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4	Revision submitted	3 Juni 2021	Submit hasil revisi 1	35
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1 message

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Dear Professor Son Radu
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We are pleased to submit an original research article entitled “Banana Resistant Starch inhibitory inflammation and Cyclooxygenase-2 in BALB/c Mice induced by Azoxymethane and Dextran Sodium Sulfate” by Syafira Noor Pratiwi, Diana Nur Afifah, Nyoman Suci Widyastiti, Vega Karlowee, Gemala Anjani, and Hermawan Istiadi.

In this manuscript, our research revealed that resistant starch from Indonesian local food, *batu* and *kepok* banana, can inhibit inflammation and supressed expression of COX-2. The treatment groups with resistant starch of batu and kepok banana flour had a significantly lower level of inflammation when compared to the positive group. The COX-2 score in the treatment group resistant starch of batu and kepok banana was significantly lower than the positive control group. The COX-2 intensity in both groups was lower than the positive group but not significant. The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups also had a lower than the positive control group.

We believe that this manuscript is appropriate for publication by the *Food Research* because it very related to aims and scope this journal. Our manuscript creates a paradigm for future studies of the evolution of functional food from Indonesian local food.

We confirm that this manuscript has not been published and is not under consideration for publication elsewhere. All authors have no conflict of interest that can affect the results of this study.

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Thank you for your attention and consideration.

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Thank you for your attention and consideration.

Sincerely,

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Manuscript Type *(Please Bold)*	**Original Article** Review Short Communication Technical Notes
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Banana Resistant Starch inhibitory inflammation and Cyclooxygenase-2 in BALB/c Mice induced by Azoxymethane and Dextran Sodium Sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. Batu bananas and kepok bananas contain resistant starch which can inhibit inflammation and Cox-2 in mice colon that induce by Azoxymethane and Dextran Sodium Sulfate. This study main object was to determine the effect of banana flour resistant starch on inflammation and Cox-2 in colon tissue. This research was experimental with post-test only design involving 20 BALB/c mice in 4 groups, namely the negative control group, the positive control group, batu banana flour treatment and kepok banana flour treatment. The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of batu and kepok banana flour had a significantly lower level of inflammation when compared to the positive group

(p = 0.035). The COX-2 score in the treatment group resistant starch of batu and kepok banana was significantly lower than the positive control group (p<0.001). The COX-2 intensity in both groups was lower than the positive group but not significant (p<0.001). The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups also had a lower than the positive control group (p<0.001). Resistant starch of batu and kepok banana can inhibit inflammation and supressed expression of COX-2.

Keywords: *batu* banana flour, colon cancer, *kepok* banana flour, resistant starch

1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and was expected to increase by 60% in 2030.(Birt and Phillips, 2014) The incidence of colon cancer would increase, especially in developing countries, including Indonesia.(Tiranda and Safitriana, 2018) It was estimated that Indonesians have a colon cancer risk of 5% or 1 in 10 Indonesians was estimated to have colon cancer.(Kemenkes RI, 2017) Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the etiology of colon cancer(Leu et al., 2007) and in one study determined that about 80% of colon cancer cases were related to diet.(Le Leu, Hu and Young, 2002) Those result studies noted that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fiber that beneficial to human health including preventing colon cancer.(Tharanathan and Mahadevamma, 2003; Hovhannisyan, Aroutiounian and Glei, 2009; Purwanti and Suhartono, 2014) In the human body, RS will not be digested but will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate.(Hu et al., 2016) Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained.(Augenlicht et al., 2002) Butyrate also has chemoprotective properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis.(Augenlicht et al., 2002; Wong et al., 2006)

The source of RS is contained in Indonesian local fruit, namely bananas where the largest component of banana fruit is starch found in the pulp. Batu banana (Musa balbisiana Colla) and Kepok banana (Musa paradisiaca formatypica) were types of banana which had high resistant starch content of 39.5% and 27.7% respectively.(Musita, 2009) Method to increase the RS content is to make banana flour using the method enzymatic autoclaving-cooling autoclaving-cooling (AC-E-AC).(Fuentes-Zaragoza et al., 2010) Current studies had revealed that kepok banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase batu banana and kepok banana up to 52.95% and 55.8% respectively.(Afifah et al., 2018)

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers such as colon cancer.(Chandrasekharan and Simmons, no date) COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane produce.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS.(Hu et al., 2016) Butyrate produced by RS may play directly to the reduction of COX-2(Jahns et al., 2011) by inhibiting COX-2 transcription elongation.(Tong et al., 2005) In addition, a mechanism that might occur was butyrate affect

inflammatory mediators that play a role in transcriptional activation of COX-2.(Usami et al., 2008),(Jung et al., 2005)

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas have the possibility to protect the colon in cancer induction effect. This study aimed to prove whether the resistant starch of batu banana flour and kepok banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Feed making was carried out at the Diponegoro University Integrated Laboratory, while animal rearing experimental animals conducted at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was conducted for 11 weeks. This study had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval Ethical Clearance under number 33 / EC / H / FK-UNDIP / IV / 2019.

2.1 Experimental animals

This study used male BALB / c mice aged 5 weeks weighing about 20 to 25 grams. Animals acclimatized beforehand for 7 days and then divided into 4 groups randomly, namely the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and without any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 was the group that was given AOM and DSS induction and given feed that had been modified with banana flour batu and kepok respectively.

Animals were caged in a room that had air conditioning and had a naturally dark light settings. Feed and drink provided ad libitum. AOM induction was given as 10 mg/kg intraperitoneally. AOM was dissolved in Phosphate Buffer Solution (PBS) at a ratio of 1: 1. AOM injection taken once after acclimatization. Giving DSS 2% performed the following day for 7 days.

Batu and kepok bananas cleaned and washed with running water then peeled. Banana pulp thin cut of about 2 mm. Banana slices dried in the sun for 3 days. Dried banana pieces that had been crushed and then sieved with a 80 mesh sieve. Banana flour treated autoclaving at 121 ° C for 15 minutes and then cooling at 4 ° C for 24 hours. The pH of banana flour was adjusted with 0.2 M acetate buffer and 2% of the pullulanase enzyme was added (v / w of banana flour) then incubated at 40 ° C for 12 hours at 150 rpm. The reaction was stopped by heating the suspension at 85 ° C for 5 minutes. Banana flour wrapped in aluminum foil then treated autoclaving at 121 ° C for 15 minutes and cooling 4 ° C for 24 hours.

2.2 Immunohistochemistry

Animals terminated by dislocation of cervical done quickly and sterile. Colon fragments were fixed in 10 % Neutral Buffer Formalin and embedded in paraffin blocks. The specimen on the paraffin block serially sectioned using rotary microtome. The sections were stained with hematoxylin eosin (HE) and immunostaining COX-2 was performed according to the manufacturer's recommendations from FineTest (Wuhan, China).

Observations inflammation and COX-2 in colon tissue was done using light microscope with a magnification of 400 times. Each colon tissue sample was captured 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, negative, minimal, mild and moderate. The score was 0 or negative if there was no inflammation. The score was 1 or mild if inflammation infiltrates the mucosa. The score was 2 or moderate if inflammation infiltrates the mucosa and submucosa. The score was 3 or moderate if the inflammation infiltrates transmural (infiltrates to the tunica intima).(Erben et al., 2014)

Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 was divided into 5 scores, the score was 0 if the estimated percentage of 0% to 5%; the score was 1 if the estimated percentage of 6% to 25%; the score was 2 if the estimated percentage of 26% to 50%; the score was 3 if the estimated percentage of 51% to 75%; and the score was 4 if the estimated percentage of 76% to 100%. The intensity of staining had 3 scores, score 1 if the slight yellowing, score 2 when the brownish yellow and score 3 when brown.(Wu and Sun, 2015)

Final scores was the sum of the staining intensity score and the cell staining percentage score. The combination of the score would had 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8).(Wu and Sun, 2015)

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results and discussion/Results

Figure 1 shows that image B (positive control) had the most inflammation among other images. Inflamed tissue in treatment 1 and 2 was not worse than the positive control but more than the negative control.

Table 2 revealed that each group had a significant difference in inflammatory values (p-Value = 0.035). Positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

Table 2 revealed that the inflammation value of the positive control group was statistically significantly different from the negative control group, both treatment groups where the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group did not had a significant difference to the both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 2 where the positive control group had the highest severity percentage which had the expression of COX-2 with the category of

strongly positive as much as 84%. The both treatment groups had the lowest severity with a positive category percentage of 48% and 50% respectively.

The results of the COX-2 percentage score in colon tissue after the intervention was there a difference in the four groups. Table 4 revealed that the positive control group had the highest COX-2 percentage score compared to other groups while the treatment group 2 had the lowest COX-2 percentage score.

Table 4 revealed that the positive control group had a different COX-2 percentage score for both treatment groups where positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 lower. Both treatment groups did not have any differences, so it could be said that the administration of batu and kepok banana resistant starch had the same effect on the COX-2.

The results of COX-2 intensity between groups showed a difference between groups. However, the inter-group mean showed that the positive control group had an intensity of COX-2 were highest when compared with other groups. Treatment group 1 had the lowest COX-2 intensity value among the other.

Test results of the further test showed that the positive control group had a different intensity value significantly with other groups where other groups had a COX-2 intensity value was lower. The negative control group had a different COX-2 intensity value from the treatment group 1 and had the same COX-2 intensity value as the treatment group 2 where both treatment groups COX-2 intensity values was lower in the negative control group. The treatment group 1 had no difference intensity value of COX-2 with 2 treatment groups.

The results of the combined value of percentages with intensity of COX-2 between groups showed a differences between groups. However, the inter-group mean showed that the positive control group had a combined value percentage of the intensity of COX-2 were highest when compared to other groups. The treatment group 1 had a combined value percentage value with the intensity of COX-2 that was lowest among the other groups.

Further test results combined value of the percentage with the intensity of COX-2 showed that the positive control group has a different intensity value significantly with other groups where other groups had combined value of the percentage with the intensity of COX-2 was lower. Negative control had different values from other groups where the combined value of the percentage with the intensity of COX-2 in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences means the administration of banana and banana batu banana resistant starch had the same effect on the combined value of the percentage with the intensity of COX-2.

4. Discussion (Please omit if you are combining results and discussion)

The above results indicate that resistant starch has a protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support the study conducted by Ying Hu et al where the inflammation score decreased in experimental animals that were induced by AOM DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the

inflammation may induce chronic immune response resulting in cellular proliferation and regeneration.(Mariani, Sena and Roncucci, 2014) If the immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation continuously.(Mariani, Sena and Roncucci, 2014)

Chronic inflammation can speed up tumor formation.(Hu et al., 2016)Therefore, individuals with ulcerative colitis are at high risk of developing Colitis Associated Cancer (CAC). This is supported by previous research that shows that RS can prevent colitis and Colorectal Cancer (CRC).4.23–25 So it can be concluded that the hospital can also prevent CAC.

The results of this study are reinforced by the results of previous studies where the positive control group that was given only a standard diet had a high inflammatory score and had the number of bacteria from the genus Fusibacterium, Escherichia and Enterococcus which were also associated with CRC in humans.(Feng et al., 2015) Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumor formation.(Zackular et al., 2013) The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumor formation emerged.(Zackular et al., 2013) The crucial role of microbiotic dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer.(Vannucci et al., 2008) Hospital administration can increase bacteria associated with RS fermentation such as Parabacteroides, Ruminococcus and Bifidobacterium.(Hu et al., 2016)In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa.29.30

SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-protein coupled receptors (GPRs) or by inhibiting deactivated hibatu.(Sebastián and Mostoslavsky, 2014) GPR43 is expressed in intestinal epithelial cells and certain immune cells but the expression of GPR43 in humans with CRC and colitis is low in expression.32.33 Previous studies have shown a positive effect that a high-fiber diet activates GPR43 and is characterized by a rapid increase in acetate.(Macia, 2015)Another study showed that RS data significantly increased the expression activity of GPR43. This indicates that GPR43 activation may have a role in intestinal homeostasis.(Hu et al., 2016)Acetate and propionate have a significant inverse correlation with tumor occurrence. Acetate and propionate modulate Treg cell and immune function. The interaction between acetate and propionate with GPR43 indicates an anti-inflammatory effect of RS.35.36

The COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia.(Hu et al., 2016) RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses.(Haenen et al., 2013)

High COX-2 expression is the beginning of tumorigenesis.(Wu and Sun, 2015) This may be because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response,

inhibiting apoptosis of tumor cells, increasing cell proliferation, regulating the cell cycle, increasing tumor angiogenesis, increasing the expression of metalloproteinases in tumor cells and stimulating the activation of precursor substances that are carcinogenic. .(Wu and Sun, 2015) The role of COX-2 is reinforced by previous studies where most colon cancers have high COX-2 expression resulting in tumor angiogenesis, immune system damage and tumor invasion.(Brown and DuBois, 2005)

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES

Table 1. Composition of experimental animal feed g/100 g

Component (g)	Mice Group			
	K-	K +	P1	P2
Batu banana flour	-	-	19	-
Kepok banana flour	-	-	-	18
Corn starch	46.57	46.57	46.57	46.57
Protein (casein)	14	14	14	14
Dectrin	15.5	15.5	5.5	5.5
Sucrose	10	10	10	10
Soybean oil	4	4	4	4
Alpacel (fiber)	5	5	5	5
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1
L-cystine	0.18	0.18	0.18	0.18
Choline	0.25	0.25	0.25	0.25

Table 2**.** Inflammation after intervention

Group	Mean ± Standard Deviation	*p*-Value
K-	1.32 ± 0.33	
K +	2.48 ± 0.50	
P1	1.56 ± 0.38	0.035 *
P2	1.40 ± 0.47	

*: there is a significant difference

a, b, c: different notations in the same column indicate a significant difference

