

The Effect of Lunasin on Inhibition of KI67, BCL-2 and C-MYC Expression in Azoxymethane and Dextran Sodium Sulfate Induced Mice Colon

(Kesan Lunasin terhadap Perencatan Ekspresi KI67, BCL-2 dan C-MYC dalam Kolon Tikus Terinduksi Azoksimetana dan Dektran Natrium Sulfat)

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ABSTRACT

Treatment of cancer using medicinal-plant based has been important due to minimal side effects, high efficiency and low cost. Lunasin from soybean is known as potential chemopreventive agent. This study aimed to study and investigate the proteins involved in the mechanisms of action of lunasin underlie its chemopreventive effects in Azoxymethane (AOM) and Dextran Sodium Sulfate (DSS) induced mice. A total 30 BLAB/c 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 were given in different doses of Low Dose Lunasin (75 mg/kgBW), Moderate Dose Lunasin (150 mg/kgBW), and High Dose Lunasin (200 mg/kgBW) to intervention groups. Immunohistochemistry was conducted to measure Ki67, C-myc, and Bcl-2 expressions 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. Further, the H-score method was used to quantify of Ki67, C-myc and Bcl-2 expressions. The results of this show that there is significant differences between the negative control and the intervention groups were found at the 75 mg/kgBW and 150 mg/kgBW ($p < 0.05$) lunasin dosage levels. This demonstrates that Lunasin inhibits proliferation and induces apoptosis in the colon mice induced by AOM and DSS.

Keywords: AOM; Bcl-2; C-myc; DSS; Ki67

ABSTRAK

Rawatan kanser berasaskan tumbuhan ubatan adalah penting kerana kesan sampingan yang minimum, kecekapan tinggi dan kos yang rendah. Lunasin daripada kacang soya dikenali sebagai agen kemohalangan yang berpotensi. Penyelidikan ini bertujuan untuk mengkaji protein yang terlibat dalam mekanisme tindakan lunasin yang mendasari kesan kemohalangnya pada tikus yang disebabkan oleh Azoksimetana (AOM) dan Dektran Natrium Sulfat (DSS). Sebanyak 30 tikus BLAB/c dipisahkan kepada enam kumpulan. Dalam lima daripada kumpulan kawalan negatif, kumpulan kawalan positif dan tiga kumpulan intervensi-karsinogenesis telah diinduksi dengan AOM dan DSS; kumpulan keenam tidak menerima campur tangan. Lunasin diberikan dalam dos berbeza Lunasin Dos Rendah (75 mg/kgBW), Lunasin Dos Sederhana (150 mg/kgBW) dan Lunasin Dos Tinggi (200 mg/kgBW) kepada kumpulan intervensi. Imunohistokimia telah dijalankan untuk mengukur ekspresi Ki67, C-myc dan Bcl-2 daripada kolon distal tikus yang telah dikorbankan. Sampel telah dinilai secara mikroskopik dan difoto dan kiraan sel dilakukan menggunakan aplikasi Image J. Selanjutnya, kaedah H-skor digunakan untuk mengukur ungkapan Ki67, C-myc dan Bcl-2. Keputusan ini menunjukkan terdapat perbezaan yang signifikan antara kawalan negatif dan kumpulan intervensi didapati pada tahap dos lunasin 75 mg/kgBW dan 150 mg/kgBW ($p < 0.05$). Ini menunjukkan bahawa Lunasin menghalang percambahan dan mendorong apoptosis dalam tikus kolon yang disebabkan oleh AOM dan DSS.

Kata kunci: AOM; Bcl-2; C-myc; DSS; Ki67

INTRODUCTION

As the third common cancer worldwide, colorectal cancer (CRC) is a malignant disease with high mortality rate nowadays (American Cancer Society 2017). Colorectal cancer events occur in developed countries due to changes in lifestyle and eating patterns leading to obesity, lack of physical activity, smoking, lack of consumption of fibrous foods, and increased alcohol consumption. CRC develops through several pathogenesis pathways including polyp, non-polyp, genetic instability pathway and CRC-associated chronic inflammation such as Inflammatory Bowel Disease (IBD), notably IBD patients have higher risk of developing CRC compared to non-IBD patients (Kim & Chang 2014; Kumar, Abbas & Aster 2013; Rawla, Sunkara & Barsouk 2019).

