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Original Article

INHIBITION OF COX-2 EXPRESSION BY LUNASIN-RICH SOYBEAN EXTRACT ON COLORECTAL CANCER

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ABSTRACT

Objective: The incidence of colorectal cancer has been growing faster than most other cancers in the past decade, especially in developing countries. One of the substances that is currently being investigated as potential chemopreventive agent is lunasin, which is contained in soybeans. This research explored the effect of lunasin on COX-2 expression in the distal colons of mice in which colorectal carcinogenesis was induced with azoxymethane (AOM) and dextran sodium sulfate (DSS).

Methods: A total of 30 Swiss Webster mice were separated into six groups. In five of the groups—a negative control group, positive control group, and three intervention groups—carcinogenesis was induced with AOM and DSS; the sixth group received no interventions. Lunasin-rich soybean extracts were given in doses of 250, 300, and 350 mg/kgBW for 6 w to the intervention groups. Immunohistochemical staining of COX-2 was then carried out on tissue samples from the distal colons of mice that had been sacrificed. The samples were microscopically assessed and photographed, and cell counts were performed using the *Image J* application. COX-2 expression is reported in the form of an optical density score (ODS).

Results: Significant differences between the negative control and the intervention groups were found at the 300 mg/kgBW ($p = 0.047$) and 350 mg/kgBW ($p = 0.016$) lunasin dosage levels.

Conclusions: This demonstrates that administration of lunasin-rich soy extracts can inhibit COX-2 expression in cryptic epithelial cells of the distal colon in mice with carcinogenesis induced by AOM and DSS.

Keywords: Azoxymethane, Colorectal cancer, COX-2, Dextran sodium sulfate, Lunasin

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INTRODUCTION

Colorectal cancer is the third most common cancer in men (746,000 cases) and the second in women (614,000 cases) worldwide [1]. Although 55% of colorectal cancer events occur in developed countries, the most rapid increases in the incidence of colorectal cancer are estimated to be occurring in developing countries. This is due to changes in lifestyle and eating patterns that are increasingly widespread, such as obesity, lack of physical activity, smoking, lack of consumption of fibrous foods, and increased consumption of alcohol [2].

Conventional treatments of colorectal cancer such as surgery, radiation therapy, chemotherapy, and immunotherapy have not been able to give desirable results. Surgical management is reported to have a risk of post-operative complications. Radiation therapy is reported to have a risk of increasing colorectal cancer recurrence [3, 4]. Chemotherapy, which works by damaging cancer cells, is also reported to damage healthy cells if given for a long time [5].

Currently, nutritional therapy is being intensively studied for the treatment of colorectal cancer. The goal of this therapy is to prevent cancer and inhibit cancer progression. Soybeans are epidemiologically proven to be beneficial to health, especially in reducing the risk of cardiovascular and cancer diseases [6]. Several studies show that the consumption of soybeans can suppress tumor growth in various tissues, such as the skin, mammary glands, bladder, and prostate [7].

The active substance in soybeans that has the potential to become a chemopreventive agent is lunasin. Lunasin is a polypeptide, composed of 43 amino acids, that has antioxidant, antihypertensive, anti-inflammatory and anticancer effects. Lunasin contains eight negatively charged Asp (D) residues on the carboxyl end, which provide a histone binding site that allows it to act as a potent inhibitor of the positively charged histone acetylations H3 and H4. In addition, the Arg-Gly-Asp (RGD) motive in lunasin also plays a role in its internalization into mammalian cells. This RGD motive can induce cell apoptosis through caspase mechanisms that are associated with cell growth and proliferation, maintenance of cell function, and cell interaction [8, 9]. In addition, there is a putative helical region in lunasin which is thought to increase its ability to bind to chromatin [10].

Studies on tumors treated with lunasin also showed a significant decrease in cell proliferation accompanied by tumor cell apoptosis induction.[8] Histologic staining in samples of tumors treated with lunasin showed that the destructive area of the tumor was replaced by necrotic and apoptotic cells [9]. Pure lunasin isolated from Bowman-Birk inhibitor (BBI) concentrates can reduce focus formation by 73% at a concentration of 100 nM. BBI is a protease inhibitor in soybeans that can increase the bioavailability of lunasin [10].