Table 3. The relationship between COX-2 expression with various treatments

Variable		COX-2				
		n	Positive	Moderately positive	Strongly positive	*p* value
Intervention						<0.001
	Control-negative	25	4 (16)	10 (40)	11 (44)	
	AOM positive control	25	0 (0)	4 (16)	21 (84)	
	AOM *batu* treatment	25	12 (48)	7 (28)	6 (24)	
	AOM *kepok* treatment	20	10 (50)	9 (45)	1 (1)	

Table 4. Expression of COX-2 after intervention

Group	COX-2 percentage score	COX-2 intensity	Score and COX-2 intensity
K-	2.52 ± 0.92a	2.48 ± 0.51a	5.00 ± 1.32a
K +	3.52 ± 0.59b	2.76 ± 0.44b	6.28 ± 0.84b
P1	1.80 ± 1.32c	2.04 ± 0.74c	3.84 ± 1.86c
P2	1.40 ± 0.88c	2.35 ± 0.49ac	3.75 ± 1.12c
p	<0.001 *	<0.001 *	<0.001 *

*: there is a significant difference

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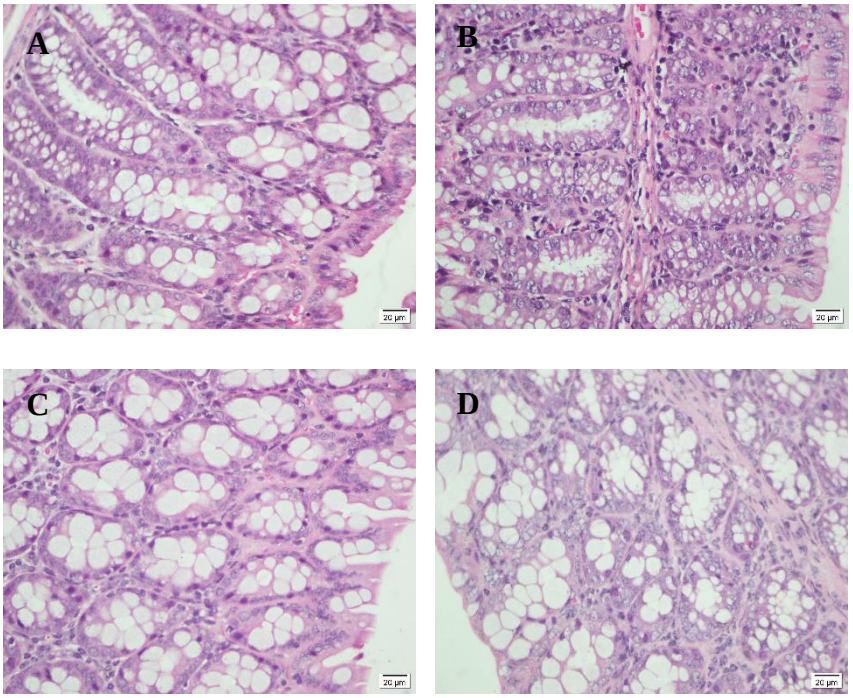

Figure 1.Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.

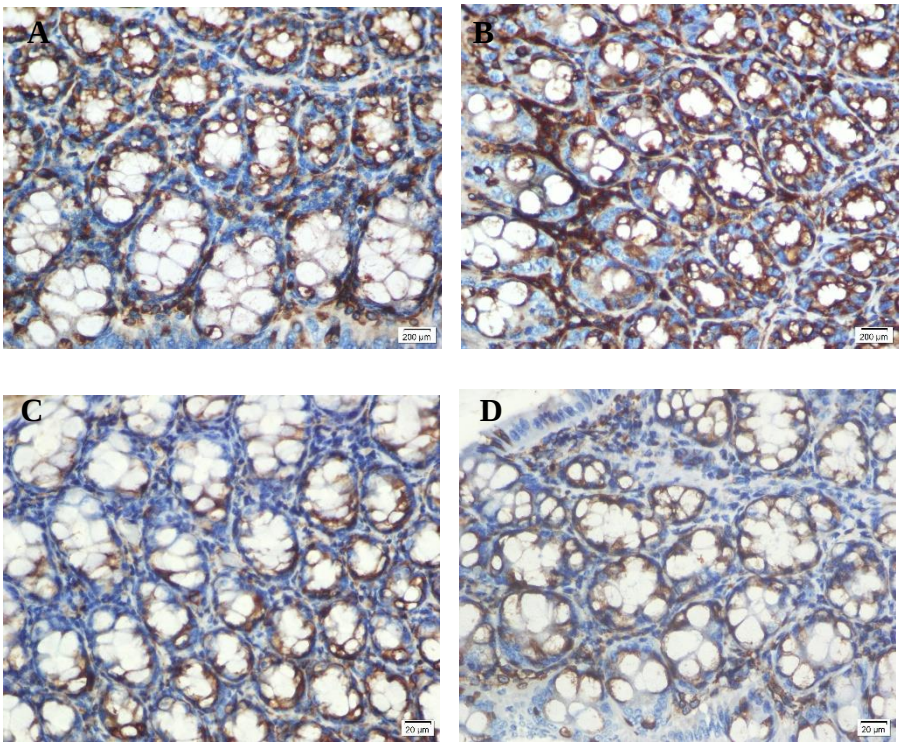

Figure 2.Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.

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Banana Resistant Starch inhibitory inflammation and Cyclooxygenase-2 in BALB/c Mice induced by Azoxymethane and Dextran Sodium Sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. Batu bananas and kepok bananas contain resistant starch which can inhibit inflammation and Cox-2 in mice colon that induce by Azoxymethane and Dextran Sodium Sulfate. This study main object was to determine the effect of banana flour resistant starch on inflammation and Cox-2 in colon tissue. This research was experimental with post-test only design involving 20 BALB/c mice in 4 groups, namely the negative control group, the positive control group, batu banana flour treatment and kepok banana flour treatment. The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of batu and kepok banana flour had a significantly lower level of inflammation when compared to the positive group ($p = 0.035$). The COX-2 score in the treatment group resistant starch of batu and kepok banana was significantly lower than the positive control group ($p<0.001$). The COX-2 intensity in both groups was lower than the positive group but not significant ($p<0.001$). The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups also had a lower than the positive control group ($p<0.001$). Resistant starch of batu and kepok banana can inhibit inflammation and suppressed expression of COX-2.

Keywords: *batu* banana flour, colon cancer, *kepok* banana flour, resistant starch

1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and was expected to increase by 60% in 2030.(Birt and Phillips, 2014) The incidence of colon cancer would increase, especially in developing countries, including Indonesia.(Tiranda and Safitriana, 2018) It was estimated that Indonesians have a colon cancer risk of 5% or 1 in 10 Indonesians was estimated to have colon cancer.(Kemenkes RI, 2017) Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the etiology of colon cancer(Leu et al., 2007) and in one study determined that about 80% of colon cancer cases were related to diet.(Le Leu, Hu and Young, 2002) Those result studies noted that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fiber that beneficial to human health including preventing colon cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan, Aroutiounian and Glei, 2009; Purwanti and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu et al., 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht et al., 2002). Butyrate also has chemoprotective properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis (Augenlicht et al., 2002; Wong et al., 2006).

The source of RS is contained in Indonesian local fruit, namely bananas where the largest component of banana fruit is starch found in the pulp. Batu banana (Musa balbisiana Colla) and Kepok banana (*Musa paradisiaca* formatypica) were types of banana which had high resistant starch content of 39.5% and 27.7% respectively.(Musita, 2009) Method to increase the RS content is to make banana flour using the method enzymatic autoclaving-cooling autoclaving-cooling (AC-E-AC).(Fuentes-Zaragoza et al., 2010) Current studies had revealed that kepok banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase batu banana and kepok banana up to 52.95% and 55.8% respectively.(Afifah et al., 2018)

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers such as colon cancer.(Chandrasekharan and Simmons, no date) COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane produce.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS.(Hu et al., 2016) Butyrate produced by RS may play directly to the reduction of COX-2(Jahns et al., 2011) by inhibiting COX-2 transcription elongation.(Tong et al., 2005) In addition, a mechanism that might occur was butyrate affect inflammatory mediators that play a role in transcriptional activation of COX-2.(Usami et al., 2008),(Jung et al., 2005)

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas have the possibility to protect the colon in cancer induction effect. This study aimed to prove whether the resistant starch of batu banana flour and kepok banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Feed making was carried out at the Diponegoro University Integrated Laboratory, while animal rearing experimental animals conducted at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was conducted for 11 weeks. This study had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval Ethical Clearance under number 33 / EC / H / FK-UNDIP / IV / 2019.

2.1 Experimental animals

This study used male BALB / c mice aged 5 weeks weighing about 20 to 25 grams. Animals acclimatized beforehand for 7 days and then divided into 4 groups randomly, namely the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and without any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 was the group that was given AOM and DSS induction and given feed that had been modified with banana flour batu and kepok respectively.

Animals were caged in a room that had air conditioning and had a naturally dark light settings. Feed and drink provided ad libitum. AOM induction was given as 10 mg/kg intraperitoneally. AOM was dissolved in Phosphate Buffer Solution (PBS) at a ratio of 1: 1. AOM injection taken once after acclimatization. Giving DSS 2% performed the following day for 7 days.

Batu and kepok bananas cleaned and washed with running water then peeled. Banana pulp thin cut of about 2 mm. Banana slices dried in the sun for 3 days. Dried banana pieces that had been crushed and then sieved with a 80 mesh sieve. Banana flour treated autoclaving at 121 ° C for 15 minutes and then cooling at 4 ° C for 24 hours. The pH of banana flour was adjusted with 0.2 M acetate buffer and 2% of the pullulanase enzyme was added (v / w of banana flour) then incubated at 40 ° C for 12 hours at 150 rpm. The reaction was stopped by heating the suspension at 85 ° C for 5 minutes. Banana flour wrapped in aluminum foil then treated autoclaving at 121 ° C for 15 minutes and cooling 4 ° C for 24 hours.

2.2 Immunohistochemistry

Animals terminated by dislocation of cervical done quickly and sterile. Colon fragments were fixed in 10 % Neutral Buffer Formalin and embedded in paraffin blocks. The specimen on the paraffin block serially sectioned using rotary microtome. The sections were stained with hematoxylin eosin (HE) and immunostaining COX-2 was performed according to the manufacturer's recommendations from FineTest (Wuhan, China).

Observations inflammation and COX-2 in colon tissue was done using light microscope with a magnification of 400 times. Each colon tissue sample was captured 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, negative, minimal, mild and moderate. The score was 0 or negative if there was no inflammation. The score was 1 or mild if inflammation infiltrates the mucosa. The score was 2 or moderate if inflammation infiltrates the mucosa and submucosa. The score was 3 or moderate if the inflammation infiltrates transmural (infiltrates to the tunica intima).(Erben et al., 2014)

Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 was divided into 5 scores, the score was 0 if the estimated percentage of 0% to 5%; the score was 1 if the estimated percentage of 6% to 25%; the score was 2 if the estimated percentage of 26% to 50%; the score was 3 if the estimated percentage of 51% to 75%; and the score was 4 if the estimated percentage of 76% to 100%. The intensity of staining had 3 scores, score 1 if the slight yellowing, score 2 when the brownish yellow and score 3 when brown.(Wu and Sun, 2015)

Final scores was the sum of the staining intensity score and the cell staining percentage score. The combination of the score would had 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8).(Wu and Sun, 2015)

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results

Figure 1 shows that image B (positive control) had the most inflammation among other images. Inflamed tissue in treatment 1 and 2 was not worse than the positive control but more than the negative control.

Table 2 revealed that each group had a significant difference in inflammatory values (p-Value = 0.035). Positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

Table 2 revealed that the inflammation value of the positive control group was statistically significantly different from the negative control group, both treatment groups where the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group did not had a significant difference to the both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 2 where the positive control group had the highest severity percentage which had the expression of COX-2 with the category of strongly positive as much as 84%. The both treatment groups had the lowest severity with a positive category percentage of 48% and 50% respectively.

The results of the COX-2 percentage score in colon tissue after the intervention was there a difference in the four groups. Table 4 revealed that the positive control group had the highest COX-2 percentage score compared to other groups while the treatment group 2 had the lowest COX-2 percentage score.

Table 4 revealed that the positive control group had a different COX-2 percentage score for both treatment groups where positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 lower. Both treatment groups did not have any differences, so it could be said that the administration of batu and kepok banana resistant starch had the same effect on the COX-2.

The results of COX-2 intensity between groups showed a difference between groups. However, the inter-group mean showed that the positive control group had an intensity of COX-2 were highest when compared with other groups. Treatment group 1 had the lowest COX-2 intensity value among the other.

Test results of the further test showed that the positive control group had a different intensity value significantly with other groups where other groups had a COX-2 intensity value was lower. The negative control group had a different COX-2 intensity value from the treatment group 1 and had the same COX-2 intensity value as the treatment group 2 where both treatment groups COX-2 intensity values was lower in the negative control group. The treatment group 1 had no difference intensity value of COX-2 with 2 treatment groups.

The results of the combined value of percentages with intensity of COX-2 between groups showed a differences between groups. However, the inter-group mean showed that the positive control group had a combined value percentage of the intensity of COX-2 were highest when compared to other groups. The treatment group 1 had a combined value percentage value with the intensity of COX-2 that was lowest among the other groups.

Further test results combined value of the percentage with the intensity of COX-2 showed that the positive control group has a different intensity value significantly with other groups where other groups had combined value of the percentage with the intensity of COX-2 was lower. Negative control had different values from other groups where the combined value of the percentage with the intensity of COX-2 in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences means the administration of banana and banana batu banana resistant starch had the same effect on the combined value of the percentage with the intensity of COX-2.