CRC-associated chronic inflammation is characterized histologically by transformation of colon and rectum normal tissue into low to high grade dysplasia. The search for an alternative novel drug with more effective, low cost and minimum side effects for the treatment of CRC-associated inflammation is importance. Nutritional therapy and medicinal-plant based nowadays such as Lunasin in Soybean extract may have high potential to become alternative novel treatment of CRC-associated inflammation with its function as chemopreventive agent. Soybeans are proven to suppress inflammation and tumor growth in various tissues, such as the mammary glands, prostate, bladder, and skin (Dia & Mejia 2011; Jiang et al. 2016; Meteoglu et al. 2018; Wan et al. 2017).

Lunasin is a bioactive peptide with a unique structure consisting of an Arginine-glycine-aspartate (RGD) motif that is similar to the RGD motif on the extracellular matrix protein (ECM). Lunasin competes with ECM protein to bind with integrin through RGD motif becoming integrin antagonists causing inactivation of FAK/Src/ERK/NF- κ B signaling pathway and decrease NF- κ B activation (Dia & Mejia 2011; Jiang et al. 2016).

NF- κ B, on its active form, functions as a transcription factor that regulates and activates the transcription of genes involved in the proliferation and survival of the tumor cells such as Ki67, C-myc, and Bcl-2. Increasing NF- κ B activation and expression are related to the high proliferation and survival rate of tumor cells (Meteoglu et al. 2018; Patel et al. 2018; Scherr 2018; Tomlinson et al. 1996).

A study from Amalia et al. (2017) shows that lunasin reduces dysplasia histologically and increases Caspase-3 expression. Lunasin also inhibits colon cancer

carcinogenesis in animal models induced by AOM and DSS through its anti-inflammation effects that suppress the inflammation process by decreasing the expression of inducible nitrite oxide (iNOS), cyclooxygenase-2 (COX-2), Tumor Necrosis Factor (TNF- α) and β -catenin (Kusmardi et al. 2019a, 2019b, 2019c, 2018a, 2018b). The proteins involved in mechanism of action of lunasin in inhibiting proliferation and promoting apoptosis as chemopreventive agent on colorectal cancer are still remain unclear, this study was designed to investigate the effect of Lunasin on Ki67, C-myc and Bcl-2 expression in Azoxymethane and Dextran Sodium Sulfate induced mice colons.

MATERIALS AND METHODS

The design of this study was experimental study using primary data in the form of quantification of Ki67, C-myc and Bcl-2 expressions. The expression of Ki67, C-myc and Bcl-2 was observed in immunohistochemically of distal colon from mice that had been induced by AOM/DSS and sacrificed, specifically the cryptic epithelium cells. The preparation was conducted using stored tissue. The observation of the preparation was carried out using a microscope at 400 times magnification in five fields of view. The preparation and observation process were conducted at the Anatomy Pathology Laboratory of the Faculty of Medicine at Universitas Indonesia, Jakarta, Indonesia.

LUNASIN

Lunasin extracted from the Grobogan soybean variant was obtained by Indonesian Legumes and Tuber Corps Research Institute Malang of East Java. The extraction process is started from the separation of oil and residue using high pressure to become dried soybean. Following the pressing process, the dried soybeans were then blended into powder and macerated using phosphate buffer saline solvent for 60 min. Afterwards, the maceration solution was filtered with filter gauze resulting extract solution that was dried at 50 °C by evaporator. Lunasin content in the soybean extract was analysed using High Performance Liquid Chromatography (HPLC) method.

EXPERIMENTAL ANIMALS

The experimental animals in this study were 12-week-old BALB/c mice kept and cared according to the ethics procedure from the commission for handling and using experimental animals from The Ethics Commission of

Faculty of Medicine, Universitas Indonesia. All BALB/c mice were male and had almost the same body weight approximately 250 grams (± 20 g). The mice were kept and cared in cages made of standard sized stainless steel with ventilation. The mice were adapted for 1 week at the Anatomy Pathology Laboratory of Faculty of Medicine at Universitas Indonesia. The hygiene was maintained routinely, and the environmental conditions such as temperature (23-24 °C) and humidity (50-60%) were always set and controlled under the ideal condition. The light was set on a cycle of 12 h of light and 12 h of dark. The mice also had access to food and drank freely in cages. The physical health of the mice, including their levels of activity, consumption of food and drink, were examined.

CARCINOGENESIS INDUCTION

Induction of colorectal carcinogenesis was conducted following the induction method by Suzuki, Kohno and Sugie (2006) and Tanaka, Kohno and Suzuki (2003). The mice that receive carcinogenesis induction were given 10 mg/kg BW of AOM diluted in NaCl 0.9% and 2% DSS. AOM was given once on the first day of the beginning of the trial week (first day) by intraperitoneal (IP) injection. After a week of AOM administration, mice were given 2% DSS dissolved in drinking water every day for a week.