The degree of COX-2 expression is a parameter that can be used to see the effect of lunasin on colorectal carcinogenesis. The COX-2 enzyme is found in some normal cells, but it is mainly induced by an inflammatory response or cancer stimulus. An increase in COX-2 expression is found in most colorectal cancers. The role of COX-2 in producing prostaglandins from arachidonic acid is also known to play an important role in cancer progression through angiogenesis and the increase of cell proliferation [11].

Through an investigation of soybeans, a food that is easily found in Indonesia at affordable prices and with no toxic effects, this study was designed to analyze the effect of lunasin extracted from soybeans on distal colon tissue in mice in which colorectal carcinogenesis was induced using azoxymethane (AOM) and dextran sodium sulfate (DSS).

MATERIALS AND METHODS

This study was designed as an experimental study using primary data in the form of quantification of COX-2 expression. The expression of COX-2 was observed in immunohistochemically stained preparations of distal colon tissue from mice that had been

sacrificed, specifically the cryptic epithelium cells. The preparations were obtained from stored tissue. Observations of the preparation was carried out using a microscope at 400 times magnification in five fields of view. This study was conducted at the Anatomy Pathology Laboratory of the Faculty of Medicine at Universitas Indonesia in Salemba, Jakarta, from January 2018 to October 2018.

Plant materials and extraction procedures

The soybeans used in this research were a Grobogan variety. These soybeans were obtained from the Indonesian Legumes and Tuber Crops Research Institute in Malang, East Java. Before lunasin extraction was carried out, the soybeans underwent a pressing process to remove their oil at the Herbs and Medicinal Plants Research Institute in Bogor, West Java. Following the pressing process, the dried soybeans were then blended into powder.

This soybean powder (1,250 g) was then macerated for 60 min using a phosphate buffer saline solvent, using 5 times as much solvent as soybean powder (6,250 ml) [12, 13]. Afterwards, the maceration solution was filtered 3 times with filter gauze, and the resulting extract solution was dried at 50°C using an evaporator.

Analysis of the lunasin content of each extract was carried out using high-performance liquid chromatography. The extract was diluted with distilled water and centrifuged at 12,000 rpm for 30 min. The resulting solution was first filtered through a 0.22 m membrane, then injected into a *high performance liquid chromatography* (HPLC) device equipped with a UV-Vis detector L-2420 (295 nm) in the car phase (5% acetonitrile and 95% distilled water). The analysis was carried out with a linear gradient for 35 min at a speed of 2 ml/minute. The lunasin level was identified based on retention time using the lunasin standard.

Animals

Male Swiss Webster mice (12 w old), approximately 250 grams, were obtained from the Agency for Health Research of the Development Ministry of Health of Republic of Indonesia. Mice were adapted for 1 w at the Anatomy Pathology Laboratory of the Faculty of Medicine at Universitas Indonesia. Mice were maintained and treated at a controlled temperature (25°C), humidity (55%), light-dark cycle (12 h), and were given standard food and drink according to the Guide for the Care and Use of Laboratory Animals from the Animal Care and Use Committee in United States, which applied universally. The physical health of the mice, including their levels of activity, consumption of food and drink, and weight, were examined.

Induction of carcinogenesis

The process of inducing colorectal carcinogenesis in mice was adopted from Kusmardi *et al.* [14], where mice given azoxymethane (AOM) dissolved in 0.9% NaCl through intraperitoneal (IP) injection in a single dose of 10 mg/kgBW at the beginning of the trial week. A week after the administration of AOM, mice were given food and drink containing 2% dextran sodium sulfate (DSS) every day for a week.