4. Discussion

The above results indicate that resistant starch has a protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support the study conducted by Ying Hu et al where the inflammation score decreased in experimental animals that were induced by AOM DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the inflammation may induce chronic immune response resulting in cellular proliferation and regeneration.(Mariani, Sena and Roncucci, 2014) If the immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation continuously.(Mariani, Sena and Roncucci, 2014)

Chronic inflammation can speed up tumor formation.(Hu et al., 2016)Therefore, individuals with ulcerative colitis are at high risk of developing Colitis Associated Cancer (CAC). This is supported by previous research that shows that RS can prevent colitis and Colorectal Cancer (CRC).4.23–25 So it can be concluded that the hospital can also prevent CAC.

The results of this study are reinforced by the results of previous studies where the positive control group that was given only a standard diet had a high inflammatory score and had the number of bacteria from the genus Fusibacterium, Escherichia and Enterococcus which were also associated with CRC in humans.(Feng et al., 2015) Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumor formation.(Zackular et al., 2013) The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumor formation emerged.(Zackular et al., 2013) The crucial role of microbiotic dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer.(Vannucci et al., 2008) Hospital administration can increase bacteria associated with RS fermentation such as Parabacteroides, Ruminococcus and Bifidobacterium.(Hu et al., 2016)In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa.29.30

SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-protein coupled receptors (GPRs) or by inhibiting deactivated hibatu.(Sebastián and Mostoslavsky, 2014) GPR43 is expressed in intestinal epithelial cells and certain immune cells but the expression of GPR43 in humans with CRC and colitis is low in expression.32.33 Previous studies have shown a positive effect that a high-fiber diet activates GPR43 and is characterized by a rapid increase in acetate.(Macia, 2015)Another study showed that RS data significantly increased the expression activity of GPR43. This indicates that GPR43 activation may have a role in intestinal homeostasis.(Hu et al., 2016)Acetate and propionate have a significant inverse correlation with tumor occurrence. Acetate and propionate modulate Treg cell and immune function. The interaction between acetate and propionate with GPR43 indicates an anti-inflammatory effect of RS.35.36

The COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia.(Hu et al., 2016) RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses.(Haenen et al., 2013)

High COX-2 expression is the beginning of tumorigenesis.(Wu and Sun, 2015) This may be because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumor cells, increasing cell proliferation, regulating the cell cycle, increasing tumor angiogenesis, increasing the expression of metalloproteinases in tumor cells and stimulating the activation of precursor substances that are carcinogenic. .(Wu and Sun, 2015) The role of COX-2 is reinforced by previous studies where most colon cancers have high COX-2 expression resulting in tumor angiogenesis, immune system damage and tumor invasion.(Brown and DuBois, 2005)

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES

Table 1. Composition of experimental animal feed g/100 g

Component (g)	Mice Group			
	K-	K +	P1	P2
Batu banana flour	-	-	19	-
Kepok banana flour	-	-	-	18
Corn starch	46.57	46.57	46.57	46.57
Protein (casein)	14	14	14	14
Dectrin	15.5	15.5	5.5	5.5
Sucrose	10	10	10	10
Soybean oil	4	4	4	4
Alpacel (fiber)	5	5	5	5
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1
L-cystine	0.18	0.18	0.18	0.18
Choline	0.25	0.25	0.25	0.25

Table 2**.** Inflammation after intervention

Group	Mean ± Standard Deviation	*p*-Value
K-	1.32 ± 0.33	
K +	2.48 ± 0.50	
P1	1.56 ± 0.38	0.035 *
P2	1.40 ± 0.47	

*: there is a significant difference

a, b, c: different notations in the same column indicate a significant difference

Table 3. The relationship between COX-2 expression with various treatments

Variable		COX-2				
		n	Positive	Moderately positive	Strongly positive	*p* value
Intervention						<0.001
	Control-negative	25	4 (16)	10 (40)	11 (44)	
	AOM positive control	25	0 (0)	4 (16)	21 (84)	
	AOM *batu* treatment	25	12 (48)	7 (28)	6 (24)	
	AOM *kepok* treatment	20	10 (50)	9 (45)	1 (1)	

Table 4. Expression of COX-2 after intervention

Group	COX-2 percentage score	COX-2 intensity	Score and COX-2 intensity
K-	2.52 ± 0.92a	2.48 ± 0.51a	5.00 ± 1.32a
K +	3.52 ± 0.59b	2.76 ± 0.44b	6.28 ± 0.84b
P1	1.80 ± 1.32c	2.04 ± 0.74c	3.84 ± 1.86c
P2	1.40 ± 0.88c	2.35 ± 0.49ac	3.75 ± 1.12c
p	<0.001 *	<0.001 *	<0.001 *

*: there is a significant difference

a, b, c: different notations in the same column indicate a significant difference

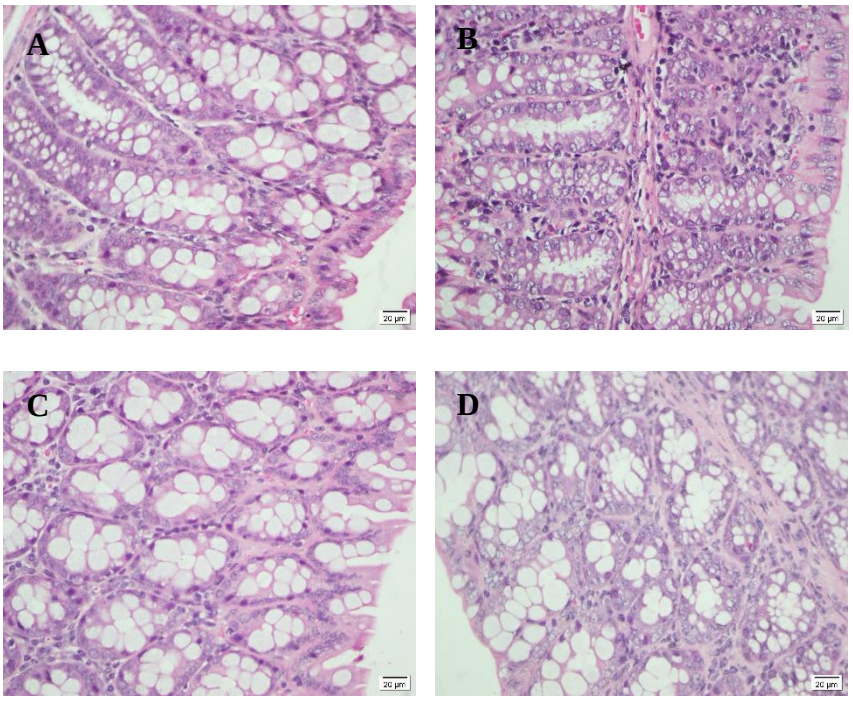

Figure 1.Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.

A B

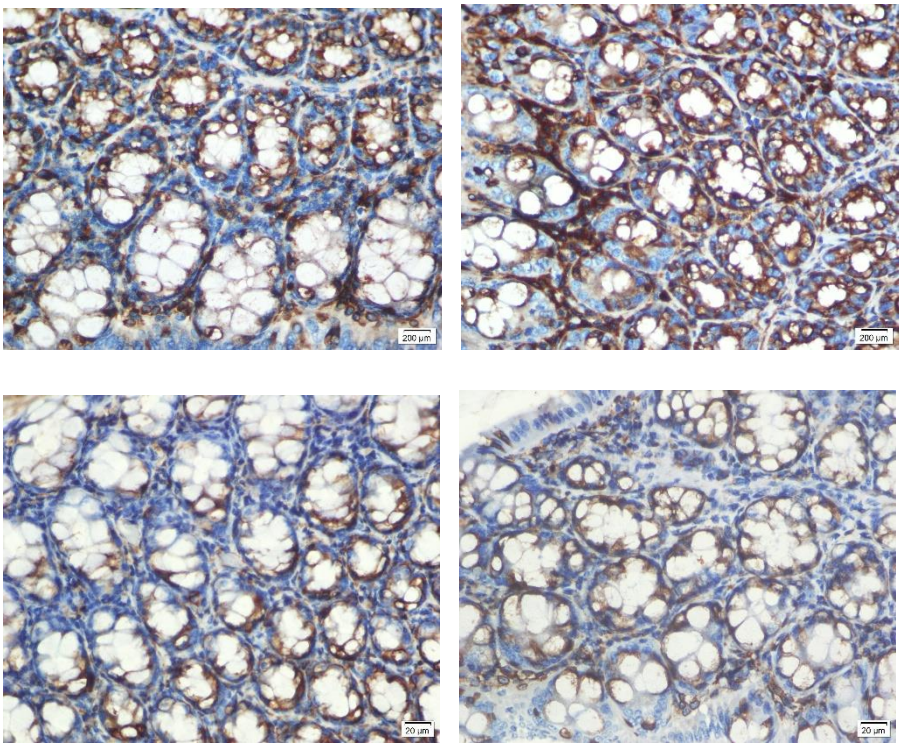

Figure 2.Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.

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Dear Professor Dr. Son Radu

Here I send the revised manuscript. I am sorry for the late reply because I need more time to revise the manuscript. Thank you.

Best regards,
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Banana ~~Resistant~~ resistant ~~Starch~~ starch inhibitory inflammation and ~~Cyclooxygenase~~cyclooxygenase-2 in BALB/c ~~Mice~~ mice induced by ~~Azoxymethane~~ azoxymethane and ~~Dextran~~ dextran ~~Sodium~~ sodium ~~Sulfate~~sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. Batu bananas and kepok bananas contain resistant starch which can inhibit inflammation and Cox-2 in mice colon that induce by Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of bananas flour toward inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice in 4 groups, negative control group, positive control group, *batu* banana treatment group, and *kepok* banana treatment group.~~This study main object was to determine the effect of banana flour resistant starch on inflammation and Cox-2 in colon tissue. This research was experimental with post-test only design involving 20 BALB/c mice in 4 groups, namely the negative control group, the positive control group, batu banana flour treatment and kepok banana flour treatment .~~ The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of *batu* and *kepok* banana flour had a significantly lower level of inflammation when compared to the positive group ($p = 0.035$). The COX-2 score in the treatment group resistant starch of *batu* and *kepok* banana was significantly lower than the positive control group ($p<0.001$). The COX-2 intensity in both groups was lower than the positive group

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but not significant (p<0.001). The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups also had a lower than the positive control group (p<0.001). Resistant starch of batu and kepok banana can inhibit inflammation and suppressed expression of COX-2.

Keywords: *batu* banana ~~flour~~, colon cancer, *kepok* banana ~~flour~~, resistant starch

1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and was expected to increase by 60% in 2030.(Birt and Phillips, 2014) The incidence of colon cancer would increase, especially in developing countries, including Indonesia ~~,~~(Tiranda and Safitriana, 2018). It was estimated that Indonesians have a colon cancer risk of 5% or 1 in 10 Indonesians was estimated to have colon cancer ~~,~~(Kemenkes RI, 2017). Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the etiology of colon cancer (Leu et al., 2007) and in one study determined that about 80% of colon cancer cases were related to diet ~~,~~(Le Leu, Hu and Young, 2002). Those result studies ~~noted~~ indicate that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fiber that beneficial to human health including preventing colon cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan, ~~*Aroutiounian and Gleiet al.*~~, 2009; Purwanti and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu *et al.*, 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht *et al.*, 2002). Butyrate also has chemoprotective properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis (Augenlicht *et al.*, 2002; Wong *et al.*, 2006).

The source of RS is contained in Indonesian local fruit, ~~namely~~ bananas where the largest component of banana fruit is starch found in the pulp. Batu banana (*Musa balbisiana* Colla) and Kepok banana (*Musa paradisiaca* formatypica) were types of banana which had high resistant starch content of 39.5% and 27.7% respectively ~~,~~(Musita, 2009). Method to increase the RS content is to make banana flour using the method enzymatic autoclaving-cooling autoclaving-cooling (AC-E-AC).(Fuentes-Zaragoza *et al.*, 2010) Current studies had revealed that kepok banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase batu banana and kepok banana up to 52.95% and 55.8% respectively.(Afifah *et al.*, 2018)

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers such as colon cancer ~~,~~(Chandrasekharan and Simmons, ~~no date~~2004). COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane produce.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS ~~,~~(Hu *et al.*, 2016). Butyrate produced by RS may play directly to the reduction of COX-2 (Jahns *et al.*, 2011) by inhibiting COX-2 transcription elongation.(Tong et al., 2005) In addition, a mechanism that might occur was butyrate affect inflammatory mediators that play a role in transcriptional activation of COX-2 ~~,~~(Usami *et al.*, 2008~~),(~~; Jung *et al.*, 2005).

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Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas have the possibility to protect the colon in cancer induction effect. This study aimed to prove whether the resistant starch of batu banana flour and kepok banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Animal food was made in the ~~Feed making was carried out at~~ the Diponegoro University Integrated Laboratory~~,~~ ~~while animal rearing e~~Experimental animals ~~conducted~~ were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was ~~conducted~~ done for 11 weeks. This ~~study~~ resesarch had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval Ethical Clearance under number 33 / EC / H / FK-UNDIP / IV / 2019.

2.1 Experimental animals

This study used male BALB / c mice aged 5 weeks weighing about 20 to 25 grams. Animals acclimatized beforehand for 7 days and then divided into 4 groups randomly, ~~namely~~ the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and without any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 was the group that was given AOM and DSS induction and given feed that had been modified with banana flour batu and kepok respectively.

Animals were caged in a room that had air conditioning and had a naturally dark light settings. ~~Feed and drink provided ad libitum.~~ They were injected by AOM induction ~~was given~~ as much as 10 mg/kg intraperitoneally~~. AOM was dissolved in Phosphate Buffer Solution (PBS) at a ratio of 1: 1. AOM injection taken once~~ after acclimatization. ~~Giving~~ DSS 2% was given~~performed~~ the following day for 7 days.