EXPERIMENTAL GROUPS

Thirty BLAB/c mice were divided into 6 sample groups: the normal group (N), a Negative Group (NEG), a Positive Group (POS), and three intervention groups. The Normal Group was not given AOM and DSS administration, but instead received IP injections of physiological saline for 4 weeks. This group represents the mice in normal condition before carcinogenesis. The negative group (NEG) was given single dose of AOM via IP injection followed by 2% DSS by oral for a week. This group represents the condition of mice after carcinogenesis, without any treatment. The Positive group (POS) was given single dose of AOM via IP injection followed by 2% DSS by oral for a week, then treated with aspirin (150 mg/kgBW) by oral for 4 weeks. This group represents the condition of mice after carcinogenesis followed by treatment as the results of carcinogenesis inhibition. The intervention groups consisted by Low Dose Lunasin (LDL), Moderate Dose Lunasin (MDL) and High Dose Lunasin (HDL). These groups 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 2% DSS for a week, then lunasin extract administration orally (LDL: 75 mg/kgBW; MDL: 150 mg/kgBW; and HDL: 200 mg/kgBW) for 4 weeks. All mice were sacrificed in the sixth week and colon as tissue samples are collected and processed becoming paraffin blocks (Figure 1).

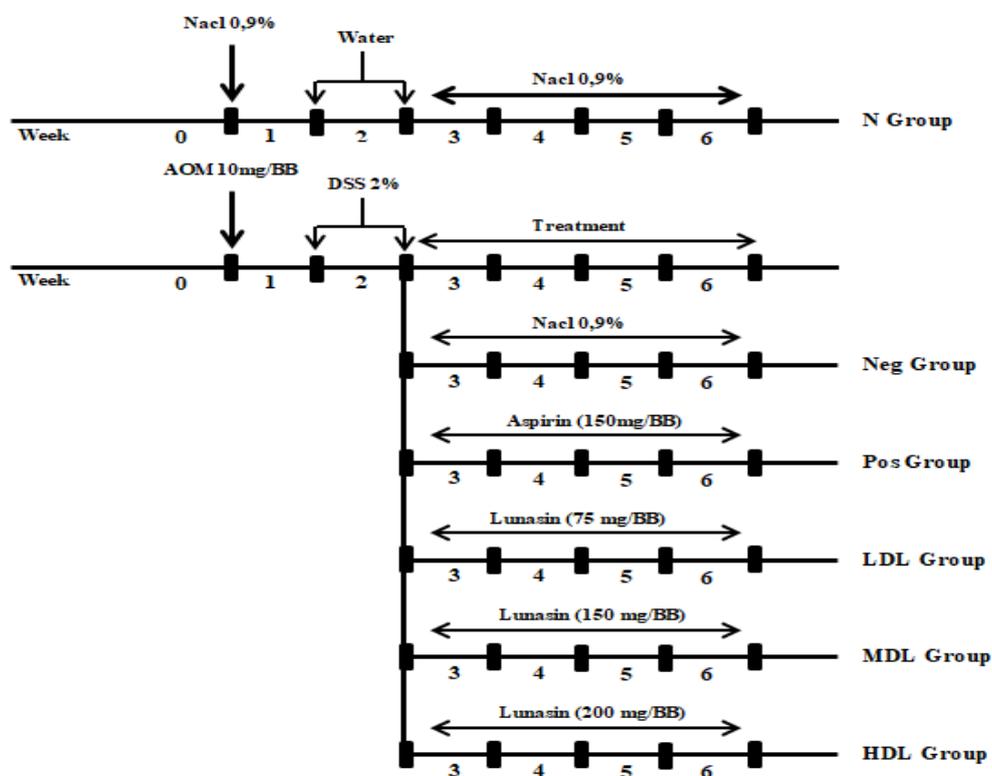


FIGURE 1. Scheme of experimental groups, N (Normal), NEG (Negative), POS (Positive), LDL (Low Dose Lunasin), MDL (Moderate Dose Lunasin), HDL (High Dose Lunasin)

The amount of sample was calculated based on Federer's formula $(n-1)(k-1) \geq 15$, with n is the number of samples in each group and k is the number of experimental groups, then $(n-1)(6-1) \geq 15$. Based on the calculation, the minimum amount of the samples in each experimental groups were 4 mice, 24 mice in total.