Treatments in experimental groups

The mice were divided into six experimental groups: the normal group, a positive control group, a negative control group, and three intervention groups. The normal group (N) represents the normal condition of the mice before the carcinogenesis. This group was not given AOM and DSS, but instead received IP injections of physiological saline for 4 w. The negative control group (KN) represents the condition of the mice after carcinogenesis, without any subsequent treatment. This group was given a single dose of AOM via IP injection followed by oral DSS for a week, then given physiological saline for 4 w. The positive control group (KP) represents the results of the most widely used treatment for inhibiting carcinogenesis. The mice in this group were given a single dose of AOM via IP injection followed by oral DSS for a week, then treated with oral aspirin (150 mg/kgBW) for 4 w. The lunasin therapy groups (L1, L2, L3) represent the results of the proposed treatments in inhibiting carcinogenesis. The mice in these groups were given a single dose of AOM via IP injection followed by oral DSS for a week, then administered lunasin-rich soybean extract orally (L1: 250 mg/kgBW; L2: 300 mg/kgBW; and L3: 350 mg/kgBW) for 4 w (fig. 1).

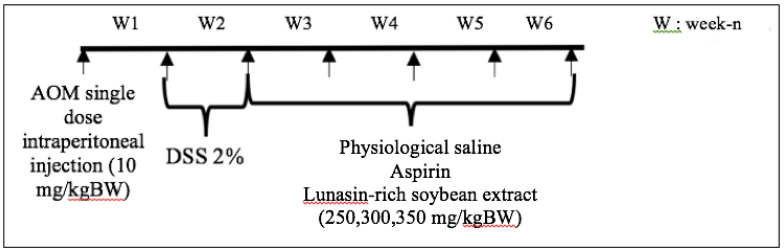

Fig. 1: Scheme of treatments in experimental groups

The minimum sample amount was calculated based on Federer's formula,

$(n-1)(k-1) \geq 15$,

With n = the number of samples in each group and k = the number of experimental groups.

$(n-1)(6-1) \geq 15$

$n-1 \geq 3$

$n \geq 4$

Based on the calculation above, the minimum number of samples in each experimental group was 4 mice, or 24 mice in total. This study, however, used 30 mice, or 5 per experimental group.

Immunohistochemistry staining

At the end of the treatment period, each mouse was anesthetized with IP injections of ketamine and xylazine, then terminated using the neck dislocation technique. Colon tissue samples obtained from the mice were made into preparations in paraffin blocks and cut across in 4 μm thick slices. Specimens were incubated with suitable secondary antibodies for 1 hour at room temperature. The incubation of the specimens was then carried out again with trekavidin conjugated with HRP for the next 30 min. Proteins were visualized using 3,3'-diaminobenzidine (DAB) for 10 min at room temperature. After that, each specimen was dipped into Lilie Mayer hematoxylin dye as a counterstain for 1–2 min, then rinsed with running water. The tissue was then dipped in lithium carbonate for 1 minute and rinsed with running water. Finally, they were dehydrated with ethanol and purified with xylol. These steps end with the closure of the specimen with aqueous mounting media. Positive control of immunohistochemical staining is liver cancer cells.

Assessment of COX-2 expression

COX-2 expression in the cytoplasm of colonic crystalline epithelial cells was assessed semiquantitatively. The bluish-colored epithelium indicates a negative result, while the brown epithelium indicates positive results. Assessment of COX-2 immunohistochemistry results was performed via a double-blind procedure. All slides were observed with a light microscope. Photos were taken at 400x magnification in five random fields of view.

Based on the color intensity, cryptic epithelial cells were divided into four groups: high positive cells, moderate positive cells, low positive cells and negative cells. High positive cells were defined as cells that contain dark brown pigments coloring all of the cytoplasm. Moderate positive cells were defined as cells that contain lighter brown pigments coloring all of the cytoplasm. Low positive cells were defined as cells that contain lighter brown pigments coloring half of the clear cytoplasms. Negative cells were defined as cells that contain slight or no brown pigments marked by cytoplasm that appear clear or bluish (fig. 2).

