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Research Article

THE EFFECT OF LUNASIN EXTRACT ON THE RECTUM HISTOPATHOLOGY OF MICE INDUCED BY AZOXYMETHANE AND DEXTRAN SODIUM SULFATE

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Abstract

Cancer is one of the diseases that cause the highest rate of morbidity and mortality in the world. And colorectal cancer as of know is ranked number 3 in the most prevalence cancer in Indonesia. Chemotherapy, radiotherapy, and surgery are still the main choice of cancer treatment. But, those therapy have various side effect, especially chemtherapy that have systemic response. Lunasin is one of the natural substance which mean less side effects, that have high anticancer effect by the inhibition of acetyl histone and from the suppression of NF-kB. This research aims to determine inhibitory potential of lunasin against the progression of the histopatological sign of colorectal cancer in rectum of mice induced by azoxymethane (AOM) and dextran sodium sulfate (DSS) that acts as cancer inducer. 24 mice was injected with AOM and DSS intraperitoneal. Then it was divided into 4 different group; control group, group that was given 20mg/kg lunasin (low dose), group that was given 30mg/kg lunasin (medium dose), group that is given 40mg/kg lunasin (high dose). And then, the rectum specimen was taken. The histopatological sign observed are inflammation, hyperplasia, and angiogenesis. The result show that lunasin extract is proven to decrease the amount of angiogenesis, hyperplasia, and inflammation. But, in inflammation and hyperplasia significant reduction is only found after increasing the dose of lunasin extract for a minimum of 20mg/kg of mice body weight. For angiogensis, the signficcant reduction of angiogenesisn is only founded after giving the high dose lunasin (40mg/kg of mice body weight). In conclusion, lunasin is able to reduce the progression of colorectal cancer in rectum of mice induced by AOM and DSS.

Keywords: Azoxymethane (AOM), Dextran sodium sulfate (DSS), Mice, Colorectal cancer, Hyperplasia, Angiogenesis, Inflammation, Rectum, Lunasin.

INTRODUCTION

Cancer is one of the diseases that cause the highest rate of morbidity and mortality in the world. There are around 14 million new cases and 8.2 million deaths because of cancer in 2012. Lung cancer, Hepatic cancer, gastric cancer, and colorectal cancer, breast cancer, and esophageal cancer is the most common cause of deaths because of cancer every year. And colorectal cancer placed third after lung cancer and breast cancer.[1]International Agency for Research on Cancer (IARC) stated that the prevalence of colorectal cancer in the world is quite the same whether in male or female. In male there are 746,000 cases (10%) and 614.000 cases (9.2%) in female.[2] Around 55% of colorectal cancer occurs in developed countries with the highest prevalence in Australia and New Zealand (male cases 44.8 per 100,000 population and female cases is 32.2 per 100,000). And the lowest ratio is in the West Africa (Male 4.5 per 100,000 and female 3.8 per 100,000).[3, 4] In Indonesia, colorectal cancer is now placed number 3 in cancer with highest prevalence in Indonesia. There were 19.1 male per 100,000 and 15.6 female per 100,000 of Indonesian people that get colorectal cancer.[5] The Incidence rate of colorectal cancer in Indonesia is still lower compared to Australia and New Zealand, but the incidence in Indonesia is categorized as high because of high population in Indonesia.[5-7] Cancer treatment as of now is still centering in surgery, chemotherapy, and radiation. Chemotherapy treatment still can not be given a satisfying result especially for cancer that has metastasis. Because of its low specificity and sensitivity, and it can cause serious side effect. That is why nowadays people try to find alternative treatment for cancer that has fewer side effects.[8-10] One of the natural substances that are tried to use to reduce colorectal cancer is lunasin in soybean. Lunasin have high anticancer effect with the inhibition of acetyl histone and from the suppression of NF-kB. That is the reason why lunasin is one of the substances that is recommended to treat cancer. Lunasin itself, are proven to have minimum of no side effect in its consumption. Recent research also stated that lunasin already proven to reduce gastric neoplasm, but no research have been done yet to know the minimum dose of lunasin and it's effect in the colorectal cancer in rectum that have quite different cell characteristic compared to gastric and the other part of colon.[11-13] Dysplasia, hyperplasia, mitosis, and inflammation are the early stage of cancer. And the progression of these disease start from chronic inflammation that lead to dysplasia, hyperplasia, and mitosis from intestines epithelial cell. Early identification is highly needed to manage the progression of cancer from the early stadium to the late stadium. This can be done with early screening in intestines epithelium.[14-15] Colorectal cancer induction with 1% or more of AOM and DSS is enough to give strong effect for the promotion of tumor cell in the laboratory rat or mouse in 14 weeks. The advantages from this model are cheap, potent carcinogen, comfortable, and valid.[16-17] AOM induction give the same result with the sporadic colorectal cancer in human in the response with promotive and preventive.[7] This study was conducted to know the efficacy of lunasin from soy fractions in reducing the progression of colorectal cancer in the rectum of mice induced by Azoxymethane (AOM) and dextran sodium