IMMUNOHISTOCHEMISTRY

At the end of trial week, each mouse was sacrificed using the neck dislocation technique. Colon tissue obtained from the mice made into paraffin blocks. Unstained slides from the sample paraffin blocks were made using a microtome (3-5 μm), then deparaffinized and rehydrated using xylol and alcohol. For the next step, the slides were put in citrate buffer and laid in Decloacking Chamber for antigen retrieval using the Heat-induced Epitope Retrieval (HIER) method at 95 °C for 20 min. The slides were washed and prepared for blocking step. Peroxidase and protein blocking reagents from Novolink Polymer Detection System were dripped on the slides followed by 15 min incubation in room temperature.

Hereafter, the slides were incubated with the primary antibody (Anti-Ki67 antibody Abcam ab15580, Anti-C-Myc Antibody Abcam ab39688, Anti-Bcl-2 Antibody Abcam ab196495) for one hour at room temperature. Each slide was incubated then with post-primary and Novolink polymer for 30 min and then counterstained by Haematoxylin Meyer subsequently immersed in Lithium Carbonate solution for one minute. The slides were washed, dehydrated, and mounted using deionized water, alcohol, and mounting medium, respectively.

All of the slides were observed using Binocular Light Microscope built-in camera on 400x magnification. Five photos were randomly taken on different areas from each slide. The area was selected based on the intact of the crypts, transverse cut area, and strongest marker expression area, and furthermore, the Ki67, C-myc and Bcl-2 expression was quantified using the H-score method.

QUANTIFICATION OF Ki67, C-myc, Bcl-2 EXPRESSIONS

The score of Ki67, C-myc and Bcl-2 expressions were determined by the brown intensity in the colon epithelial cells. Ki67 and C-myc are expressed in the nucleus, while Bcl-2 is expressed in the cytoplasm. The dark brown nucleus or cytoplasm cells are identified as High

Positive cells (HP). In contrast, the light brown nucleus or cytoplasm cells are identified as Moderate Positive cells (MP). Moreover, cells with lighter brown nuclei and cytoplasm than MP cells are identified as Low Positive cells (LP) and blue nuclei and cytoplasm are identified as negative cells (NC). The number of cells with different intensities was calculated using the ImageJ-cell counter program. The result of cell calculation was entered in the H-score formula (below) to get the H-score of Ki67 expression. A blind procedure is conducted to avoid subjectivity in the result interpretation and the H-score calculation.

$$\frac{\% \text{ of HP} \times 4 + \% \text{ of MP} \times 3 + \% \text{ of LP} \times 2 + \% \text{ of NC} \times 1}{100}$$

where % of HP is the percentage contribution of high positive cells; % of MP is the percentage contribution of moderate positive cells; % of LP is the percentage contribution of low positive cells; % of NC is the percentage contribution of negative cell.

STATISTICAL ANALYSIS

The H-score data obtained are displayed in tables describing the Ki67, C-myc and Bcl-2 distribution. The normality test was done using the Shapiro Wilk test and followed by the homogeneity test. The data was analysed using the analysis of variance test (ANOVA), continued with the Post Hoc test if the data are normally distributed and homogeneous. However, if the data are not normally distributed and homogeneous, the data analysis was conducted using the Kruskal-Wallis test followed by the LSD or Mann-Whitney U test. The analysis used 95% confidence level ($\alpha = 0.05$). All tests used Statistical Product and Service Solutions (SPSS) version 23.0.

RESULTS AND DISCUSSION

The effect of lunasin on Ki67, C-myc and Bcl-2 expressions in the cryptic epithelial cells of the distal colon tissue in mice that had colorectal carcinogenesis induced by AOM and DSS administration is shown in Table 1. Based on the H-Score data, the highest expression of Ki67, C-myc and Bcl-2 was found in the Negative Group (NEG), while the lowest expression was found in the Positive Group (POS). In the Lunasin treatment groups, the expression of Ki67, C-myc, and Bcl-2 was found to be dose-dependent.

TABLE 1. Descriptive analysis of Ki67, C-myc and Bcl-2 expressions in control groups and lunasin therapy groups

Experimental groups	Median (Minimum-Maximum)		
	Ki67	C-myc	Bcl-2
Normal (N)	1.85 (1.3-2.23)	2.02 (1.80-2.42)	2.06 (1.77-2.39)
Negative (NEG)	2.92 (2.18-3.28)	3.14 (2.13-3.40)	3.04 (2.66-3.30)
Positive (POS)	1.61 (1.31-1.88)	1.95 (1.38-2.31)	1.79 (1.61-2.13)
Low dose lunasin (LDL)	2.60 (2.26-3.10)	2.63 (1.91-3.16)	2.54 (2.29-2.87)
Moderate dose lunasin (MDL)	2.09 (1.57-3.51)	2.23 (1.81-2.54)	2.24 (1.97-2.62)
High dose lunasin (HDL)	1.84 (1.52-2.23)	1.81 (1.39-2.30)	1.99 (1.73-2.21)