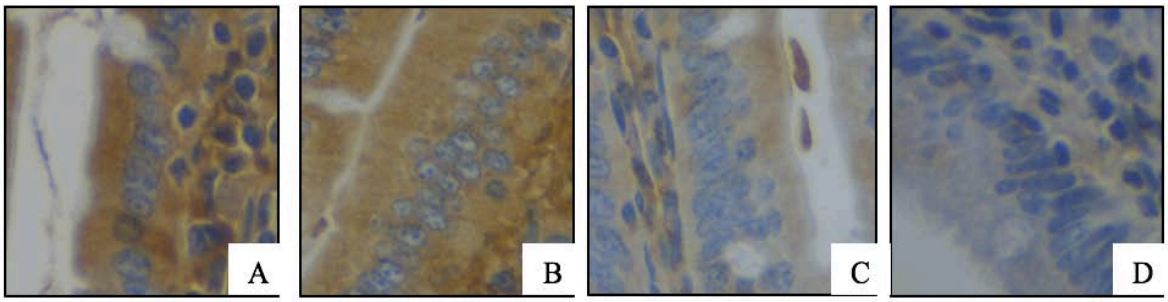

Fig. 2: Standard for determining color intensity in preparations In this figure, A–D illustrate the following: A) high positive cells, B) moderate positive cells, C) low positive cells, D) negative cells

The total number of positive and negative cells in each photo was calculated manually by marking the cryptic epithelial cells using the *Image J* application. Based on the intensity of the color, positive results were next categorized as high positive, marked with a dark blue dot; moderate positive, marked with a light blue dot; and low positive, marked with a light green dot. Negative results are marked with a purple dot. The interpretation results were quantified as optical density scores (ODS) and expressed in percentages.

Management and analysis of data

Statistical analysis of this study used SPSS for Mac, version 24.0. Because the number of samples was less than 50, we performed the Shapiro-Wilk normality test. Furthermore, the Levene test was conducted to determine the homogeneity of the variance of the data.

If the data had a normal distribution and homogenous variance, parametric variance analysis (ANOVA) was carried out to find out whether there was a significant difference between groups. ANOVA tests were used because the number of groups being compared was more than two. If there was a significant difference, the two mean analyses were done via a post hoc test to find out which groups had significant differences.

If the data were abnormally distributed, or if they were normally distributed but the variance was not homogeneous, a Kruskal-Wallis nonparametric test was used to determine the presence of significant differences between groups. If a significant difference was found, the Mann Whitney nonparametric test was conducted to identify which groups had significant differences.

RESULTS

The effect of lunasin-rich soybean extract on COX-2 expression in the cryptic epithelial cells of the distal colon tissue in mice that had colorectal carcinogenesis induced via administration of AOM and DSS is shown in table 1. The highest COX-2 expression was found in the negative control group, while the lowest expression was found in the positive control group. The decrease of COX-2 expression in the groups given lunasin-rich soybean extract was found to be dose-dependent.

Table 1: Descriptive analysis of COX-2 expression in the control groups and lunasin therapy groups

Groups	Median (Min–Max)
Normal	234.95 (228.42–264.50)
Negative control	293.38 (276.06–375.48)
Positive control	259.83 (246.85–268.96)
Lunasin 250	280.31 (270.47–301.04)
Lunasin 300	269.18 (267.79–297.19)
Lunasin 350	261.96 (247.68–277.56)

The Shapiro Wilk normality test showed that the data was not normally distributed ($p = 0.003$). Data transformation with the log function was performed to normalize the data, but the results of normality tests show that the data was still not normally distributed. Furthermore, the Levene homogeneity test indicated that the variance of the data was not homogeneous ($p = 0.028$). Because the data obtained were not normally distributed and not homogeneous, the analysis was continued with a Kruskal-Wallis nonparametric test, which determined that there were significant differences between groups ($p = 0.001$).