Batu and *kepok* bananas ~~cleaned~~were ~~-~~peeled and washed ~~with~~ by ~~running~~ water then peeled. Banana were ~~pulp thin~~ sliced~~cut of~~ about 2 mm~~. Banana slices~~ then were dried in the sun for 3 days. Dried banana pieces ~~that had been~~were crushed and ~~then~~ were sieved with a 80 mesh sieve. Banana flour were treated autoclaving at 121 ° C for 15 minutes and ~~then cooling~~were cooled at 4 ° C for 24 hours. The pH of banana flour was adjusted with 0.2 M acetate buffer ~~and~~ then 2% of the pullulanase enzyme was added (v / w of banana flour)~~. then~~ The banana flour were incubated at 40 ° C for 12 hours at 150 rpm. The reaction was stopped by heating the suspension at 85-~~°~~C for 5 minutes. Banana flour were wrapped in aluminum foil then ~~treated~~ were autoclaving at 121-~~°~~C for 15 minutes and ~~cooling~~ were cooled at 4~~°~~C for 24 hours.

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2.2 Immunohistochemistry

Animals were terminated by dislocation of cervical ~~done~~ quickly and sterile. The colon ~~Colon fragments~~ were fixed in 10 % Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the paraffin block serially sectioned using rotary microtome. The colon sections were stained with hematoxylin eosin (HE)~~. and immunostaining~~ Immunostaining COX-2 ~~was~~ were performed according to the manufacturer's recommendations ~~from~~ (Fine Test, (Wuhan, China).

~~Observations inflammation and COX-2 in~~in the observation of colon tissues ~~was~~ were done using light microscope with ~~a magnification of~~ 400 times magnification~~.~~ Each colon tissue sample was captured 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, ~~negative, minimal, mild and moderate. T~~the score was 0 or negative if there was no inflammation~~. T~~; the score was 1 or mild if inflammation infiltrates the mucosa~~. T~~; the score was 2 or moderate if inflammation infiltrates the mucosa and submucosa~~. T~~; the score was 3 or moderate if the inflammation infiltrates transmural (infiltrates to the tunica intima) ~~.~~(Erben *et al.*, 2014).

Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 was divided into 5 scores, the score was 0 if the estimated percentage of 0% to 5%; the score was 1 if the estimated percentage of 6% to 25%; the score was 2 if the estimated percentage of 26% to 50%; the score was 3 if the estimated percentage of 51% to 75%; and the score was 4 if the estimated percentage of 76% to 100%. The intensity of staining had 3 scores, score 1 if the slight yellowing, score 2 when the brownish yellow and score 3 when have brown colour~~.~~ (Wu and Sun, 2015).

Final scores was the sum of the staining intensity score and the cell staining percentage score. The combination of the score would had 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8)~~.~~ (Wu and Sun, 2015).

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results

Figure 1 shows that image B (positive control) had the most inflammation among other images. Tissue in treatment 1 and treatment 2 more inflamed than positive control but the negative control had the least amount of inflammation. ~~Inflamed tissue in treatment 1 and 2 was not worse than the positive control but more than the negative control.~~

Table 2 revealed that each group had a significant difference in inflammatory values (p-Value = 0.035). Positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

Table 2 revealed that the inflammation value of the positive control group was statistically significantly different from the negative control group, both treatment groups where the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group did not had a significant difference to the both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 2 where the positive control

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group had the highest severity percentage which had the expression of COX-2 with the category of strongly positive as much as 84%. The both treatment groups had ~~the~~ lower~~st~~ severity with a positive category percentage of 48% and 50% respectively.

~~The results of the COX-2 percentage score in colon tissue after the intervention was there a difference in the four groups. Table 4 revealed that the positive control group had the highest COX-2 percentage score compared to other groups while the treatment group 2 had the lowest COX-2 percentage score.~~

Table 4 revealed that the positive control group had a different COX-2 percentage score and intensity for both treatment groups where positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 lower. Both treatment groups did not have any differences, so it could be said that the administration of batu and kepok banana resistant starch had the same effect on the COX-2.

~~The results of COX-2 intensity between groups showed a difference between groups. However, the inter-group mean showed that the positive control group had an intensity of COX-2 were highest when compared with other groups. Treatment group 1 had the lowest COX-2 intensity value among the other.~~

~~Test results of the further test showed that the positive control group had a different intensity value significantly with other groups where other groups had a COX-2 intensity value was lower. The negative control group had a different COX-2 intensity value from the treatment group 1 and had the same COX-2 intensity value as the treatment group 2 where both treatment groups COX-2 intensity values was lower in the negative control group. The treatment group 1 had no difference intensity value of COX-2 with 2 treatment groups.~~

The results of the combined value ~~of~~ between percentages ~~with~~ and intensity of COX-2 between groups showed a differences ~~between groups. However, the inter-group mean showed that the positive~~ Positive control group had the highest ~~a~~ combined value ~~percentage of the intensity of COX-2 were highest~~ when compared to other groups. ~~T~~ and the treatment group 1 had the lowest ~~a~~ combined value ~~percentage value with the intensity of COX-2 that was lowest~~ among the other groups. Both treatment groups did not have any differences in combined value means the administration of banana and banana batu banana resistant starch had the same effect.

~~Further test results combined value of the percentage with the intensity of COX-2 showed that the positive control group has a different intensity value significantly with other groups where other groups had combined value of the percentage with the intensity of COX-2 was lower. Negative control had different values from other groups where the combined value of the percentage with the intensity of COX-2 in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences means the administration of banana and banana batu banana resistant starch had the same effect on the combined value of the percentage with the intensity of COX-2.~~

4. Discussion

The above results indicate that resistant starch in *batu* and *kepok* banana flours ~~has~~ may have ~~a~~ protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit

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inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support the study conducted by Ying Hu *et al* (2016) where the inflammation score decreased in experimental animals that were induced by AOM DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the inflammation may induce chronic immune response resulting in cellular proliferation ~~and regeneration.~~ ~~-~~(Mariani, ~~Sena and Roncucci~~*et al.*, 2014). If the immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation. ~~continuously.~~ (Mariani, *et al.*~~Sena and Roncucci~~, 2014).

~~Chronic inflammation can speed up tumor formation.(Hu *et al.*, 2016)Therefore, individuals with ulcerative colitis are at high risk of developing Colitis Associated Cancer (CAC). This is supported by previous research that shows that RS can prevent colitis and Colorectal Cancer (CRC).4.23–25 So it can be concluded that the hospital can also prevent CAC.~~

The results of this study are reinforced by the results of previous studies where the positive control group that was given only a standard diet had a high inflammatory score and had the number of bacteria from the genus *Fusibacterium*, *Escherichia* and *Enterococcus* which were also associated with CRC in humans ~~-~~(Feng *et al.*, 2015). Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumor formation ~~-~~(Zackular *et al.*, 2013). The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumor formation emerged ~~-~~(Zackular *et al.*, 2013). The crucial role of microbiotic dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer ~~-~~(Vannucci *et al.*, 2008). Hospital administration can increase bacteria associated with RS fermentation such as *Parabacteroides*, *Ruminococcus* and *Bifidobacterium* ~~-~~(Hu *et al.*, 2016). In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa (Wong, *et al.*, 2013; Nava and Stappenbeck, 2011).~~29.30~~

SCFAs produced by the colonic microbiota ~~-~~can stimulate cell function through activation of G-protein coupled receptors (GPRs) or ~~by inhibiting deactivated hibatu~~histone deacetylation inhibition ~~-~~(Sebastián and Mostoslavsky, 2014). GPR43 is GPR that expressed in intestinal epithelial cells and certain immune cells, but the expression is lower when ~~of GPR43 in humans with~~individual have CRC and colitis ~~is low in expression~~condition (Tang, *et al.*, 2011; Maslowski, *et al.*, 2009).~~32.33~~ Previous studies ~~have~~ had showed~~n~~ a positive effect ~~that a~~ of high-fiber diet that can increase ~~activates~~ activation of GPR43. ~~and is characterized by a rapid increase in acetate.~~(Macia, 2015). Another study showed that RS ~~data~~ can increased the activation of GPR43 expression significantly ~~increased the expression activity of GPR43~~. ~~This~~ Those studies indicates that GPR43 activation may have a role in intestinal homeostasis ~~-~~(Hu *et al.*, 2016). Furthermore, ~~A~~acetate and propionate have a significant inverse correlation with tumor occurrence~~. Acetate and propionate~~ by modulate Treg cell and immune function. ~~The interaction between acetate and propionate with GPR43~~Those indicates an anti-inflammatory effect of SCFA produced by RS fermentation by colonic microbiota ~~RS~~(Smith, *et al.*, 2013; Fukuda, *et al.*, 2011).~~35.36~~

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High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This may be because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumor cells, increasing cell proliferation, regulating the cell cycle, increasing tumor angiogenesis, increasing the expression of metalloproteinases in tumor cells and stimulating the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 was reinforced by previous studies where most colon cancers have high COX-2 expression and resulting in tumor angiogenesis, immune system damage and tumor invasion (Brown and DuBois, 2005).

~~The~~ COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia ~~.~~(Hu *et al*., 2016). RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses~~.~~(Haenen *et al*., 2013).

~~High COX-2 expression is the beginning of tumorigenesis.(Wu and Sun, 2015) This may be because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumor cells, increasing cell proliferation, regulating the cell cycle, increasing tumor angiogenesis, increasing the expression of metalloproteinases in tumor cells and stimulating the activation of precursor substances that are carcinogenic. .(Wu and Sun, 2015) The role of COX-2 is reinforced by previous studies where most colon cancers have high COX-2 expression resulting in tumor angiogenesis, immune system damage and tumor invasion.(Brown and DuBois, 2005)~~

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Conflict of interest

The authors declare no conflict of interest.

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Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES

Table 1. Composition of experimental animal feed g/100 g

Component (g)	Mice Group			
	K-	K +	P1	P2
Batu banana flour	-	-	19	-
Kepok banana flour	-	-	-	18
Corn starch	46.57	46.57	46.57	46.57
Protein (casein)	14	14	14	14
Dectrin	15.5	15.5	5.5	5.5
Sucrose	10	10	10	10
Soybean oil	4	4	4	4
Alpacel (fiber)	5	5	5	5
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1
L-cystine	0.18	0.18	0.18	0.18
Choline	0.25	0.25	0.25	0.25

Table 2**.** Inflammation after intervention

Group	Mean ± Standard Deviation	*p*-Value
K-	1.32 ± 0.33	
K +	2.48 ± 0.50	
P1	1.56 ± 0.38	0.035 *
P2	1.40 ± 0.47	

*: there is a significant difference

a, b, c: different notations in the same column indicate a significant difference

Table 3. The relationship between COX-2 expression with various treatments

Variable	COX-2				
	n	Positive	Moderately positive	Strongly positive	*p* value
Intervention					<0.001
Control-negative	25	4 (16)	10 (40)	11 (44)	
AOM positive control	25	0 (0)	4 (16)	21 (84)	
AOM *batu* treatment	25	12 (48)	7 (28)	6 (24)	
AOM *kepok* treatment	20	10 (50)	9 (45)	1 (1)	

Table 4. Expression of COX-2 after intervention

Group	COX-2 percentage score	COX-2 intensity	Score and COX-2 intensity
K-	2.52 ± 0.92a	2.48 ± 0.51a	5.00 ± 1.32a
K +	3.52 ± 0.59b	2.76 ± 0.44b	6.28 ± 0.84b
P1	1.80 ± 1.32c	2.04 ± 0.74c	3.84 ± 1.86c
P2	1.40 ± 0.88c	2.35 ± 0.49ac	3.75 ± 1.12c
p	<0.001 *	<0.001 *	<0.001 *

*: there is a significant difference

a, b, c: different notations in the same column indicate a significant difference

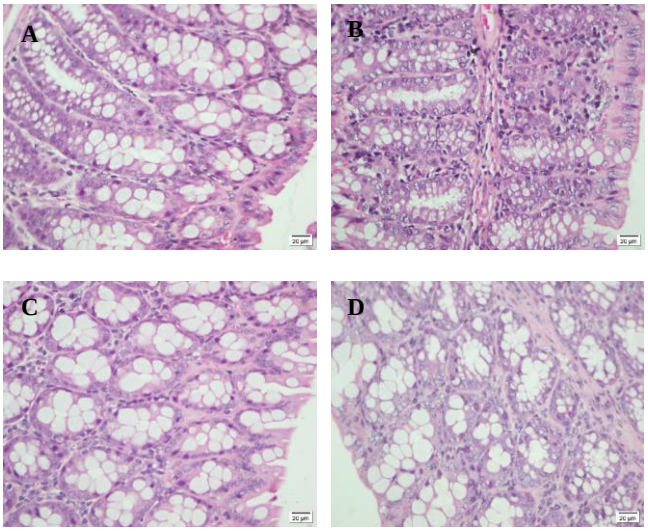

Figure 1. Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.

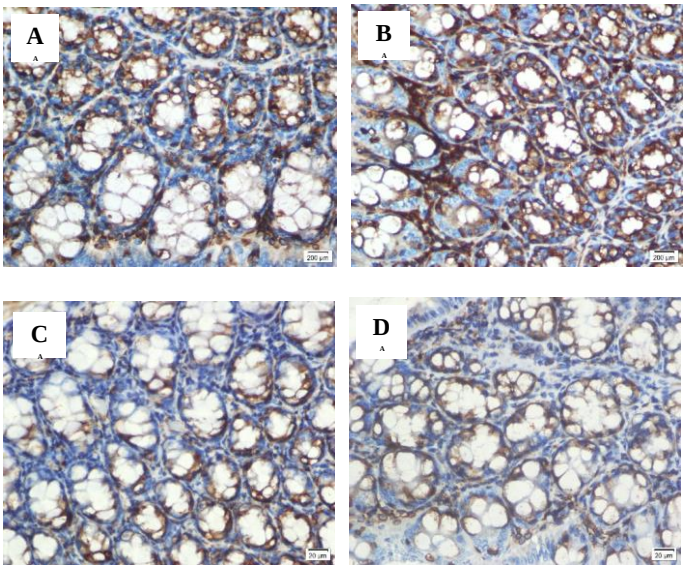

Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.

