest intensity of brown color occurred in the negative control group, while the lowest intensity was found in the normal and LSE 350 mg/kg BW groups. In the LSE group, TNF-α expression was decreased progressively, as shown in D, E, and F of fig. 3.

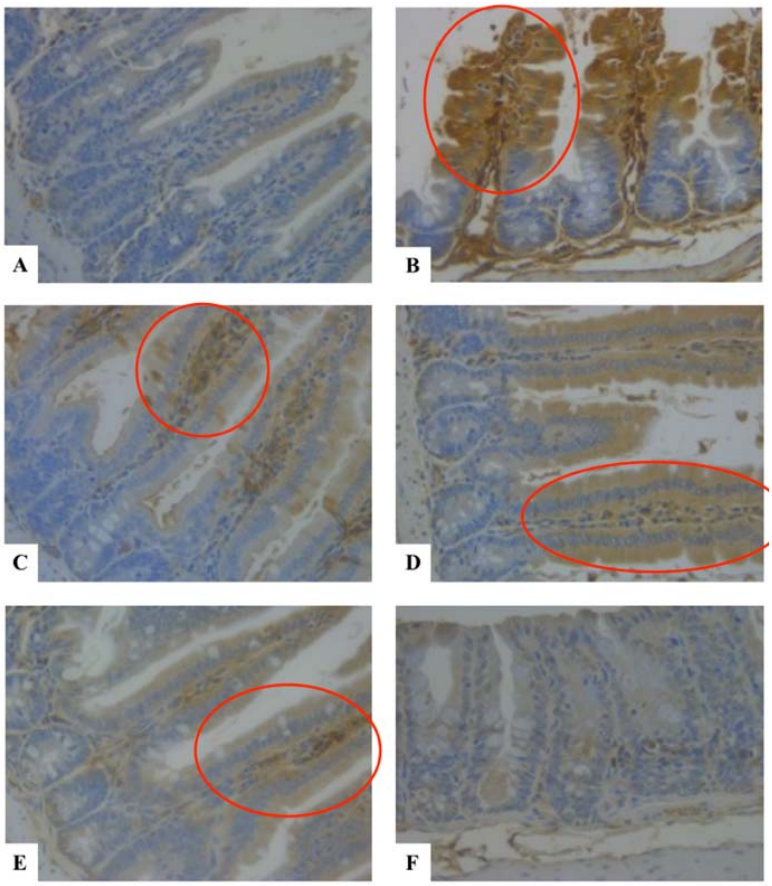

Fig. 3: Expression of TNF-α in colonic epithelial cells after immunohistochemical staining (400X magnification). Normal (A); negative control (B); positive control (C); LSE-250 (D); LSE-300 (E); LSE-350 (F)

A statistically significant difference was seen in the index expression of TNF-α between the normal and negative control (p=0.009), LSE-250 (p=0.028), and LSE-300 (p=0.009) groups; between the negative control and positive control (p=0.009), LSE-300 (p=0.016), and LSE-350 (p=0.009) groups; between the positive control and LSE-300 (p=0.028) group; between the LSE-250 and LSE-350 (p=0.047) groups; and between the LSE-300 and LSE-350 (p=0.009) groups.