The normality test showed that Ki67, C-myc and Bcl-2 H-Score data was not normally distributed ($p < 0.05$). Furthermore, the Levene homogeneity test result showed that the variance of the data was not homogenous ($p < 0.05$). Based on those results, the analysis of the data was carried out using Kruskal-Wallis nonparametric test, which determined that there were significant differences of Ki67, Bcl-2, and C-myc expressions between groups ($p < 0.05$). The Mann Whitney test was carried out to find out which groups had significant differences. The result showed that there were significant differences of Ki67, C-myc, and Bcl-2 expressions between many of the groups. Ki67 expression indicated that there were significant differences between groups, in particular: N with NEG group ($p = 0.000$), N with LDL group ($p=0.000$), NEG with POS group ($p=0.000$), NEG with MDL group ($p=0.043$), NEG with HDL ($p=0.000$), POS with LDL group ($p=0.000$), POS with MDL ($p=0.000$), and LDL and HDL group ($p=0.000$).

The significant differences between groups of C-myc expressions in particular: N with NEG group ($p = 0.000$), N with LDL group ($p=0.007$), NEG with POS group ($p=0.000$), NEG with MDL group ($p=0.002$), NEG with HDL ($p=0.000$), POS with LDL group ($p=0.000$), POS with MDL ($p=0.000$), and LDL and HDL group ($p=0.008$). The significant differences between groups of Bcl-2 expressions in particular: N with NEG group ($p = 0.000$), N with LDL group ($p=0.001$), NEG with POS group ($p=0.000$), NEG with MDL group ($p=0.002$), NEG with HDL ($p=0.000$), POS with LDL group ($p=0.000$), POS with MDL ($p=0.000$), and LDL and HDL group ($p=0.021$).

The comparison of the immunohistochemical staining results of the Ki67, C-myc and Bcl-2 expression between the control groups and lunasin treatment groups can be seen in Figures 2 and 3. The difference of brown color intensity in the nucleus (Ki67, C-myc) and cytoplasm (Bcl-2) of colon epithelial cells indicates the Ki67, C-myc, and Bcl-2 expression levels. Strong brown color indicates high expression level, while light brown color indicates a lower expression level. The stronger the brown color, the higher the expression level. The colon cells with blue nuclei or cytoplasm represent no expression of Ki67, C-myc, and Bcl-2. The clear/bluish (negative cells) or faint brown (low positive cells) were found mostly in the normal group (N). In the negative group (NEG), epithelial cells with high intensity of brown colors were the most common. Meanwhile, there was a gradual decrease in brown color intensity as dosage increased in the lunasin intervention groups.

Based on statistical tests, it shows clearly that lunasin has effect on the Ki67, C-myc and Bcl-2 expression in the mice colon induced by AOM and DSS. Lunasin had a significant effect on Ki67, C-myc and Bcl-2 expressions on the highest dose (200 mg/kgBW). AOM causes mutations on K-ras, PI3KCA or beta-catenin genes, then DSS induces colon inflammation in the NEG group. DNA damage and pro-inflammatory cytokines are stimuli required for activating FAK/Src/ERK/Nf- κ B signaling pathway heading to the increase of NF- κ B activation (Montovani et al. 2008). Overexpression and excessive activation of NF- κ B increases the expression of genes involved in cell cycle promotion, proliferation,