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Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. Batu bananas and kepok bananas contain resistant starch which can inhibit inflammation and Cox-2 in mice colon that induce by Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of bananas flour toward inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice in 4 groups, negative control group, positive control group, *batu* banana treatment group, and *kepok* banana treatment group. The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of *batu* and *kepok* banana flour had a significantly lower level of inflammation when compared to the positive group ($p = 0.035$). The COX-2 score in the treatment group resistant starch of *batu* and *kepok* banana was significantly lower than the positive control group ($p<0.001$). The COX-2 intensity in both groups was lower than the positive group but not significant ($p<0.001$). The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups also had a lower than the positive control group ($p<0.001$). Resistant starch of batu and kepok banana can inhibit inflammation and suppressed expression of COX-2.

Keywords: *batu* banana, colon cancer, *kepok* banana, resistant starch

1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and was expected to increase by 60% in 2030.(Birt and Phillips, 2014) The incidence of colon cancer would increase, especially in developing countries, including Indonesia (Tiranda and Safitriana, 2018). It was estimated that Indonesians have a colon cancer risk of 5% or 1 in 10 Indonesians was estimated to have colon cancer (Kemenkes RI, 2017). Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the etiology of colon cancer (Leu et al., 2007) and in one study determined that about 80% of colon cancer cases were related to diet (Le Leu, Hu and Young, 2002). Those result studies indicate that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fiber that beneficial to human health including preventing colon cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan, *et al.*, 2009; Purwanti and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu *et al*., 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht *et al*., 2002). Butyrate also has chemoprotective properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis (Augenlicht *et al*., 2002; Wong *et al*., 2006).

The source of RS is contained in Indonesian local fruit, bananas where the largest component of banana fruit is starch found in the pulp. Batu banana (*Musa balbisiana* Colla) and Kepok banana (*Musa paradisiaca* formatypica) were types of banana which had high resistant starch content of 39.5% and 27.7% respectively (Musita, 2009). Method to increase the RS content is to make banana flour using the method enzymatic autoclaving-cooling autoclaving-cooling (AC-E-AC).(Fuentes-Zaragoza *et al*., 2010) Current studies had revealed that kepok banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase batu banana and kepok banana up to 52.95% and 55.8% respectively.(Afifah *et al*., 2018)

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers such as colon cancer (Chandrasekharan and Simmons, 2004). COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane produce.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS (Hu *et al*., 2016). Butyrate produced by RS may play directly to the reduction of COX-2 (Jahns *et al*., 2011) by inhibiting COX-2 transcription elongation.(Tong et al., 2005) In addition, a mechanism that might occur was butyrate affect inflammatory mediators that play a role in transcriptional activation of COX-2 (Usami *et al*., 2008; Jung *et al*., 2005).

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas have the possibility to protect the colon in cancer induction effect. This study aimed to prove whether the resistant starch of batu banana flour and kepok banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

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2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Animal food was made in the the Diponegoro University Integrated Laboratory. Experimental animals were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was done for 11 weeks. This resesarch had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval Ethical Clearance under number 33 / EC / H / FK-UNDIP / IV / 2019.

2.1 Experimental animals

This study used male BALB / c mice aged 5 weeks weighing about 20 to 25 grams. Animals acclimatized beforehand for 7 days and then divided into 4 groups randomly, the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and without any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 was the group that was given AOM and DSS induction and given feed that had been modified with banana flour batu and kepok respectively.

Animals were caged in a room that had air conditioning and had a naturally dark light setting. They were injected by AOM induction as much as 10 mg/kg intraperitoneally after acclimatization. DSS 2% was given the following day for 7 days.

Batu and *kepok* bananas were peeled and washed by water then peeled. Banana were sliced about 2 mm then were dried in the sun for 3 days. Dried banana pieces were crushed and were sieved with a 80 mesh sieve. Banana flour were treated autoclaving at 121 ° C for 15 minutes and were cooled at 4 ° C for 24 hours. The pH of banana flour was adjusted with 0.2 M acetate buffer then 2% of the pullulanase enzyme was added (v / w of banana flour). The banana flour were incubated at 40 ° C for 12 hours at 150 rpm. The reaction was stopped by heating the suspension at 85°C for 5 minutes. Banana flour were wrapped in aluminum foil then were autoclaving at 121°C for 15 minutes and were cooled at 4°C for 24 hours.

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2.2 Immunohistochemistry

Animals were terminated by dislocation of cervical quickly and sterile. The colons were fixed in 10 % Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the paraffin block serially sectioned using rotary microtome. The colon sections were stained with hematoxylin eosin (HE). Immunostaining COX-2 were performed according to the manufacturer's recommendations (Fine Test, Wuhan, China).

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in the observation of colon tissues were done using light microscope with 400 times magnification Each colon tissue sample was captured 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, the score was 0 or negative if there was no inflammation; the score was 1 or mild if inflammation infiltrates the mucosa; the score was 2 or moderate if inflammation infiltrates the mucosa and submucosa; the score was 3 or moderate if the inflammation infiltrates transmural (infiltrates to the tunica intima) (Erben *et al*., 2014).

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Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 was divided into 5 scores, the score was 0 if the estimated percentage of 0% to 5%; the score was 1 if the estimated percentage of 6% to 25%; the score was 2 if the estimated percentage of 26% to 50%; the score was 3 if the

estimated percentage of 51% to 75%; and the score was 4 if the estimated percentage of 76% to 100%. The intensity of staining had 3 scores, score 1 if the slight yellowing, score 2 when the brownish yellow and score 3 when have brown colour (Wu and Sun, 2015).

Final scores was the sum of the staining intensity score and the cell staining percentage score. The combination of the score would had 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8) (Wu and Sun, 2015).

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results

Figure 1 shows that image B (positive control) had the most inflammation among other images. Tissue in treatment 1 and treatment 2 more inflamed than positive control but the negative control had the least amount of inflammation.

Table 2 revealed that each group had a significant difference in inflammatory values (p-Value = 0.035). Positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

Table 2 revealed that the inflammation value of the positive control group was statistically significantly different from the negative control group, both treatment groups where the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group did not had a significant difference to the both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 2 where the positive control group had the highest severity percentage which had the expression of COX-2 with the category of strongly positive as much as 84%. The both treatment groups had lower severity with a positive category percentage of 48% and 50% respectively.

Table 4 revealed that the positive control group had a different COX-2 percentage score and intensity for both treatment groups where positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 lower. Both treatment groups did not have any differences, so it could be said that the administration of batu and kepok banana resistant starch had the same effect on the COX-2.

The results of the combined value between percentages and intensity of COX-2 between groups showed a differences. Positive control group had the highest combined value when compared to

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other groups and the treatment group 1 had the lowest combined value among the other groups. Both treatment groups did not have any differences in combined value means the administration of banana and banana batu banana resistant starch had the same effect.

4. Discussion

The above results indicate that resistant starch in *batu* and *kepok* banana flours may have protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support the study conducted by Ying Hu *et al* (2016) where the inflammation score decreased in experimental animals that were induced by AOM DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the inflammation may induce chronic immune response resulting in cellular proliferation. (Mariani, *et al*., 2014). If the immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation. (Mariani, *et al*., 2014).

The results of this study are reinforced by the results of previous studies where the positive control group that was given only a standard diet had a high inflammatory score and had the number of bacteria from the genus *Fusibacterium*, *Escherichia* and *Enterococcus* which were also associated with CRC in humans (Feng *et al*., 2015). Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumor formation (Zackular *et al*., 2013). The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumor formation emerged (Zackular *et al*., 2013). The crucial role of microbiotic dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer (Vannucci *et al*., 2008). Hospital administration can increase bacteria associated with RS fermentation such as *Parabacteroides*, *Ruminococcus* and *Bifidobacterium* (Hu *et al*., 2016). In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa (Wong, *et al*., 2013; Nava and Stappenbeck, 2011).

SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-protein coupled receptors (GPRs) or histone deacetylation inhibition (Sebastián and Mostoslavsky, 2014). GPR43 is GPR that expressed in intestinal epithelial cells and certain immune cells, but the expression is lower when individual have CRC and colitis condition (Tang, *et al*., 2011; Maslowski, *et al*., 2009). Previous studies had showed a positive effect of high-fiber diet that can increase activation of GPR43.(Macia, 2015). Another study showed that RS can increased the activation of GPR43 expression significantly. Those studies indicates that GPR43 activation may have a role in intestinal homeostasis (Hu *et al*., 2016). Furthemore, acetate and propionate have a significant inverse correlation with tumor occurrence by modulate Treg cell and immune function. Those indicates an anti-inflammatory effect of SCFA produced by RS fermentation by colonic microbiota (Smith, *et al*., 2013; Fukuda, *et al*., 2011).

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High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This may be because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumor cells, increasing cell proliferation, regulating the cell cycle, increasing tumor angiogenesis, increasing the expression of metalloproteinases in tumor cells and stimulating the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 was reinforced by previous studies where most colon cancers have high COX-2 expression and resulting in tumor angiogenesis, immune system damage and tumor invasion (Brown and DuBois, 2005).

COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia (Hu *et al*., 2016). RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses (Haenen *et al*., 2013).

Conflict of interest

The authors declare no conflict of interest.

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Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES

Table 1. Composition of experimental animal feed g/100 g

Component (g)	Mice Group			
	K-	K +	P1	P2
Batu banana flour	-	-	19	-
Kepok banana flour	-	-	-	18
Corn starch	46.57	46.57	46.57	46.57
Protein (casein)	14	14	14	14
Dectrin	15.5	15.5	5.5	5.5
Sucrose	10	10	10	10
Soybean oil	4	4	4	4
Alpacel (fiber)	5	5	5	5
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1
L-cystine	0.18	0.18	0.18	0.18
Choline	0.25	0.25	0.25	0.25

Table 2**.** Inflammation after intervention

Group	Mean ± Standard Deviation	*p*-Value
K-	1.32 ± 0.33	
K +	2.48 ± 0.50	
P1	1.56 ± 0.38	0.035 *
P2	1.40 ± 0.47	

*: there is a significant difference

a, b, c: different notations in the same column indicate a significant difference

Table 3. The relationship between COX-2 expression with various treatments

Variable	COX-2				
	n	Positive	Moderately positive	Strongly positive	*p* value
Intervention					<0.001
Control-negative	25	4 (16)	10 (40)	11 (44)	
AOM positive control	25	0 (0)	4 (16)	21 (84)	
AOM *batu* treatment	25	12 (48)	7 (28)	6 (24)	
AOM *kepok* treatment	20	10 (50)	9 (45)	1 (1)	

Table 4. Expression of COX-2 after intervention

Group	COX-2 percentage score	COX-2 intensity	Score and COX-2 intensity
K-	2.52 ± 0.92a	2.48 ± 0.51a	5.00 ± 1.32a
K +	3.52 ± 0.59b	2.76 ± 0.44b	6.28 ± 0.84b
P1	1.80 ± 1.32c	2.04 ± 0.74c	3.84 ± 1.86c
P2	1.40 ± 0.88c	2.35 ± 0.49ac	3.75 ± 1.12c
p	<0.001 *	<0.001 *	<0.001 *

*: there is a significant difference

a, b, c: different notations in the same column indicate a significant difference

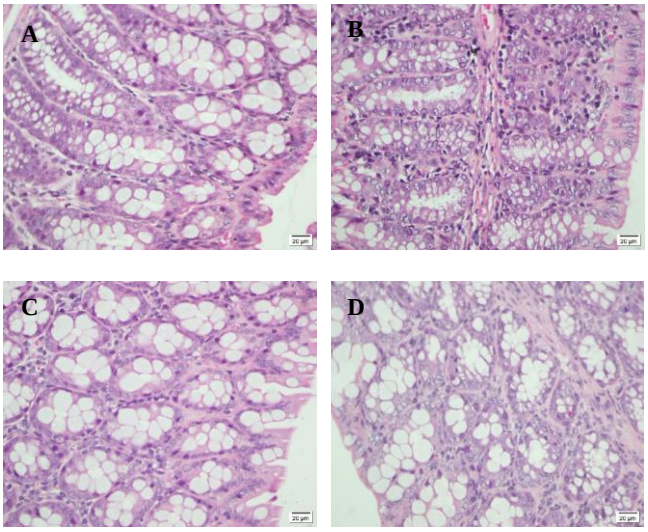
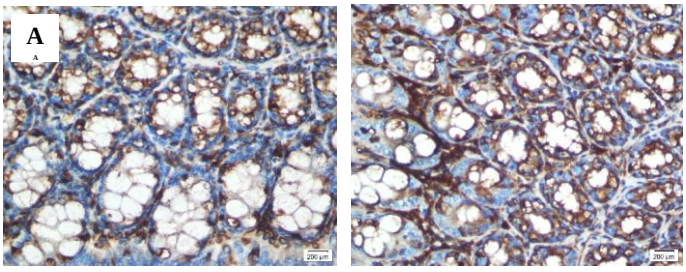


Figure 1**.** Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.



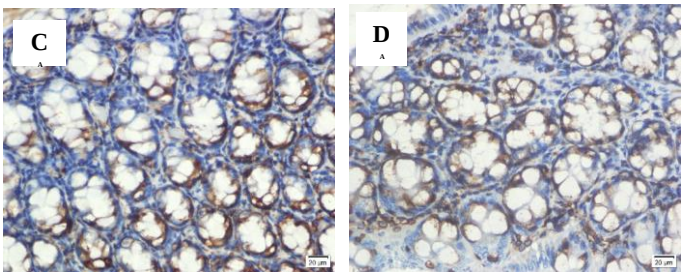


Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.

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Dear Prof. Son Radu

I already revised the manuscript based on the Food Research format. Here is the revised manuscript. Thank you.