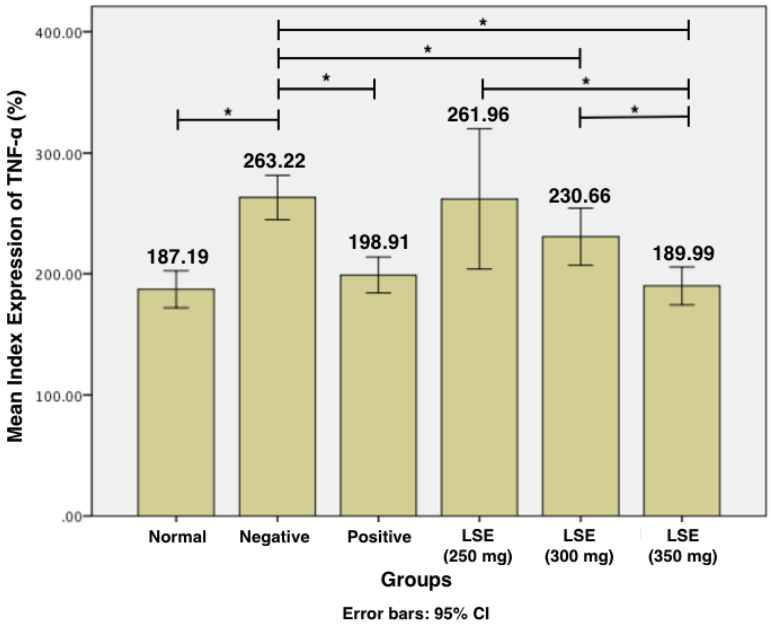

Fig. 4: Mean index expression of TNF-α (p=0.001); *p<0.05 is statistically significant

DISCUSSION

Colorectal carcinogenesis with AOM/DSS

Chronic inflammation, as occurs in inflammatory bowel disease (IBD), has a role in carcinogenesis [14, 15]. Okayasu *et al.*15 simulated colorectal carcinogenesis as it occurs in humans by injecting mice once with 7.4 mg/kgBW of AOM and giving them a drink containing 3% DSS for four cycles to trigger chronic ulcerative colitis. Several colon mucosal tumors, the majority of which were seen in the left colon (descending, sigmoid, and rectum) and transverse colon, were found in the AOM/DSS group. AOM demonstrates genotoxic (i.e., mutagenic) activity which is ideal to initiate carcinogenesis. Meanwhile, DSS creates chronic inflammation (colitis). Thus, both AOM and DSS play a role in the initiation and progression of carcinogenesis in animal models [16]. In this study, we attempted to mimic colorectal carcinogenesis with AOM/DSS as well, but with the dosage and method adopted by Kusmardi *et al.* [17].

Cytokines are produced due to carcinogen stimuli, infections, and inflammation. Among cytokines, TNF-α is the main mediator of inflammation [14]. The focus of this study was to assess TNF-α expression in colonic epithelial cells. TNF-α is a pro-inflammatory cytokine not only produced by inflammatory cells but also by other cells, including epithelial cells [18]. The results of this study indicate that there are significant differences in TNF-α expression between the normal and AOM/DSS-induced (negative control) groups. This suggests that AOM/DSS successfully creates an inflammatory condition so that TNF-α increases, as evidenced by the dark brown epithelial cells that were observed.

The activation of the NF-κB pathway can explain why the expression of TNF-α increases during inflammation. NF-κB is a transcription factor found in cytoplasm that plays a role in inflammation and carcinogenesis. In unstimulated conditions, NF-κB is in an inactive state because it binds to the protein inhibitor κB (IκB) [14, 18]. NF-κB can be activated if IκB becomes dissociated when there are stimuli, such as IL-1, TNF-α, LPS, DNA damage, double-stranded RNA (RNAds), cyclic adenosine monophosphate (cAMP), toll-like receptor (TLR) due to tissue damaged by microorganisms, hypoxia, and solid tumors. NF-κB regulates genes associated with inflammation, one of which is TNF-α, as well as proliferation; angiogenesis; survival; and tumor promotion and metastasis [14, 18]. Rogler *et al.* [19]. Observed the results of the biopsy of human intestinal mucosa that was undergoing inflammation and found that activated NF-κB was not only found in macrophages but also in epithelial cells.

Although not given AOM/DSS, the colonic epithelial cytoplasm in the normal group was not completely blue (indicating a negative expression of TNF-α) but instead tended to be light brown in intensity. Considering that NF-κB can be activated by microorganisms through TLR, it is possible that microbiota (bacteria) in the mice's colon stimulates the activation of NF-κB so that TNF-α is formed in small amounts.