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sulfate (DSS). The use of AOM and DSS in the induction aims to make the process of carcinogenesis of sporadic colorectal cancers occur through inflammation.[17]

METHODS

Research Design

This study was an experimental study *in vivo* using experimental animals (mice). *In vivo* study to look at the barriers to colon carcinogenesis conducted at the Laboratory of Anatomical Pathology, Faculty of Medicine, University of Indonesia.

Soybean Sources

The original Indonesian soybean (*Glycine max* L.) is obtained from soybean varieties Bogor Agricultural University. Soybean harvest at 75-100 days. As for soybeans that have been matured in physiological characteristics is largely leaves (90-95%) of yellowish brown and fall, but not because of pests or diseases; the trunk is dry, as well as the fruit begins to change color from green to yellow lightly browned and cracked, or pods already look old. Soybean harvesting should be done in the morning when the weather is sunny and soybeans still somewhat hand-over that is not easily broken. Yields then dried about 1-2 hours in the hot sun or using the dryer with a maximum temperature of 60 ° C. After drying, the next process is the defoliation of soybean seeds from the pods can be done traditionally by theser pedal or by machine. Furthermore, after cleaning and sorting soy beans, as well as drying the seeds. Drying is carried out in the sun until it reaches 12-13% moisture content. If all the process has been carried out soy beans are ready to be made extracts used in this study. Commercial soybean meal derived from soybean oil processing company

Experimental animals

Animals used in this study are the Balb/c mice males with an average weight ± 20 grams were obtained from the Laboratory Animal Pathological Anatomy University of Indonesia. Male mice Balb/c was 20 weeks with an average weight ± 20 grams were obtained from the Animal Pathology Laboratory of the Faculty of medicine. The sample is a colon of mice populations that have been induced AOM 10 mg/kg body weight of mice intraperitoneally. A week later the mice were fed with standard and beverages that contain DSS 2% every day for a week. Post-AOM and DSS, mice were given a solution every day for 6 weeks

Determination of lunasin content by HPLC Method

Analysis of lunasin content of each faction performed by HPLC method. Stages as follows: Fraction obtained is filtered through a membrane filter of 0.22 μm before injection. A total of 100 mL injected into a C4 fraction Vydac® (25 cm x 4.6 mm, particle size 10 m) using detector diode array (215 nm) with a mobile phase (buffer A: 5% acetonitrile and 0.08% trifluoroacetic acid and buffer B: 95% acetonitrile and 0.1% trifluoroacetic acid) in a linear gradient for 30 minutes at 1 mL/min. Each faction identified the retention time using a standard commercial lunasin. Fractions containing high concentrations of lunasin to the active fraction will hereinafter be used in this study. In this study, the experimental group number is 4, the number of replicates in each group is: $n \geq 6$. So this study used 24 mice Balb/c males were divided into 4 groups (each group consisted of 6 mice), namely:

1. The group of mice was induced AOM + DSS (AOM injection of one dose of 10 mg/kg i.p and DSS solution of 2% w/v daily for 7 days) called control
2. AOM + DSS group (one injection of AOM dose of 10 mg/kg ip and DSS solution of 2% w/v daily for 7 days) + solution of soybean lunasin low dose (20 mg/kg) w/v called group 1
3. AOM + DSS group (one injection of AOM dose of 10 mg/kg ip and DSS solution of 2% w/v daily for 7 days) + solution of soybean lunasin medium dose (30 mg/kg) w/v called group 2
4. AOM + DSS group (one injection of AOM dose of 10 mg/kg ip and DSS solution of 2% w/v daily for 7 days) + solution of soybean lunasin high dose (40 mg/kg) w/v called group 3