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Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. Batu bananas and kepok bananas contain resistant starch which can inhibit inflammation and Cox-2 in mice colon that induce by Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of bananas flour toward inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice in 4 groups, negative control group, positive control group, *batu* banana treatment group, and *kepok* banana treatment group. The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of *batu* and *kepok* banana flour had a significantly lower level of inflammation when compared to the positive group ($p = 0.035$). The COX-2 score in the treatment group resistant starch of *batu* and *kepok* banana was significantly lower than the positive control group ($p<0.001$). The COX-2 intensity in both groups was lower than the positive group but not significant ($p<0.001$). The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups also had a lower than the positive control group ($p<0.001$). Resistant starch of batu and kepok banana can inhibit inflammation and suppressed expression of COX-2.

Keywords: *batu* banana, colon cancer, *kepok* banana, resistant starch

1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and was expected to increase by 60% in 2030.(Birt and Phillips, 2014) The incidence of colon cancer would increase, especially in developing countries, including Indonesia (Tiranda and Safitriana, 2018). It was estimated that Indonesians have a colon cancer risk of 5% or 1 in 10 Indonesians was estimated to have colon cancer (Kemenkes ~~RI~~, 2017). Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the etiology of colon cancer (Leu et al., 2007) and in one study determined that about 80% of colon cancer cases were related to diet (Le Leu, Hu and Young, 2002). Those result studies indicate that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fiber that beneficial to human health including preventing colon cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan~~,~~ *et al.*, 2009; Purwanti and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu *et al*., 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht *et al*., 2002). Butyrate also has chemoprotective properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis (Augenlicht *et al*., 2002; Wong *et al*., 2006).

The source of RS is contained in Indonesian local fruit, bananas where the largest component of banana fruit is starch found in the pulp. Batu banana (*Musa balbisiana* Colla) and Kepok banana (*Musa paradisiaca* formatypica) were types of banana which had high resistant starch content of 39.5% and 27.7% respectively (Musita, 2009). Method to increase the RS content is to make banana flour using the method enzymatic autoclaving-cooling autoclaving-cooling (AC-E-AC).(Fuentes-Zaragoza *et al*., 2010) Current studies had revealed that kepok banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase batu banana and kepok banana up to 52.95% and 55.8% respectively~~.~~ (Afifah *et al*., 2018).

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers such as colon cancer (Chandrasekharan and Simmons, 2004). COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane produce.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS (Hu *et al*., 2016). Butyrate produced by RS may play directly to the reduction of COX-2 (Jahns *et al*., 2011) by inhibiting COX-2 transcription elongation.(Tong et al., 2005) In addition, a mechanism that might occur was butyrate affect inflammatory mediators that play a role in transcriptional activation of COX-2 (~~Usami *et al*., 2008;~~ Jung *et al*., 2005; Usami *et al*., 2008).

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas have the possibility to protect the colon in cancer induction effect. This study aimed to prove whether the resistant starch of batu banana flour and kepok banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

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2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Animal food was made in the the Diponegoro University Integrated Laboratory. Experimental animals were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was done for 11 weeks. This resesarch had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval Ethical Clearance under number 33 / EC / H / FK-UNDIP / IV / 2019.

2.1 Experimental animals

This study used male BALB / c mice aged 5 weeks weighing about 20 to 25 grams. Animals acclimatized beforehand for 7 days and then divided into 4 groups randomly, the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and without any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 was the group that was given AOM and DSS induction and given feed that had been modified with banana flour batu and kepok respectively.

Animals were caged in a room that had air conditioning and had a naturally dark light setting. They were injected by AOM induction as much as 10 mg/kg intraperitoneally after acclimatization. DSS 2% was given the following day for 7 days.

Batu and *kepok* bananas were peeled and washed by water then peeled. Banana were sliced about 2 mm then were dried in the sun for 3 days. Dried banana pieces were crushed and were sieved with a 80 mesh sieve. Banana flour were treated autoclaving at 121 ° C for 15 minutes and were cooled at 4 ° C for 24 hours. The pH of banana flour was adjusted with 0.2 M acetate buffer then 2% of the pullulanase enzyme was added (v / w of banana flour). The banana flour were incubated at 40 ° C for 12 hours at 150 rpm. The reaction was stopped by heating the suspension at 85°C for 5 minutes. Banana flour were wrapped in aluminum foil then were autoclaving at 121°C for 15 minutes and were cooled at 4°C for 24 hours.

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2.2 Immunohistochemistry

Animals were terminated by dislocation of cervical quickly and ~~sterile~~sterilized. The colons were fixed in 10 % Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the paraffin block serially sectioned using rotary microtome. The colon sections were stained with hematoxylin eosin (HE). Immunostaining COX-2 were performed according to the manufacturer's recommendations (Fine Test, Wuhan, China).

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~~In~~ In the observation of colon tissues were done using light microscope with 400 times magnification. Each colon tissue sample was captured 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, the score was 0 or negative if there was no inflammation; the score was 1 or mild if inflammation infiltrates the mucosa; the score was 2 or moderate if inflammation infiltrates the mucosa and submucosa; the score was 3 or moderate if the inflammation infiltrates transmural (infiltrates to the tunica intima) (Erben *et al*., 2014).

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Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 was divided into 5 scores, the score was 0 if the estimated percentage of 0% to 5%; the score was 1 if the estimated percentage of 6% to 25%; the score was 2 if the estimated percentage of 26% to 50%; the score was 3 if the

estimated percentage of 51% to 75%; and the score was 4 if the estimated percentage of 76% to 100%. The intensity of staining had 3 scores, score 1 if the tissue have slight ~~yellowing~~yellow colour, score 2 when the brownish yellow and score 3 when have brown colour (Wu and Sun, 2015).

Final scores was the sum of the staining intensity score and the cell staining percentage score. The combination of the score would had 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8) (Wu and Sun, 2015).

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results

Figure 1 shows that image B (positive control) had the most inflammation among other images. Tissue in treatment 1 and treatment 2 more inflamed than positive control but the negative control had the least amount of inflammation. Table 2 revealed that each group had a significant difference in inflammatory values (p-Value = 0.035). Positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

~~Table 2 revealed that each group had a significant difference in inflammatory values (p-Value = 0.035). Positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.~~

~~Table 2 revealed~~Furthermore, that the inflammation value of the positive control group was statistically significantly different from the negative control group, both treatment groups where the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group did not had a significant difference to the both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 2 where the positive control group had the highest severity percentage which had the expression of COX-2 with the category of strongly positive as much as 84%. ~~The b~~Both treatment groups had lower severity with a positive category percentage of 48% and 50% respectively.

Table 4 revealed that the positive control group had a different COX-2 percentage score and intensity for both treatment groups where positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 lower. Both treatment groups did not have any differences, so it could

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be said that the administration of batu and kepok banana resistant starch had the same effect on the COX-2.

The results of the combined value between percentages and intensity of COX-2 between groups showed ~~a~~ differences. Positive control group had the highest combined value when compared to other groups and the treatment group 1 had the lowest combined value among the other groups. Both treatment groups did not have any differences in combined value means the administration of banana and banana batu banana resistant starch had the same effect.

4. Discussion

The above results indicate that resistant starch in *batu* and *kepok* banana flours may have protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support the study conducted by ~~Ying~~ Hu *et al*, (2016) where the inflammation score decreased in experimental animals that were induced by AOM DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the inflammation may induce chronic immune response resulting in cellular proliferation. (Mariani, *et al*., 2014). If the immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation. (Mariani, *et al*., 2014).

The results of this study are reinforced by the results of previous studies where the positive control group that was given only a standard diet had a high inflammatory score and had the number of bacteria from the genus *Fusibacterium*, *Escherichia* and *Enterococcus* which were also associated with CRC in humans (Feng *et al*., 2015). Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumor formation (Zackular *et al*., 2013). The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumor formation emerged (Zackular *et al*., 2013). The crucial role of microbiotic dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer (Vannucci *et al*., 2008). Hospital administration can increase bacteria associated with RS fermentation such as *Parabacteroides*, *Ruminococcus* and *Bifidobacterium* (Hu *et al*., 2016). In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa (Wong, *et al*., 2013; Nava and Stappenbeck, 2011).

SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-protein coupled receptors (GPRs) or histone deacetylation inhibition (Sebastián and Mostoslavsky, 2014). GPR43 is GPR that expressed in intestinal epithelial cells and certain immune cells, but the expression is lower when individual have CRC and colitis condition (Tang, *et al*., 2011; Maslowski, *et al*., 2009). Previous studies had showed a positive effect of high-fiber diet that can increase activation of GPR43.(Macia, 2015). Another study showed that RS can increased the activation of GPR43 expression significantly. Those studies indicates that GPR43 activation may have a role in

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intestinal homeostasis (Hu *et al*., 2016). Furthemore, acetate and propionate have a significant inverse correlation with tumor occurrence by modulate Treg cell and immune function. Those indicates an anti-inflammatory effect of SCFA produced by RS fermentation by colonic microbiota (Smith, *et al*., 2013; Fukuda, *et al*., 2011).

High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This may be because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumor cells, increasing cell proliferation, regulating the cell cycle, increasing tumor angiogenesis, increasing the expression of metalloproteinases in tumor cells and stimulating the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 was reinforced by previous studies where most colon cancers have high COX-2 expression and resulting in tumor angiogenesis, immune system damage and tumor invasion (Brown and DuBois, 2005).

COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia (Hu *et al*., 2016). RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses (Haenen *et al*., 2013).

Conflict of interest

The authors declare no conflict of interest.

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Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES

Table 1. Composition of experimental animal feed g/100 g

Component (g)	Mice Group			
	K-	K +	P1	P2
Batu banana flour	-	-	19	-
Kepok banana flour	-	-	-	18
Corn starch	46.57	46.57	46.57	46.57
Protein (casein)	14	14	14	14
Dectrin	15.5	15.5	5.5	5.5
Sucrose	10	10	10	10
Soybean oil	4	4	4	4
Alpacel (fiber)	5	5	5	5
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1
L-cystine	0.18	0.18	0.18	0.18
Choline	0.25	0.25	0.25	0.25

Table 2**.** Inflammation after intervention

Group	Mean ± Standard Deviation	*p*-Value
K-	1.32 ± 0.33	
K +	2.48 ± 0.50	
P1	1.56 ± 0.38	0.035 *
P2	1.40 ± 0.47	

*: there is a significant difference

a, b, c: different notations in the same column indicate a significant difference

Table 3. The relationship between COX-2 expression with various treatments

Variable	COX-2				
	n	Positive	Moderately positive	Strongly positive	*p* value
Intervention					<0.001
Control-negative	25	4 (16)	10 (40)	11 (44)	
AOM positive control	25	0 (0)	4 (16)	21 (84)	
AOM *batu* treatment	25	12 (48)	7 (28)	6 (24)	
AOM *kepok* treatment	20	10 (50)	9 (45)	1 (1)	

Table 4. Expression of COX-2 after intervention

Group	COX-2 percentage score	COX-2 intensity	Score and COX-2 intensity
K-	2.52 ± 0.92a	2.48 ± 0.51a	5.00 ± 1.32a
K +	3.52 ± 0.59b	2.76 ± 0.44b	6.28 ± 0.84b
P1	1.80 ± 1.32c	2.04 ± 0.74c	3.84 ± 1.86c
P2	1.40 ± 0.88c	2.35 ± 0.49ac	3.75 ± 1.12c
p	<0.001 *	<0.001 *	<0.001 *

*: there is a significant difference

a, b, c: different notations in the same column indicate a significant difference

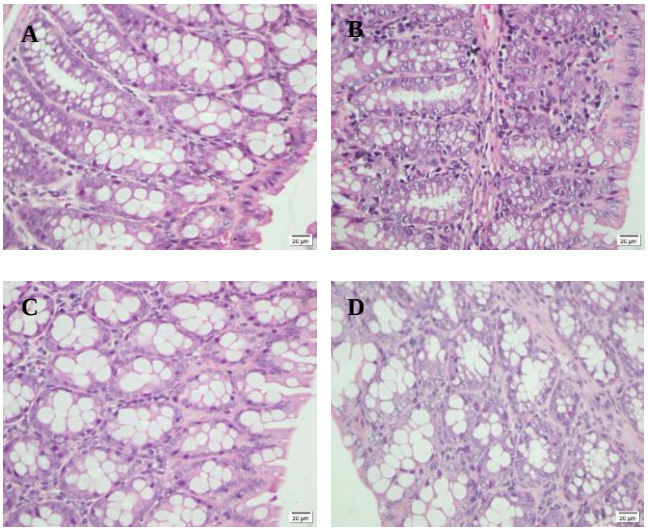
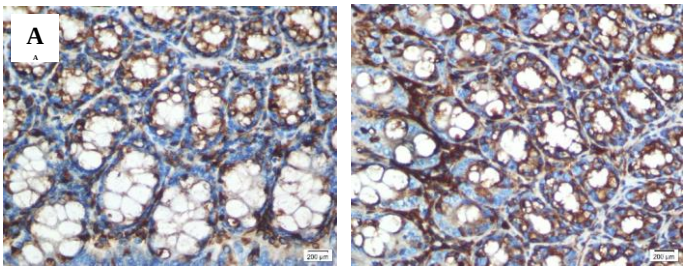


Figure 1**.** Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.



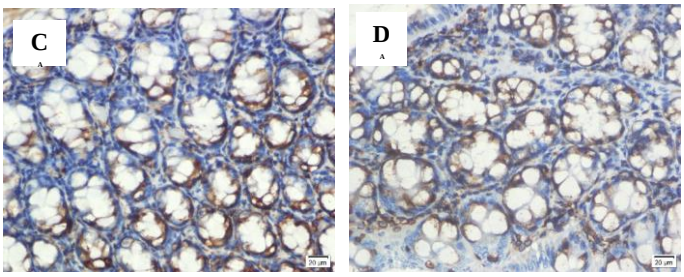


Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.

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Thank you for your fine contribution. We look forward to your continued contributions to the Journal.

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Food Research, is pleased to inform you that the following manuscript has been accepted for publication in Food Research journal.