Aspirin therapy in colorectal carcinogenesis models

This study used a dose of 150 mg/kgBW of aspirin, according to previous studies by Amalia *et al.* [17]. In this study, no significant differences in TNF-α expression were seen between the aspirin (positive control) group and the normal group, but a significant difference was seen between the positive control group and negative control group. This suggests that aspirin succeeded in reducing the amount of TNF-α in epithelial cells that had undergone colorectal carcinogenesis. Moreover, the TNF-α expression was similar to that of the normal group.

Some studies have proven the relationship between aspirin use and a reduced risk of cancer. Low-dose aspirin (i.e., 81–160 mg) can reduce the risk of developing an adenoma [20]. However, the mechanism by which aspirin prevents CRC is still unknown. There are several possible mechanisms, including cyclooxygenase (COX)-dependent pathways and COX-independent pathways. In COX-dependent pathways, aspirin and its metabolites (sodium salicylate) can permanently inactivate COX-1 and COX-2, resulting in apoptosis and the inhibition of angiogenesis. Meanwhile, in COX-independent pathways, aspirin or salicylates inhibit the activity of IκB kinase (IKK) to prevent NF-κB activation, both *in vivo* and *in vitro* [20, 21]. As a result of NF-κB inhibition, TNF-α expression decreased in the positive control group.

LSE therapy in colorectal carcinogenesis model

After mice had undergone colorectal carcinogenesis, mice were treated with LSE at three different doses (250, 300, and 350 mg/kgBW). These doses were determined based on previous studies by Amalia *et al.*, [17]. Which showed that soybean extracts at doses of 150 and 200 mg/kgBW were able to increase apoptosis and significantly reduce dysplasia in the colon against negative controls.

Soybeans were chosen because they have the highest lunasin content among other sources, at about 0.5–8.1 mg/g [22]. Lunasin content in this study was 0.823 mg/g of soybean extract, which falls within the projected range.

The LSE-250 group did not show significant differences in TNF-α expression with negative controls. However, at higher doses (i.e., LSE-300 and LSE-350), significant differences with negative controls were observed. The higher the LSE doses was, the lower the TNF-α expression was, indicating a dose-dependent effect. A study by Hernández-Ledesma *et al.* [10] proved that the inhibitory effect of TNF-α on RAW 64.7 macrophage cells, in which inflammation was induced using LPS, was greater as the dose of lunasin increased. At 200 μM lunasin, TNF-α decreased by 23%. Previous studies by de Mejia *et al.* [23]. showed that lunasin derived from soybeans can inhibit the inflammatory process through the NF-κB pathway.

LSE-350 reduced the TNF-α expression significantly when compared to the negative control group but insignificantly when compared to the normal group. The result of immunohistochemical staining between these two groups was almost identical. This indicates that LSE-350 mg/kgBW can restore TNF-α expression to resemble that of the normal group.

Although the results of LSE therapy showed the restoration of TNF-α expression, this result does not rule out the contribution of other contents in the soybean extract, which could also work as an anti-inflammatory, such as isoflavones. Isoflavones have been known for their use in the treatment of hormone-related cancers, such as breast, cervical, and prostate cancers, by inducing apoptosis through mitochondrial pathways and NF-κB [9].

CONCLUSION

LSE inhibits TNF-α expression in the colonic epithelium of Swiss Webster mice in which carcinogenesis has been induced with AOM/DSS, with significant inhibition at doses of 300 and 350 mg/kgBW. The inhibition of TNF-α expression increases as the dose of LSE increases (i.e., in a dose-dependent manner). At a dose of 350 mg/kgBW, TNF-α expression is very similar to that of the normal group.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

There are no conflicts of interest to declare

REFERENCES

1. World Health Organization. Cancer. Available from: http://www.who.int/mediacentre/factsheets/fs297/en/ [Last accessed on 04 Jan 2018]
2. GLOBOCAN. Estimated cancer incidence, mortality and prevalence in. International Agency for Research on Cancer:

WHO; 2012. Available from: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx [Last accessed on 02 Dec 2012]