Methods for Data Collection

Balb/c mice 20 week old male ± acclimatized beforehand for one week before induced AOM. Mice were maintained and treated in accordance Guide for the Care and Use of Laboratory Animals of the Animal Care and Use Committee. Mice were maintained in a controlled temperature conditions of 25 ° C, humidity 55% with a cycle of 12 hours light/dark. The whole of mice fed with standard and drinking mineral water ad libitum. Induction of colorectal carcinogenesis do adopt the method Kusmardi et al (2014): All the mice induced with AOM dose of 10 mg/kg dissolved in 0.9% NaCl at a dose of 10 mg/kg body weight by intraperitoneal early as one (except K-1 induced only with solvent AOM). One week after induction of AOM, mice were fed a standard and a drink containing DSS 2% daily for one week. Post-induction of AOM and DSS is completed, all the experimental animals were randomized (except K-1) was then placed in a separate enclosure which is maintained such that do not interact. Post-induction of AOM and DSS, the test group was given a test solution fraction lunasin-rich soy and soy commercial native Indonesia with low, medium, and high dose per mouse every day for 6 weeks. At the end of the study, mice were sacrificed with ether one day after the 6-week intervention with the test solution. Colorectal mice were taken and then the rest of the dirt cleaned from the lumen of the colon by rinsing with water. Tissue sections were fixed with buffered formalin and staining to see barriers to colorectal carcinogenesis through observation of the histopathological parameters dysplasia, hyperplasia, inflammation, and mitosis.

Staining of hematoxylin and eosin

Colorectal tissue pieces made paraffin blocks. Paraffin blocks made 4 μm thick slices and mounted on glass objects for HE staining stages as follows. Preparations deparafinitation using xylol I, II and II, respectively 5 minutes. Then preparations rehydrated using absolute alcohol, 96% and 70% respectively for 5 minutes, and washed in running water for 5 minutes. The preparation is then inserted into hematoxylin (Meyer solution) for 7 minutes, and rinsed in running water for 10 minutes. After that, the stocks dipped into a saturated lithium carbonate 2-3 or 1-2 minutes dip soaked and rinsed with running water for 5 minutes. Preparations controlled if the blue color is sufficient, if it is not put back into solution Meyer

(hematoxylin) for 2 minutes, then rinsed in running water, soaked in eosin for 1-2 minutes, dehydrated in alcohol 70%, 80% and 96% respectively in absolute -masing for 3 minutes, clearing with xylol I, II, and III, and the last drops of the entelan and covered with a glass lid. Preparations viewed using a light microscope with 400x magnification.

Assessment of histopathology

Histopathologic examination was performed on samples of colon of mice. Analysis is done by reading the histopathological changes in the outward appearance of a double-blind, by counting the number of cells showing the criteria of a variable parameter dependent per 10 visual fields were taken at random, and viewed with a light microscope magnification of 400x.

Statistical Analysis

Research data analysis performed using SPSS statistical test version 20. The difference in the results of various measurements between the control group and the test group, were tested with a significance level of $p < 0.05$ (confidence interval/CI = 95%). Objective measurement includes the quantification of the amount of rectum who had dysplasia, hyperplasia, mitosis and inflammation. Results obtained in the form of numerical data scaled ratio. If the data were normally distributed, then the statistical analysis performed one-way ANOVA followed by Tukey's test. Before the test data is determined by the distribution of Kolmogorov-Smirnov test and homogeneity variants performed with Levene test. When the resulting data distribution is not normal, it is necessary to transform the data in accordance monogram Altman in 1991 with the logarithm or other means before it can test the hypothesis. Another way is to change the continuous variables into ordinal or nominal variable, so it can be done non-parametric analysis (Tumbelaka *et al.,* 2011), the Kruskal-Wallis test.

RESULTS

Inflammation

In table 1 above shows that there is a constant decrease in the average focal inflammation of the rectum of mice induced by AOM and DSS after administration of lunasin. The amount of impairment is also directly proportional to the dose given lunasin.