Manuscript Title	:	Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate
Authors	:	Pratiwi, S.N., Afifah, D.N., Widyastiti, S.W., Karlowee, V., Anjani, G. and Istiadi, H.

We thank you for your fine contribution to the Food Research journal and encourage you to submit other articles to the Journal.

Yours sincerely,

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Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. *Batu* bananas and *kepok* bananas

contain resistant starch which can inhibit inflammation and Cox-2 in mice colon that are induced by Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of banana flour on inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice in 4 groups, negative control group, positive control group, *batu* banana treatment group, and *kepok* banana treatment group. The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of *batu* and *kepok* banana flour had a significantly lower level of inflammation when compared to the positive group ($p = 0.035$). The COX-2 score in the treatment group resistant starch of *batu* and *kepok* banana was significantly lower than the positive control group ($p<0.001$). The COX-2 intensity in both groups was lower than the positive group but not significant ($p<0.001$). The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups was also had lower than the positive control group ($p<0.001$). Resistant starch of *batu* and *kepok* banana can inhibit inflammation and suppressed the expression of COX-2.

Keywords: *batu* banana, Colon cancer, *kepok* banana, Resistant starch

1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and was expected to increase by 60% in 2030 (Birt and Phillips, 2014). The incidence of colon cancer would increase, especially in developing countries, including Indonesia (Tiranda and Safitriana, 2018). It was estimated that Indonesians have a colon cancer risk by 5% or 1 in 10 Indonesians was estimated to have colon cancer (Kemenkes, 2017). Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the aetiology of colon cancer (Le Leu *et al*., 2007) and one study determined that about 80% of colon cancer cases were related to diet (Le Leu *et al.*, 2002). The results indicate that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fibre that is beneficial to human health including preventing colon cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan *et al.*, 2009; Purwanti and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu *et al*., 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht *et al*., 2002). Butyrate also has chemoprotective

properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis (Augenlicht *et al*., 2002; Wong *et al*., 2006).

The source of RS is contained in the Indonesian local bananas where the largest component of its starch was found in the pulp. *Batu* banana (*Musa balbisiana* Colla) and *Kepok* banana (*Musa paradisiaca* formatypica) were types of banana that had high resistant starch content of 39.5% and 27.7%, respectively (Musita, 2009). The method used to increase the RS content in banana flour was through autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) (Fuentes-Zaragoza *et al*., 2010). Current studies had revealed that *kepok* banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase the RS in *batu* banana and *kepok* banana up to 52.95% and 55.8% respectively (Afifah *et al*., 2018).

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers (Chandrasekharan and Simmons, 2004). COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane production.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS (Hu *et al*., 2016). Butyrate produced by RS may directly influence the reduction of COX-2 (Jahns *et al*., 2011) by inhibiting COX-2 transcription elongation (Tong *et al*., 2005). In addition, a mechanism that might occur was the effect of inflammatory mediators that play a role in the transcriptional activation of COX-2 (Jung *et al*., 2005; Usami *et al*., 2008).

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas can protect the colon in the cancer induction effect. This study aimed to prove whether the resistant starch of *batu* banana flour and *kepok* banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Animal food was made in the Diponegoro University Integrated Laboratory. Experimental animals were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was done for 11 weeks. This research had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval for Ethical Clearance under number 33/EC/H/FK-UNDIP/IV/2019.

2.1 Experimental animals

This study used male BALB/c mice aged 5 weeks weighing about 20 to 25 g. The animals were acclimatized beforehand for 7 days, then divided into 4 groups randomly, the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 were groups given AOM and DSS induction and feed that had been modified with banana flour *batu* and *kepok* respectively.

Animals were caged in a room that had air conditioning and had a naturally dark setting. They were injected by AOM induction as much as 10 mg/kg intraperitoneally after acclimatization. DSS 2% was given the following day for 7 days.

Batu and *kepok* bananas were peeled and washed with water. The bananas were sliced to about 2 mm thin then dried in the sun for 3 days. Dried banana pieces were crushed and sieved with an 80 mesh sieve. Banana flour was treated by autoclaving it at 121°C for 15 mins and were cooled at 4°C for 24 hrs. The pH of banana flour was adjusted with 0.2 M acetate buffer then 2% of pullulanase enzyme was added (v/w of banana flour). The banana flour was incubated at 40°C for 12 hrs at 150 rpm. The reaction was stopped by heating the suspension at 85°C for 5 mins. Banana flour was wrapped in aluminium foil then were autoclaved at 121°C for 15 mins and were cooled at 4°C for 24 hrs.

2.2 Immunohistochemistry

Animals were quickly terminated by dislocation of the cervical and sterilized. The colons were fixed in 10% Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the paraffin block was serially sectioned using a rotary microtome. The colon sections were stained with hematoxylin-eosin (HE). Immunostaining COX-2 was performed according to the manufacturer's recommendations (Fine Test, Wuhan, China).

The observation of colon tissues was done using a light microscope with 400× magnification. Each colon tissue sample was captured with 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, the score was 0 or negative if there was no inflammation; the score was 1 or mild if inflammation infiltrates the mucosa; the score was 2 or moderate if inflammation infiltrates the mucosa and submucosa; the score was 3 or moderate if the inflammation infiltrates transmural (infiltrates to the tunica intima) (Erben *et al*., 2014).

Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 were divided into 5 scores, the score was 0 if the estimated percentage was 0% to 5%; the score was 1 if the estimated percentage was 6% to 25%, the score was 2 if the estimated percentage was 26% to 50%, the score was 3 if the estimated percentage was 51% to 75% and the score was 4 if the estimated percentage was 76% to 100%. The intensity of staining had 3 scores, score 1 if the tissue has a slightly yellow colour, score 2 when it has a brownish-yellow colour and score 3 when it is brown (Wu and Sun, 2015).

The final scores were the sum of the staining intensity score and the cell staining percentage score. The combination of the score would have 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8) (Wu and Sun, 2015).

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by using the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results

Figure 1 shows that image B (positive control) was the most inflamed among other images. Tissue in treatment 1 and treatment 2 was more inflamed than the positive control but the negative control had the least amount of inflammation. Table 2 revealed that each group had a significant difference in inflammatory values (p-Value = 0.035). The positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

Furthermore, the inflammation value of the positive control group was significantly different from the negative control group, both treatment groups displayed the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group

did not have a significant difference in both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow colour in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 2 where the positive control group had the highest severity percentage which had the expression of COX-2 with the category of strongly positive as much as 84%. Both treatment groups had lower severity with a positive category percentage of 48% and 50%, respectively.

Table 4 reveals that the positive control group had a different COX-2 percentage score and intensity for both treatment groups where the positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences, thus, it could be said that the administration of *batu* and *kepok* banana resistant starch had the same effect on the COX-2.

The results of the combined value between percentages and intensity of COX-2 between groups showed differences. The positive control group had the highest combined value when compared to other groups and treatment group 1 had the lowest combined value among the other groups. Both treatment groups did not have any differences in combined value means the administration of *kepok* banana and *batu* banana resistant starch had the same effect.

4. Discussion

The above results indicate that resistant starch in *batu* and *kepok* banana flours may have a protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support Hu *et al.* (2016) where the inflammation score decreased in experimental animals that were induced by AOM and DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the inflammation may induce chronic immune response resulting in cellular proliferation (Mariani *et al*., 2014). If the immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation (Mariani *et al*., 2014).

The results of this study are reinforced by previous studies where the positive control group that was given only a standard diet had a high inflammatory score and the number of bacteria from the genus *Fusobacterium*, *Escherichia* and *Enterococcus* were also associated with CRC in humans (Feng *et al*., 2015). Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumour formation (Zackular *et al*., 2013). The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumour formation emerged (Zackular *et al*., 2013). The crucial role of microbiota dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer (Vannucci *et al*., 2008). Hospital administration can increase bacteria associated with RS fermentation such as *Parabacteroides*, *Ruminococcus* and *Bifidobacterium* (Hu *et al*., 2016). In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa (Nava and Stappenbeck, 2011; Wong, *et al*., 2013).

SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-protein coupled receptors (GPRs) or histone deacetylation inhibition (Sebastián and Mostoslavsky, 2014). GPR43 is GPR that was expressed at a lower concentration in the intestinal epithelial cells and certain immune cells when an individual has CRC and colitis condition (Maslowski, *et al*., 2009; Tang *et al*., 2011). Previous studies had shown that a high-fibre diet has a positive effect that can increase the activation of GPR43 (Macia *et al.*, 2015). Another study showed that RS can increase the activation of GPR43 expression significantly. Those studies indicate that GPR43 activation may have a role in intestinal homeostasis (Hu *et al*., 2016). Furthermore, acetate and propionate have a significant inverse correlation with tumour occurrence by modulating Treg cell and immune function. Those indicate an anti-inflammatory effect of SCFA produced by RS fermentation by colonic microbiota (Fukuda, *et al*., 2011; Smith, *et al*., 2013).

High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This is because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumour cells, increasing cell proliferation, regulating the cell cycle, increasing tumour angiogenesis, increasing the expression of metalloproteinases in tumour cells and stimulating the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 was reinforced by previous studies where most colon cancers have high COX-2 expression and result in tumour angiogenesis, immune system damage and tumour invasion (Brown and DuBois, 2005).

COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia (Hu *et al.,* 2016). RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses (Haenen *et al.,* 2013).

Conflict of interest

The authors declare no conflict of interest.

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Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES

Table 1. Composition of experimental animal feed g/100 g

Component (g)	Mice Group			
	K-	K +	P1	P2
Batu banana flour	-	-	19	-
Kepok banana flour	-	-	-	18
Corn starch	46.57	46.57	46.57	46.57
Protein (casein)	14	14	14	14
Dectrin	15.5	15.5	5.5	5.5
Sucrose	10	10	10	10
Soybean oil	4	4	4	4
Alpacel (fiber)	5	5	5	5
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1
L-cystine	0.18	0.18	0.18	0.18
Choline	0.25	0.25	0.25	0.25

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Table 2**.** Inflammation after intervention

Group	Mean ± Standard Deviation	*p*-Value
K-	1.32 ± 0.33	
K +	2.48 ± 0.50	
P1	1.56 ± 0.38	0.035 *
P2	1.40 ± 0.47	

*Statistically significant difference ($p<0.05$)

a, b, c: different notations in the same column indicate a significant difference

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Table 3. The relationship between COX-2 expression with various treatments

Variable		COX-2			
	n	Positive	Moderately positive	Strongly positive	*p* value
Intervention					<0.001
Control-negative	25	4 (16)	10 (40)	11 (44)	
AOM positive control	25	0 (0)	4 (16)	21 (84)	
AOM *batu* treatment	25	12 (48)	7 (28)	6 (24)	
AOM *kepok* treatment	20	10 (50)	9 (45)	1 (1)	

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Table 4. Expression of COX-2 after intervention

Group	COX-2 percentage score	COX-2 intensity	Score and COX-2 intensity
K-	2.52 ± 0.92a	2.48 ± 0.51a	5.00 ± 1.32a
K +	3.52 ± 0.59b	2.76 ± 0.44b	6.28 ± 0.84b
P1	1.80 ± 1.32c	2.04 ± 0.74c	3.84 ± 1.86c
P2	1.40 ± 0.88c	2.35 ± 0.49ac	3.75 ± 1.12c
p	<0.001 *	<0.001 *	<0.001 *

*Statistically significant difference

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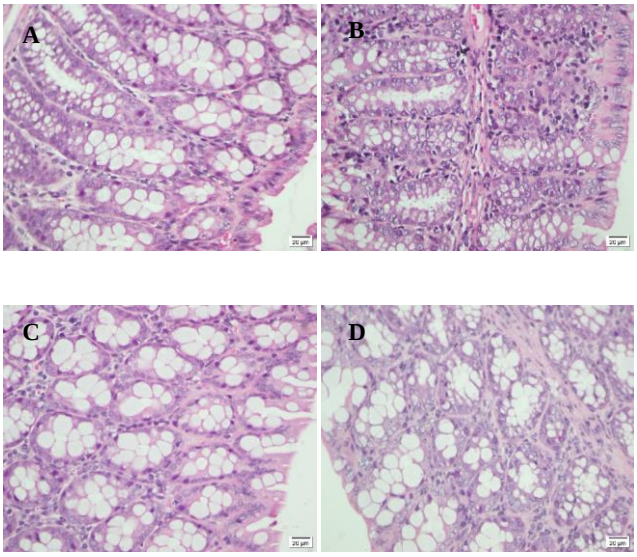

Figure 1**.** Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.

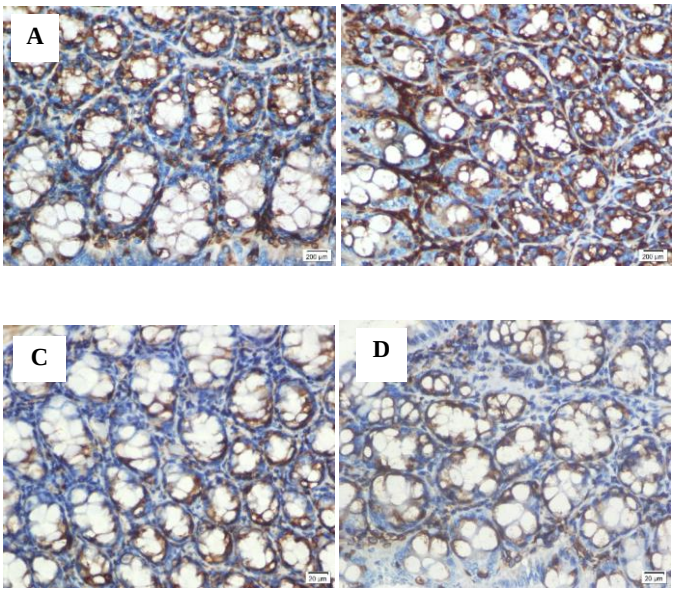

Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.