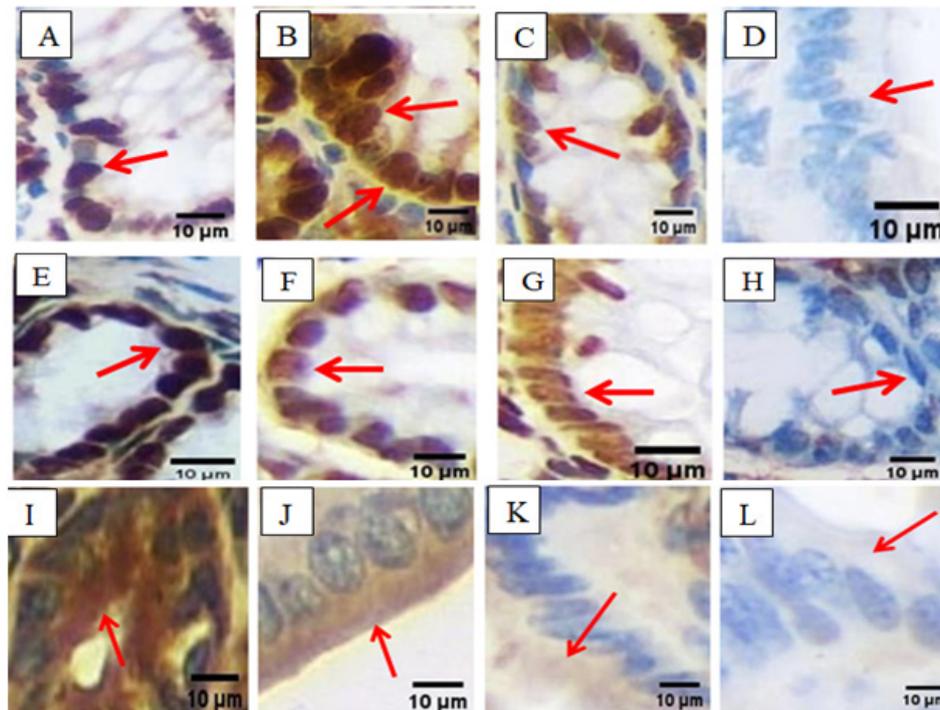


FIGURE 2. Differences of color intensity on the immunohistochemistry results of Ki67, C-myc, and Bcl-2 (400× magnification). (A) Ki67 high positive cell (HP), (B) Ki67 moderate positive cell (MP), (C) Ki67 low positive cell (LP), (D) Ki67 negative cell (NC), (E) C-myc high positive cell, (F) C-myc moderate positive cell, (G) C-myc low positive cell, (H) C-myc negative cell, (I) Bcl-2 high positive cell, (J) Bcl-2 moderate positive cell, (K) Bcl-2 low positive cell, (L) Bcl-2 negative cell. The red arrows indicate the epithelial cell nuclei and cytoplasm stained with difference intensity of brown to blue color

and apoptotic regulators such as Ki67, C-myc, and Bcl-2. The combination of AOM and DSS treatment causes the increase of proliferation and dysplasia leading to colorectal carcinogenesis (Robertis et al. 2011; Hirano et al. 2020; Lin et al. 2020).

An increased proliferation rate after AOM induction characterized by Ki67 and C-myc overexpression is also found in a study conducted by Robertis et al. (2011). A study by Lin et al. (2020) shows that Ki67 overexpression also occurred in animal models induced only by AOM and DSS, furthermore, AOM and DSS treatments create a condition that is similar to the early step of colorectal carcinogenesis where Bcl-2 overexpression is also reported on that carcinogenesis step.

Ki67 is a protein used as a proliferation marker in tumor, while C-myc is a transcription factor that regulates >15% of gene expression in human including genes involved in proliferation, cell cycle, survival and cell metabolism. Overexpression of Ki67 and C-myc is

commonly found in breast, colon, pancreatic and lung cancers (He et al. 2018; Ibadawy et al. 2019; Menon et al. 2019). In cancer, the balance between proliferation and apoptosis is disrupted characterized by the increase of proliferation and decrease of apoptosis. Apoptosis is regulated by a balance of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) protein expression. Bcl-2 overexpression in colorectal cancer is associated with poor differentiation, prognosis, and tumor cell survival rate (Lu et al. 1996; Mohan et al. 2012).

The expression of Ki67, C-myc, and Bcl-2 in the Positive Groups (POS) was not significantly different from Normal Groups (N). However, Ki67, C-myc, and Bcl-2 expressions in POS are lower than Negative Group (NEG). POS group is a sample group treated by AOM and DSS followed by aspirin which is an anti-inflammatory, analgesic and antiplatelet aggregation agent. Aspirin contains acetylsalicylic acid that inhibits cyclin A2, HMGB1 and the NF- κ B signaling pathway.