3. Tuan J, Chen YX. Dietary and lifestyle factors associated with colorectal cancer risk and interactions with microbiota: fiber, red or processed meat and alcoholic drinks. Gastrointest Tumors 2016;3:17–24.
4. Johnson CM, Wei C, Ensor JE, Smolenski DJ, Amos CI, Levin B, *et al.* Meta-analyses of colorectal cancer risk factors. Cancer Causes Control 2013;24:1207–22.
5. Siegel RL, Miller KD, Fedewa SA, Ahnen DJ, Meester RGS, Barzi A, *et al.* Colorectal cancer statistics. CA Cancer J Clin 2017;67:177–93.
6. Bhutia SK, Maiti TK. Targeting tumors with peptides from natural sources. Trends Biotechnol 2008;26:210–7.
7. Galvez AF, Chen N, Macasieb J, de Lumen BO. Chemopreventive property of a soybean peptide (lunasin) that binds to deacetylated histones and inhibits acetylation. Cancer Res 2001;61:7473–8.
8. Mejia EGD, Dia VP. The role of nutraceutical proteins and peptides in apoptosis, angiogenesis, and metastasis of cancer cells. Cancer Metastasis Rev 2010;29:511–28.
9. Aluko RE. Functional foods and nutraceuticals. 1st ed. New York (NY): Springer; 2012. p. 92–5.
10. Hernández Ledesma B, Hsieh C, Lumen BO. Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. Biochem Biophys Res Commun 2009;390:803–8.
11. Seber LE, Barnett BW, McConnell EJ, Hume SD, Cai J, Boles K, *et al.* Scalable purification and characterization of the anticancer lunasin peptide from soybean. PLoS One 2012;7:e35409.
12. Dia VP, Wang W, Oh VL, de Lumen BO, de Mejia EG. Isolation, purification and characterisation of lunasin from defatted soybean flour and *in vitro* evaluation of its anti-inflammatory activity. Food Chem 2009;114:108–15.
13. Kusmardi, Priosoeryanto B, Harlina E, Cornain S. Inhibition activities of fish oil in iNOs, COX-2, and β-Catenin expressions in colorectal preneoplasia of mice induced by azoxymethane and dextran sodium sulfate. J Appl Biotechnol 2014;2:91.
14. Costa D, Carvalho J. Molecular link mechanisms between inflammation and cancer. Curr Pharm Des 2012;18:3831–52.
15. Okayasu I, Ohkusa T, Kajiura K, Kanno J, Sakamoto S. Promotion of colorectal neoplasia in experimental murine ulcerative colitis. Gut 1996;39:87–92.
16. Parang B, Barrett CW, Williams CS. AOM/DSS model of colitis-associated cancer. Methods Mol Biol 2016;1422:297–307.
17. Amalia AW, Kusmardi, Elya B, Arsianti A. Inhibition of carcinogenesis by seed and soybean meal extract in colon of mice: apoptosis and dysplasia. Asian J Pharm Clin Res 2017;10:123.
18. Monteleone G, Pallone F, Stolfi C. The dual role of inflammation in colon carcinogenesis. Int J Mol Sci 2012;13:11071–84.
19. Rogler G, Brand K, Vogl D, Page S, Hofmeister R, Andus T, *et al.* Nuclear factor κB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. Gastroenterology 1998;115:357–69.
20. Garcia Albeniz X, Chan AT. Aspirin for the prevention of colorectal cancer. Best Pract Res Clin Gastroenterol 2011;25:461–72.
21. Alfonso L, Ai G, Spitale RC, Bhat GJ. Molecular targets of aspirin and cancer prevention. Br J Cancer 2014;111:61–7.
22. Liu J, Jia S, Kirberger M, Chen N. Lunasin as a promising health-beneficial peptide. Eur Rev Med Pharmacol Sci 2014;18:2070–5.
23. de Mejia EG, Dia VP. Lunasin and lunasin-like peptides inhibit inflammation through suppression of NF-κB pathway in the macrophage. Peptides 2009;30:2388–98.