To find out which groups had significant differences, the Mann Whitney test was used, and showed that there were significant differences between many of the groups, in particular: the N group with KN group ($p = 0.009$); the L1 group ($p = 0.009$) and L2 group ($p = 0.009$); the KN group with the KP group (0.009); the L2 group ($p = 0.047$) and L3 group ($p = 0.016$); the KP group with the L1 group ($p = 0.009$) and L2 group ($p = 0.016$) (fig. 3).

The comparison of the immunohistochemical staining results of COX-2 expression between the control groups and intervention groups can be seen in fig. 4. Higher-intensity brown colors in epithelial cells show higher positive results produced by COX-2 expression in these cells. In the N group, most epithelial cells were clear/bluish (negative) or faint brown (low positive). In the KN group, higher intensities of brown were observed in most epithelial cells. In the KP group, low–medium intensity browns were the most common (low–medium positive). Meanwhile, in the lunasin intervention groups, there was a gradual decrease in brown color intensity as dosage increased.

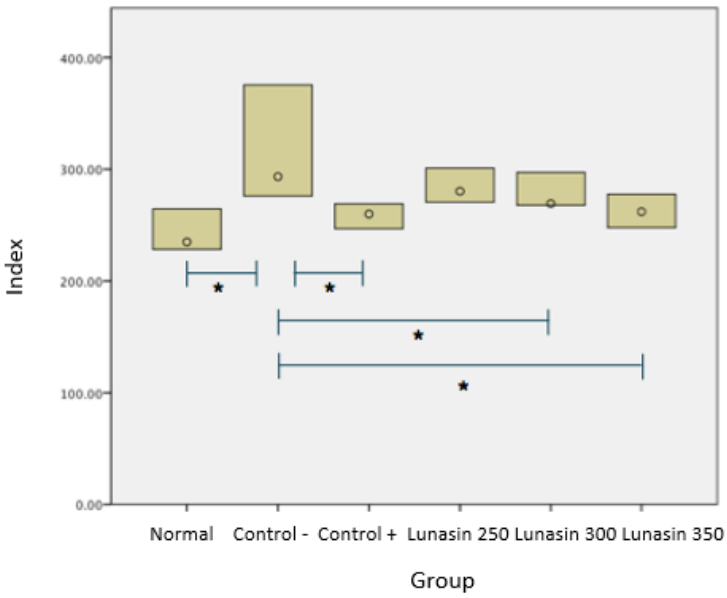

Fig. 3: Graph of descriptive analysis of COX-2 expression in the control groups and lunasin therapy group