Table 1. Comparation of inflammation focus from the rectum of mice induced by AOM and DSS that was given different dose of lunasin

Dose groups	n	Mean (focus/lpf)	p value
Control (without lunasin extract)	6	1.6667	<0.001
Low dose (20mg/kg)	6	0.7333	
Medium dose (30mg/kg)	6	0.5167	
High dose (40mg/kg)	6	0.4500	

One wayanova test. Post hoc bonferine analysist: control vs low dose p<0.001; Control vs medium dose p<0.001; Control vs high dose p<0.001; Low dose vs medium dose p=0.482; Low dose vs high dose p=0.155; Medium dose vs high dose P=1.0

Then, statistical comparison of the control group who did not receive insulin injections with each group of mice given lunasin with a low dose (20mg/kg), medium dose (30mg/kg) and high dose (40mg/kg), indicating significant results, Whereas the increase in 10mg/kg lunasin not demonstrated a significant reduction, it was proven with the results of post hoc p> 0.05 in the comparison between the low dose with medium dose and medium dose with high dose. While the addition of insulin doses as much as 20mg/kg showed better inflammation reduction, yet the p value is still <0.05. So, different dose doesn't show any significant statistical difference.

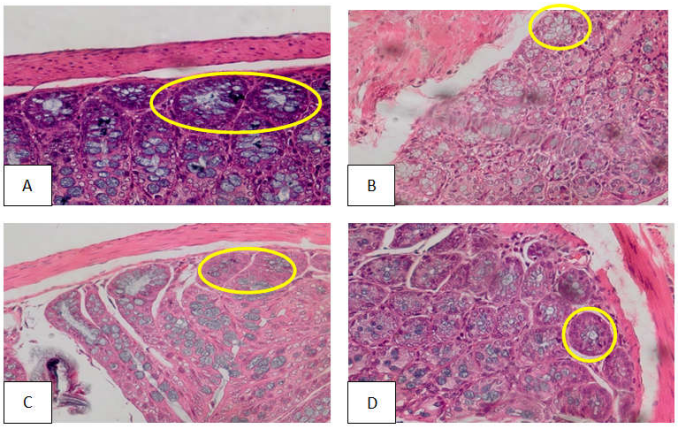

Figure 1. Inflammation comparition of control group and lunasin group. picture A shows Inflammation in control group, B low dose lunasin, C medium dose lunasin, and D high dose lunasin

Hyperplasia

Table 2 indicated that there is a decrease in the average number of focal hyperplasia in the rectum mice dinduksi by AOM and DSS after administration of lunasin. The control group showed a significant reduction when in comparison with the group that was given lunasin 20mg/kg, 30mg/kg, or 40 mg/kg. But increasing doses of lunasin as much as 10 mg/kg showed no meaningful decreased in hyperplasia because of p> 0.05. Statistically significant decrease can only be observed after increasing the dose of lunasin 20mg/kg, we can see itu from the comparation between low dose with high dose that have p=0.009.

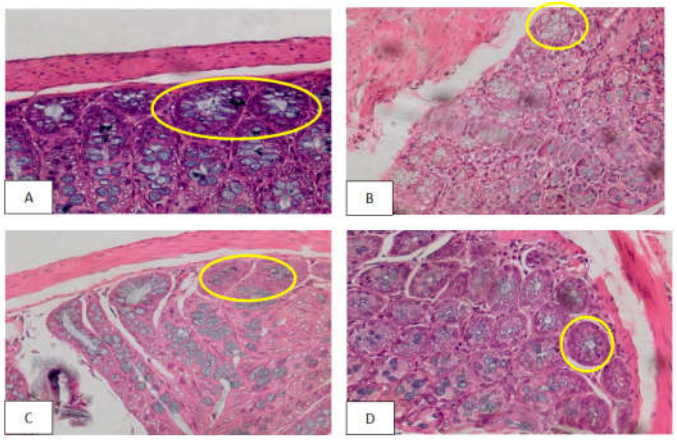

Figure 2. Hyperplasia comparition of control group and lunasin group. picture A shows hyperplasia in control group, B low dose lunasin, C medium dose lunasin, and D high dose lunasin

Angiogenesis

Angiogenesis also showed a decrease in the average after injections of lunasin. However, there are different things that indicated by a decrease in angiogenesis compared with hyperplasia and inflammatory.

Table 2 Comparation of hyperplasia focus from the rectum of mice induced by AOM and DSS that was given different dose of lunasin

Dose groups	n	Mean (focus/lpf)	p value
Control (without lunasin extract)	6	1.4000	<0.001
Low dose (20mg/kg)	6	0.7833	
Medium dose (30mg/kg)	6	0.5167	
High dose (40mg/kg)	6	0.3000	

One wayanova test. Post hoc bonferroni analysist: control vs low dose p=0.001; Control vs medium dose p<0.001; Control vs high dose p<0.001; Low dose vs medium dose p=0.336; Low dose vs high dose p=0.009; Medium dose vs high dose P=0.689.