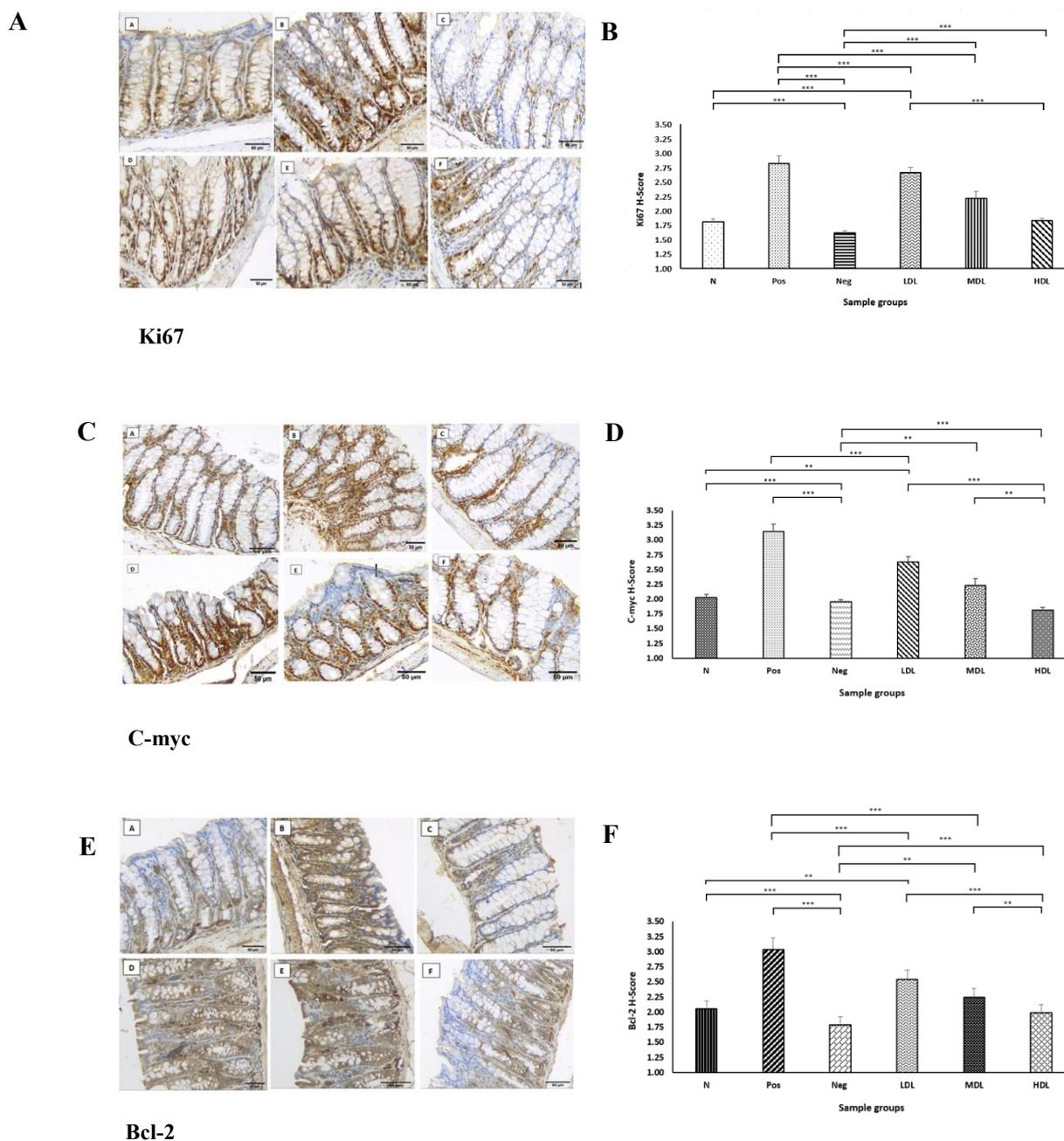


FIGURE 3. The comparison of the immunohistochemical staining results of Ki67, C-myc and Bcl-2 expression. Lunasin decrease Ki67, C-myc and Bcl-2 expression of colon epithelial cells in mice induced by AOM and DSS. The difference intensity of brown color from immunohistochemistry result showed in left panels (A, C, E). The quantification of Ki67, C-myc and Bcl-2 using H-Score from each sample group showed in right panels (B, D, F). The expression of Ki67, C-myc and Bcl-2 is highest in the NEG group (Left panels, inside figure B) and while the lowest expression is in POS Group (left panels, inside figure C). Statistical differences were analyzed with Kruskal Wallis and Post Hoc Mann Whitney Tests. The statistical test indicated that there is difference of Ki67, C-myc and Bcl-2 expression among the sample groups showed by p-value $* < 0.05$, p-value $** < 0.01$ and p-value $*** < 0.001$. Ki67, C-myc and Bcl-2 expressions respectively decrease depending on the dose of lunasin (right panels). Inside figures in left panels: (A) Normal Group (N), (B) Negative Group (NEG), (C) Positive Group (POS), (D) Low Dose Lunasin (LDL), (E) Moderate Dose Lunasin (MDL), (F) High Dose Lunasin (HDL) (400× magnification)

The acetyl group in aspirin acetylates serine residues causing cyclooxygenase (COX) inhibition in converting arachidonic acid into prostaglandin H₂ and its derivatives that play an essential role in the proliferation (Bagher et al. 2018).