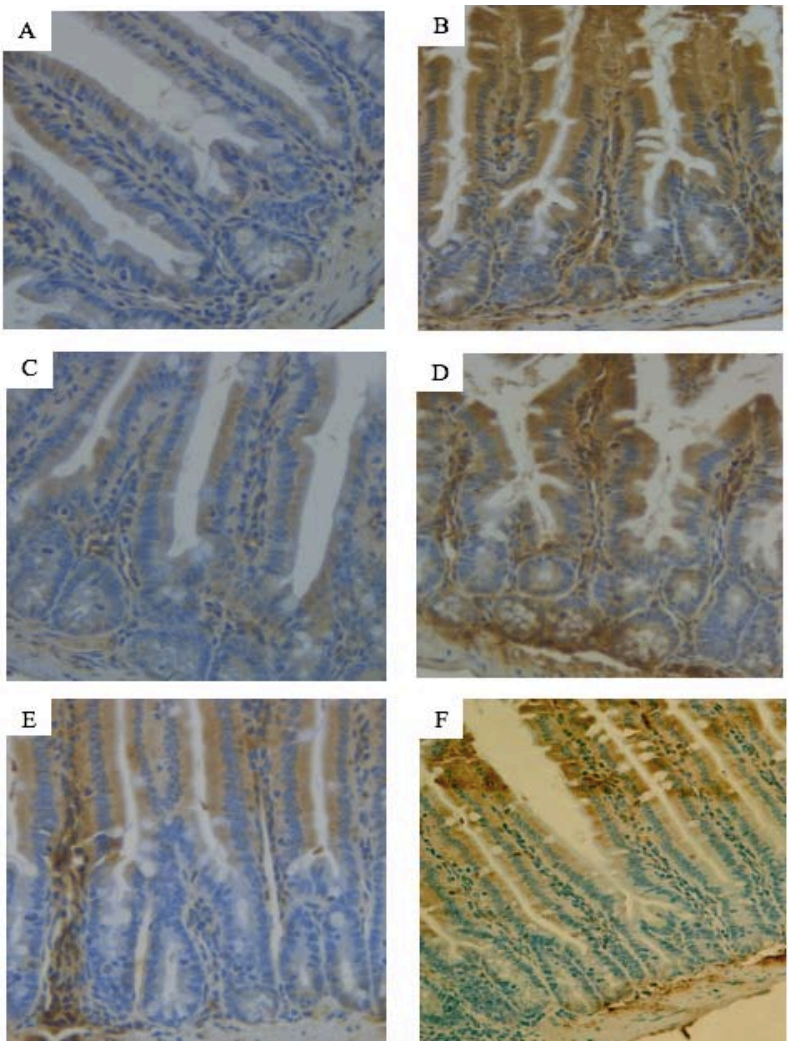

Fig. 4: Immunohistochemical staining results of COX-2 expression in each treatment group, In this fig. A–F illustrate the following: A) normal, B) negative control, C) positive control, D) lunasin dose 250 mg/kgBW, E) lunasin dose 300 mg/kgBW, F) lunasin dose 350 mg/kgBW

DISCUSSION

Based on the statistical tests that were carried out, it became clear that lunasin-rich soybean extract had a significant effect on COX-2 expression at doses of 300 mg/kgBW (p = 0.047) and 350 mg/kgBW (p = 0.016). These findings are in line with the findings of Liu *et al.* [8] regarding the role of lunasin as an anti-inflammatory and anticancer agent. As an anti-inflammatory, lunasin plays an important role in inhibiting pro-inflammatory cytokines, such as TNF-and IL-6, without damaging cell viability. Dia *et al.* [15] showed that lunasin can also reduce iNOS and COX-2 expression, thereby reducing NO and PGE2 production.

The use of the term lunasin-rich soybean extract in this study was intended to show that soybeans are a rich source of lunasin. Liu *et al.* [8] showed that soybeans are the food sources with the highest lunasin content, with a range of 0.5–8.1 mg/g. The result of the HPLC analysis showed that the lunasin content of the soybean extract used in this study was 0.823 mg/g.

The dosages of lunasin-rich soybean extract in this study—250, 300, and 350 mg/kgBW—were determined based on previous work by Amalia *et al.* [16], which also used soybean extract in doses of 150 mg/kgBW and 200 mg/kgBW. This study's dose difference of 50 mg/kgBW between the intervention groups was determined according to the previous study.

Although there were no significant differences between the lunasin intervention groups (L1, L2 and L3), the results showed a gradual decrease in COX-2 expression at doses of 300 and 350 mg/kgBW compared to KN group. Therefore, inhibition of COX-2 expression can be said to be dose-dependent: higher doses of lunasin will result in greater inhibition of COX-2. These findings are in line with those of Dia *et al.* [15], which showed that increasing the dose of lunasin from 100 M to 200 M and then 300 M resulted in greater inhibition of COX-2 expression in HT-29 colorectal cancer cells.

Factors that play an important role in the pathogenesis of colitis-related colorectal cancer are the NF-B pathways, both in immune cells and epithelial cells, as well as activation of local cytokines and lymphokines. NF-B is a pro-inflammatory transcription factor that found in the cytoplasm in inactive form. Stimulus and extracellular signals in the form of inflammation will trigger a series of phosphorylation, ubiquitination, and degradation of NF-kB inhibitors that can activate NF-kB. When activated, NF-kB will translocate to the nucleus and trigger the transcription genes that encode cytokines, adhesion molecules, apoptotic regulators, cell cycle regulators, other transcription factors, and enzymes, including COX-2 [15].