The decrease in angiogenesis did not show significance p>0.05 when comparing control group and the group lunasn low dose (20 mg/kg) and lunasin medium dose (30 mg/kg). New meaningful value obtained when comparing the control group with lunasin high dose (40 mg/kg). In addition, increase lunasin dose of 10 mg/kg also showed a decline in the number tdak angiogenesis meaningful, since the comparison between the low dose with medium dose and medium dose with high dose showed non-significant results.

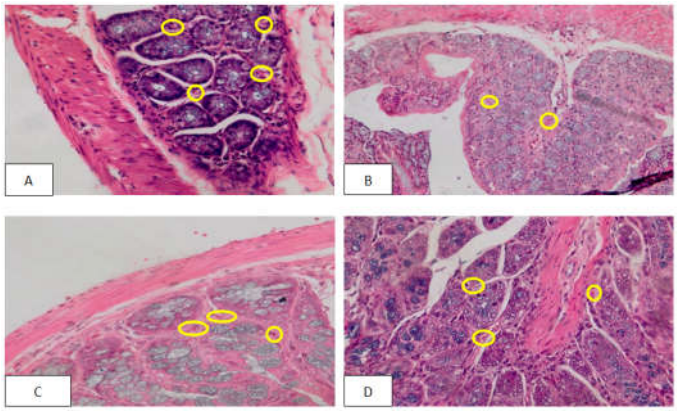

Figure 3. Angiogenesis comparition of control group and lunasin group. picture A shows angiogenesis in control group, B low dose lunasin, C medium dose lunasin, and D high dose lunasin

Table 3. Comparation of angiogenesis focus from the rectum of mice induced by AOM and DSS that was given different dose of lunasin

Dose groups	n	Mean (focus/lpf)	p value
Control (without lunasin extract)	6	0.9500	<0.001
Low dose (20mg/kg)	6	0.9000	
Medium dose (30mg/kg)	6	0.7167	
High dose (40mg/kg)	6	0.5333	

One wayanova test. Post hoc bonferroni analysist: control vs low dose p=1.00; Control vs medium dose p=0.150; Control vs high dose p=0.002; Low dose vs medium dose p=0.429; Low dose vs high dose p=0.007; Medium dose vs high dose P=0.429

DISCUSSION

Lunasin and inflammation

From the previous study done by (17), we can see that lunasin have anti-inflamatory effect. In this experiment, the result is coherent with the previous study, and from this experiment. The inflammatory reaction is showing consistent reduction after injection of lunasin extract. The inflammatory focus observed from the control group is much more compared to the inflammatory focus observeede in the 3 groups that was injected by lunasin.

This prove the anti-inflammatory activity of lunasin that works by inhibiting liposaccharide (LPS), which induces the production of pro-inflammatory mediators interleukine (IL-6), tumor necrosis factor (TNF-α) and prostaglandin E2 (PGE2) in cells RAW 264.7 through the suppression of nuclear factor (NF)-κB. It has been reported also that this protein activates anti-inflammatory activity via modulation of cyclooxygenase-2 (COX-2)/PGE2 and inducible nitric oxide synthase (iNOS)/nitric oxide pathway. Additionally, lunasin has strong antioxidant properties, reducing ROS induction by LPS-producing cells macrophages act as a catcher (scavenger) is a potent free radical.[17] Giving administration of lunasin in mice induced AOM and DSS as much as 20 mg/kg has been shown to reduce the signs of inflammation significantly compared to mice that were not given injections with lunasin. But an increase in the dosage of lunasin as much as 30 mg/kg (medium dose) showed no statistically significant difference in the reduction in the number of focal inflammation was observed compared with injection of as much as 20 mg/kg. The same result is also shown by the increase of 30 mg.kg lunasin dose to 40 mg/kg, once observed no significant differences in the amount of inflammation focus that can be observed. However, when compared to the amount of decrease in the inflammatory focus on providing lunasin low doses (20mg/kg) with lunasinadministration of high doses (40mg/kg) there was a decrease in the number of inflammatory focussigniikan statistically. This suggests that the anti-inflammatory mechanism of lunasin to inhibit lipopolysaccharide lines can indeed be improved with increased doses of lunasin, but to achieve significant reduction, dose needs to be added by more than 20mg/kg BW.