The dosages of lunasin in this study were determined based on previous work by Amalia et al. (2017). Ki67, C-myc and Bcl-2 expression levels in sample groups treated by Lunasin decrease depending on the dose of lunasin. The expression of Ki67, C-myc and Bcl-2 in lunasin treatment sample group with high dose of Lunasin is similar with Normal and Positive group. The higher the dose of lunasin, the lower the Ki67, C-myc and Bcl-2 expression levels. It shows that Lunasin has mechanisms to inhibit proliferation and induce apoptosis in colon of mice induced AOM/DSS. Arginine-Glycine-Aspartate (RGD) motif in Lunasin structure allows lunasin to compete with extracellular matrix (ECM) proteins for binding to integrin receptors.

The interaction between lunasin and integrin receptors via the RGD motif decreases phosphorylation or activation of FAK/Src/ERK/NF- κ B signaling pathway downstream proteins. A study by Dia and Mejia (2011) shows that lunasin decreases the phosphorylation of focal adhesion kinase (FAK), Src, and ERK leading to ERK staying in the inactive form. ERK in its active form is required to activate inhibitor kappa B kinase (IkK), an enzyme that phosphorylates Inhibitor kappa B (I κ B) to undergo ubiquitination. If IkK remains inactive or unphosphorylated forms, then I κ B ubiquitination will not occur, so NF- κ B is still inactive in the cytoplasm and not be able to act as a transcription factor that activates the expression of genes involved in cell cycle, proliferation and apoptosis regulators such as Ki67, C-myc, and Bcl-2.

The association between inflammation and colon cancer development is clear, where colon inflammation, like inflammatory bowel disease (IBD), increases the risk of developing colon cancer called colon cancer-associated inflammation. Lunasin, as an anti-inflammatory and antioxidant agent, inhibits the colon inflammation process leading to the prevention of colorectal carcinogenesis in animal models induced by AOM and DSS. Kusmardi et al. (2019a, 2019b, 2019c, 2018a, 2018b) stated that the expression of TNF- α , COX-2, beta-catenin and iNOS in the sample group without lunasin treatment is higher than in the sample group with lunasin treatment leading to decreased NF- κ B activation so that lunasin potentially inhibits colorectal carcinogenesis-associated inflammation.

Lunasin also decreases IL-6 expression, causing a reduction of STAT3 phosphorylation, which is a protein regulating apoptosis-related gene expressions like Bcl-2. The effect of lunasin on inhibiting proliferation *in vitro* has been demonstrated in several studies. Jia et al. (2015) found that lunasin reduces proliferation activity in fibroblast cell culture. Lunasin also inhibits colon cancer growth and induces apoptosis of colon cancer cell lines (KM12L4, RKO, HCT-16, HT-29) in a study by Dia and Mejia (2011). In addition, Hsieh et al. (2010) shows that administration of lunasin (20 mg/kg BW and 4 mg/kg BW) reduced Ki67 expression (37%) in breast cancer xenograft animal model. Study conducted by Amalia, et al. (2017) show that lunasin increases apoptosis and histologically reduces dysplasia grade in the colon of mice induced by AOM/DSS.

The effects of lunasin on c-myc expression in colorectal cancer are still unclear, yet several other studies show controversial results. Study conducted by Jia et al. (2015) showed that lunasin and genistein have effects as a chemopreventive agents by decreasing beta-catenin expression and its target gene expressions, cyclin D1 and c-myc in HC11 mammalian cancer cell cultures. However, Pabona et al. (2012) showed different results where the expression of c-myc in MCF-7 and HC11 cell cultures treated with lunasin did not show significant differences with the control. Lunasin does not strongly inhibit Wnt-1 in Wnt-1-induced HC11, so lunasin cannot reduce C-myc expression and is not effective in inhibiting proliferation. Nevertheless, lunasin is still effective in inducing apoptosis in HC11 and MCF-7 cell cultures.

Dia and Mejia (2009) shows that lunasin decreases Bcl-2 expression and increases Bax expression *in vitro* using HT-29 cell culture. Furthermore, Mohan et al. (2012) stated that NF- κ B plays a role in regulating the expression of Bcl-2 and Bax in MCF-7 cell culture. The suppression of NF- κ B expression and activation increases apoptosis in MCF-7 cell culture. Jia, et al. (2015) also states that lunasin reduces IL-6 expression in fibroblast cell culture leading to inhibition of NF- κ B expression and activation.

CONCLUSIONS

Lunasin inhibits the expression of Ki67, C-myc, and Bcl-2 in mouse colon epithelial cells induced by AOM and DSS, and consequently Lunasin potentially inhibits colorectal carcinogenesis by reducing proliferation and inducing apoptosis.

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