According to Liu *et al.* [17], COX-2 expression is one of the factors that play an important role in the progression of colorectal cancer. This is because COX-2 mediates the formation of PGE2, which triggers the activation of the Wnt/β-catenin pathway; this, in turn, initiates a series of processes that lead to the abnormal proliferation of colon cells.

An *in vitro* study conducted by Dia *et al.* [18] showed that the administration of lunasin can inhibit this proliferation and trigger apoptosis of HT-29 colorectal cancer cells. According to this study, the inhibition of cell proliferation due to lunasin occurred because cells are inhibited from entering the mitotic phase (G2/M), which is controlled by a regulator kinase called cyclin-dependent kinase 1 (CDK1).

In addition to proliferation inhibition, apoptosis induction is an anticancer mechanism that is induced by lunasin. The increase in apoptosis is characterized by the modification of B-cell lymphoma 2 proteins (Bcl-2) and Bcl-2 associated X proteins (Bax). Supression of Bcl-2 protein expression that was anti-apoptotic and induction of Bax protein that was pro-apoptosis was found upon administration of lunasin [18]

The significant differences between the KN and N groups indicate that the AOM and DSS given in the KN group were effective in inducing carcinogenesis through inflammatory pathways, including increasing COX-2 expression. The success of this induction of colorectal carcinogenesis is in line with the study by Rosenberg *et al.* [19] where administration of a single dose of AOM through IP injection followed by DSS in drinking water for a week showed the onset of tumors about 6 w after administration. Tanaka *et al.* [20] also showed that the administration of AOM and DSS using the same protocol caused distortion of the cryptic epithelium, irregular cryptic spread in lamina propria and infiltration of inflammatory cells about 3–4 w after administration. AOM and DSS were also known to cause severe colitis about 12 w after administration.

The Mann Whitney test results also showed that there was a significant difference between the KP and KN groups (p = 0.009). This is in line with research conducted by Rothwell *et al.* [21] where long-term aspirin administration every day for 5 y is known to be useful in reducing the risk of death caused by all types of cancer. In addition, several cohort studies have also shown that the risk of death from colorectal cancer can decrease significantly (by 40%) after long-term aspirin treatment.[18] In this study, the use of aspirin at a dose of 150 mg/kgBW was based on a previous study by Amalia *et al.* [22] that analyzed the effect of soybean extract on apoptosis and dysplasia in the colons of mice that had carcinogenesis induced with AOM and DSS.

COX-2 is the main pharmacological target of aspirin as a chemopreventive agent. This is based on the finding that aspirin can only reduce the risk of colorectal cancer in individuals with increased COX-2 expression. This finding is also associated with a reduced risk of mortality [23]. In colonic mucosal tissue, COX-2 is more commonly found in tumor tissue, including epithelial, endothelial, stromal, and mononuclear cells. The impact of increased COX-2 expression on colorectal cancer is related to increased prostaglandin production which causes apoptotic inhibition, stimulation of tumor growth, and angiogenesis [24].

Most of the previous research on lunasin has been done in *in vitro* studies. The advantage of this study is the ability to analyze the effect of several different dosage levels of lunasin on colorectal cancer in mice *in vivo*. Possible visual bias due to manual observation and quantification of immunohistochemical staining results might be a disadvantage of this study. Nevertheless, the subjectivity can be minimized by using an optical density scoring system.

CONCLUSION

The administration of lunasin-rich soybean extract inhibited COX-2 expression in cryptic epithelial cells of the distal colon in mice in which colorectal carcinogenesis was induced by AOM and DSS. A statistically significant decrease in COX-2 expression was found at doses of 300 and 350 mg/kgBW in a dose-dependent manner. For the further studies, the authors recommend a longer period of carcinogenesis induction to be carried out to determine the efficacy of lunasin in a more severe model of colorectal cancer. This recommendation is based on some studies which show that it takes about 20 w for AOM and DSS to induce mice to progress to colorectal carcinoma [21].

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

There are no conflicts of interest to declare

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