Hyperplasia

Focal hyperplasia also decreased significantly when comparing the number of focus hyperplasia in control group that was not given injection with the group that received injections of lunasin. In post hoc test all comparisons with the control group showed a significant comparaition (P <0.05).This proves the theory that lunasin can reduce the number of hyperplasia with cell apoptosis inducer and increasing the number of pro-apoptotic proteins and decrease the number of anti-apoptotic proteins. This results in increased activity of caspase-9 and caspase-3 activity (executor of apoptosis) in colon cancer cells.[17, 18] Studies with colon cancer cells HT-29 and KM12L4 show lunasin cause phase of the cell cycle G2/M arrest and induces mitochondrial apoptosis pathway .(17) Phase of the cell cycle G2/M arrest caused by the concomitant increase in p21 protein expression in colon cancer cells, HT-29, while the protein expression of p21 and p27 are both governed by the provision of lunasin in colon cancer cells KM12L4. Measurement of protein expression with the mitochondrial apoptosis pathway showed Bac lunasin affects the ratio of Bcl-2 by up-regulating pro-apoptosis and down-regulating anti-apoptotic Bcl-2. Dengna it is linked to increased expression of pro-apoptotic clusterin, which has a positive effect on the increased expression of p21. Translocation of Bax into the mitochondrial membrane resulted in cytochrome c release is indicated by increased expression of cytosolic cytochrome c in colon cancer cells KM12L4 by lunasin. This results in increased activity of caspase-9 and caspase-3 activity (executor of apoptosis) in colon cancer cells, H-29 and KM12L4 by lunasin. This mechanism shows that lunasin can induce apoptosis in human colon cancer cells.[17]

Lunasin injected dose escalation have an effect of decrease in the amount of focus hyperplasia shown. However, as seen in the appearance of focal inflammation, hyperplasia demonstrated a decrease in focus also not statistically significant ($p > 0.05$) while adding a dose of only 10 mg/kg. But the addition of a dose of 20mg/kg showed a statistically significant decrease ($p < 0.05$). This proves that the number of pro-apoptosis induced by lunasin and activity of the mitochondrial apoptosis that appears directly proportional to the level of the injected lunasin.

Angiogenesis

Angiogenesis is the physiological process through the which new blood vessels form from pre-existing vessels. Tumors induce blood vessel growth (angiogenesis) by secreting various growth factors like VEGF. Growth factors such as bFGF and VEGF can induce capillary growth into the tumor, the which some Researchers suspect supply required nutrients, allowing for tumor expansion. Unlike normal blood vessels, tumor blood vessels are dilated with an irregular shape.[18] The nature of lunasin that can reduce inflammation and act as a tumor suppressor that induces apoptosis of tumor cells in theory can also inhibit the angiogenesis by decreasing pro-inflammatory substances and apoptosis of tumor cells. However, the experimental results showed that the lunasin injection of 20mg/kg memnunjukkan no statistically significant decrease in the angiogenesis occurring ($p > 0.05$). When lunasin doses increased up to 30 mg/kg, we also have not found a significant decrease. A significant reduction happens only when lunasin dose increased up to 40 mg/kg. This suggests that the effect of lunasin in preventing angiogenesis in mice rectum induced by AOM and DSS not sepoten ability to inhibit inflammation and hyperplasia. Until now, this has not discovered lunasin mechanism that directly prevents angiogenesis. No statistically significant differences in the reduction in angiogenesis focus on giving insulin injections with a low dose (20mg/kg) or medium dose (30mg/kg). Differences arise when given a high dose (40mg/kg). This proves that lunisin may reduce angiogenesis, as described previously, but not with the same potency as an ability of lunasinto reduce inflammation and hyperplasia in the rectum.

Conclusion

After lunasintreatment is given, there was a decline of inflammation, hyperplasia, and angiogenesis in mice rectum induced by AOM and DSS. However, the addition of the treatment lunasin concentration of 10 mg/kg did not show any significant difference to the histopathology of rectum that can be observed. The difference will be significant after treated of doseis increased by a minimum of 20mg/kg for hyperplasia and inflammation, and by the minimum of 40 mg/kg for angiogenesis.

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