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Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. *Batu* bananas and *kepok* bananas

contain resistant starch which can inhibit inflammation and Cox-2 in mice colon that are induced by Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of banana flour on inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice in 4 groups, negative control group, positive control group, *batu* banana treatment group, and *kepok* banana treatment group. The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of *batu* and *kepok* banana flour had a significantly lower level of inflammation when compared to the positive group ($p = 0.035$). The COX-2 score in the treatment group resistant starch of *batu* and *kepok* banana was significantly lower than the positive control group ($p<0.001$). The COX-2 intensity in both groups was lower than the positive group but not significant ($p<0.001$). The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups was also had lower than the positive control group ($p<0.001$). Resistant starch of *batu* and *kepok* banana can inhibit inflammation and suppressed the expression of COX-2.

Keywords: *batu* banana, Colon cancer, *kepok* banana, Resistant starch

1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and was expected to increase by 60% in 2030 (Birt and Phillips, 2014). The incidence of colon cancer would increase, especially in developing countries, including Indonesia (Tiranda and Safitriana, 2018). It was estimated that Indonesians have a colon cancer risk by 5% or 1 in 10 Indonesians was estimated to have colon cancer (Kemenkes, 2017). Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the aetiology of colon cancer (Le Leu *et al*., 2007) and one study determined that about 80% of colon cancer cases were related to diet (Le Leu *et al.*, 2002). The results indicate that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fibre that is beneficial to human health including preventing colon cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan *et al.*, 2009; Purwanti and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu *et al*., 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht *et al*., 2002). Butyrate also has chemoprotective

properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis (Augenlicht *et al*., 2002; Wong *et al*., 2006).

The source of RS is contained in the Indonesian local bananas where the largest component of its starch was found in the pulp. *Batu* banana (*Musa balbisiana* Colla) and *Kepok* banana (*Musa paradisiaca* formatypica) were types of banana that had high resistant starch content of 39.5% and 27.7%, respectively (Musita, 2009; Afifah *et al.*, 2020). The method used to increase the RS content in banana flour was through autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) (Fuentes-Zaragoza *et al*., 2010; Ratnasari *et al*., 2018; Afifah *et al*., 2018). Current studies had revealed that *kepok* banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase the RS in *batu* banana and *kepok* banana up to 52.95% and 55.8% respectively (Afifah *et al*., 2021a).

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers (Chandrasekharan and Simmons, 2004). COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane production.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS (Hu *et al*., 2016). Butyrate produced by RS may directly influence the reduction of COX-2 (Jahns *et al*., 2011; Afifah *et al*., 2021b) by inhibiting COX-2 transcription elongation (Tong *et al*., 2005). In addition, a mechanism that might occur was the effect of inflammatory mediators that play a role in the transcriptional activation of COX-2 (Jung *et al*., 2005; Usami *et al*., 2008).

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas can protect the colon in the cancer induction effect. This study aimed to prove whether the resistant starch of *batu* banana flour and *kepok* banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Animal food was made in the Diponegoro University Integrated Laboratory. Experimental animals were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was done for 11 weeks. This research had ethical approval from the Ethics

Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval for Ethical Clearance under number 33/EC/H/FK-UNDIP/IV/2019.

2.1 Experimental animals

This study used male BALB/c mice aged 5 weeks weighing about 20 to 25 g. The animals were acclimatized beforehand for 7 days, then divided into 4 groups randomly, the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 were groups given AOM and DSS induction and feed that had been modified with banana flour *batu* and *kepok* respectively. The composition of feed for each group can be seen within the Table 1.

Animals were caged in a room that had air conditioning and had a naturally dark setting. They were injected by AOM induction as much as 10 mg/kg intraperitoneally after acclimatization. DSS 2% was given the following day for 7 days.

Batu and *kepok* bananas were peeled and washed with water. The bananas were sliced to about 2 mm thin then dried in the sun for 3 days. Dried banana pieces were crushed and sieved with an 80 mesh sieve. Banana flour was treated by autoclaving it at 121°C for 15 mins and were cooled at 4°C for 24 hrs. The pH of banana flour was adjusted with 0.2 M acetate buffer then 2% of pullulanase enzyme was added (v/w of banana flour). The banana flour was incubated at 40°C for 12 hrs at 150 rpm. The reaction was stopped by heating the suspension at 85°C for 5 mins. Banana flour was wrapped in aluminium foil then were autoclaved at 121°C for 15 mins and were cooled at 4°C for 24 hrs.

2.2 Immunohistochemistry

Animals were quickly terminated by dislocation of the cervical and sterilized. The colons were fixed in 10% Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the paraffin block was serially sectioned using a rotary microtome. The colon sections were stained with hematoxylin-eosin (HE). Immunostaining COX-2 was performed according to the manufacturer's recommendations (Fine Test, Wuhan, China).

The observation of colon tissues was done using a light microscope with 400× magnification. Each colon tissue sample was captured with 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, the score was 0 or negative if there was no inflammation; the score

was 1 or mild if inflammation infiltrates the mucosa; the score was 2 or moderate if inflammation infiltrates the mucosa and submucosa; the score was 3 or moderate if the inflammation infiltrates transmural (infiltrates to the tunica intima) (Erben *et al*., 2014).

Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 were divided into 5 scores, the score was 0 if the estimated percentage was 0% to 5%; the score was 1 if the estimated percentage was 6% to 25%, the score was 2 if the estimated percentage was 26% to 50%, the score was 3 if the estimated percentage was 51% to 75% and the score was 4 if the estimated percentage was 76% to 100%. The intensity of staining had 3 scores, score 1 if the tissue has a slightly yellow colour, score 2 when it has a brownish-yellow colour and score 3 when it is brown (Wu and Sun, 2015).

The final scores were the sum of the staining intensity score and the cell staining percentage score. The combination of the score would have 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8) (Wu and Sun, 2015).

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by using the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results

Figure 1 shows that image B (positive control) was the most inflamed among other images. Tissue in treatment 1 and treatment 2 was more inflamed than the positive control but the negative control had the least amount of inflammation. Table 2 revealed that each group had a significant difference in inflammatory values (p-Value = 0.035). The positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

Furthermore, the inflammation value of the positive control group was significantly different from the negative control group, both treatment groups displayed the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group did not have a significant difference in both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow colour in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 3 where the positive control group had the highest severity percentage which had the expression of COX-2 with the category of strongly positive as much as 84%. Both treatment groups had lower severity with a positive category percentage of 48% and 50%, respectively.

Table 4 reveals that the positive control group had a different COX-2 percentage score and intensity for both treatment groups where the positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences, thus, it could be said that the administration of *batu* and *kepok* banana resistant starch had the same effect on the COX-2.

The results of the combined value between percentages and intensity of COX-2 between groups showed differences. The positive control group had the highest combined value when compared to other groups and treatment group 1 had the lowest combined value among the other groups. Both treatment groups did not have any differences in combined value means the administration of *kepok* banana and *batu* banana resistant starch had the same effect.

4. Discussion

The above results indicate that resistant starch in *batu* and *kepok* banana flours may have a protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support Hu *et al.* (2016) where the inflammation score decreased in experimental animals that were induced by AOM and DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the inflammation may induce chronic immune response resulting in cellular proliferation (Mariani *et al*., 2014). If the

immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation (Mariani *et al*., 2014).

The results of this study are reinforced by previous studies where the positive control group that was given only a standard diet had a high inflammatory score and the number of bacteria from the genus *Fusobacterium*, *Escherichia* and *Enterococcus* were also associated with CRC in humans (Feng *et al*., 2015). Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumour formation (Zackular *et al*., 2013). The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumour formation emerged (Zackular *et al*., 2013). The crucial role of microbiota dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer (Vannucci *et al*., 2008). Hospital administration can increase bacteria associated with RS fermentation such as *Parabacteroides*, *Ruminococcus* and *Bifidobacterium* (Hu *et al*., 2016). In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa (Nava and Stappenbeck, 2011; Wong, *et al*., 2013).

SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-protein coupled receptors (GPRs) or histone deacetylation inhibition (Sebastián and Mostoslavsky, 2014). GPR43 is GPR that was expressed at a lower concentration in the intestinal epithelial cells and certain immune cells when an individual has CRC and colitis condition (Maslowski, *et al*., 2009; Tang *et al*., 2011). Previous studies had shown that a high-fibre diet has a positive effect that can increase the activation of GPR43 (Macia *et al.*, 2015). Another study showed that RS can increase the activation of GPR43 expression significantly. Those studies indicate that GPR43 activation may have a role in intestinal homeostasis (Hu *et al*., 2016). Furthermore, acetate and propionate have a significant inverse correlation with tumour occurrence by modulating Treg cell and immune function. Those indicate an anti-inflammatory effect of SCFA produced by RS fermentation by colonic microbiota (Fukuda, *et al*., 2011; Smith, *et al*., 2013).

High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This is because COX-2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumour cells, increasing cell proliferation, regulating the cell cycle, increasing tumour angiogenesis, increasing the expression of metalloproteinases in tumour cells and stimulating the activation of

precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 was reinforced by previous studies where most colon cancers have high COX-2 expression and result in tumour angiogenesis, immune system damage and tumour invasion (Brown and DuBois, 2005).

COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia (Hu *et al.*, 2016). RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses (Haenen *et al*., 2013).

Conflict of interest

The authors declare no conflict of interest.

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Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES

Table 1. Composition of experimental animal feed g/100 g

Component (g)	Mice Group			
	K-	K +	P1	P2
Batu banana flour	-	-	19	-
Kepok banana flour	-	-	-	18
Corn starch	46.57	46.57	46.57	46.57
Protein (casein)	14	14	14	14
Dectrin	15.5	15.5	5.5	5.5
Sucrose	10	10	10	10
Soybean oil	4	4	4	4
Alpacel (fiber)	5	5	5	5
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1
L-cystine	0.18	0.18	0.18	0.18
Choline	0.25	0.25	0.25	0.25

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Table 2**.** Inflammation after intervention

Group	Mean ± Standard Deviation	*p*-Value
K-	1.32 ± 0.33[a]	
K +	2.48 ± 0.50[b]	
P1	1.56 ± 0.38[c]	0.035 *
P2	1.40 ± 0.47[c]	

*Statistically significant difference (p<0.05)

a, b, c: different notations in the same column indicate a significant difference

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Table 3. The relationship between COX-2 expression with various treatments

Variable		COX-2			*p* value
	n	Positive	Moderately positive	Strongly positive	
Intervention					<0.001
Control-negative	25	4 (16)	10 (40)	11 (44)	
AOM positive control	25	0 (0)	4 (16)	21 (84)	
AOM *batu* treatment	25	12 (48)	7 (28)	6 (24)	
AOM *kepok* treatment	20	10 (50)	9 (45)	1 (1)	

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Table 4. Expression of COX-2 after intervention

Group	COX-2 percentage score	COX-2 intensity	Score and COX-2 intensity
K-	2.52 ± 0.92[a]	2.48 ± 0.51[a]	5.00 ± 1.32[a]
K +	3.52 ± 0.59[b]	2.76 ± 0.44[b]	6.28 ± 0.84[b]
P1	1.80 ± 1.32[c]	2.04 ± 0.74[c]	3.84 ± 1.86[c]
P2	1.40 ± 0.88[c]	2.35 ± 0.49[ac]	3.75 ± 1.12[c]
p	<0.001 *	<0.001 *	<0.001 *

*Statistically significant difference

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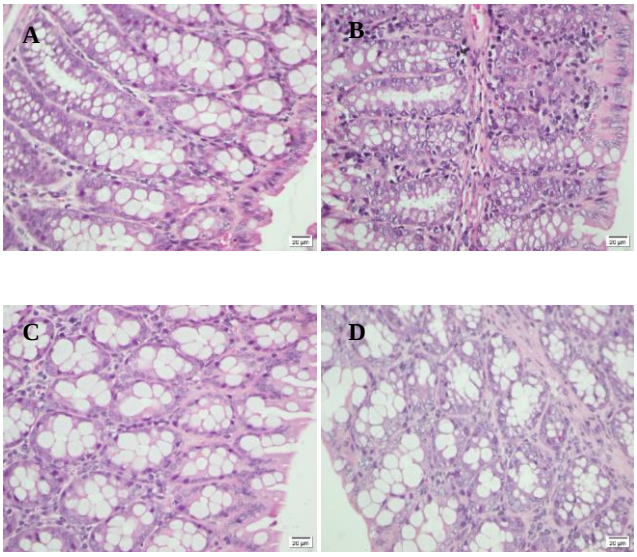

Figure 1**.** Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.

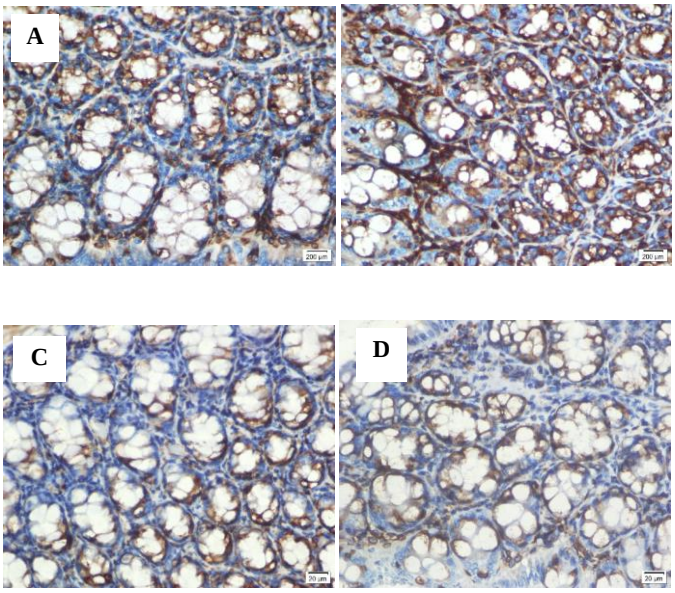

Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification 400x; B: positive control group, magnification 400x; C: treatment group 1, magnification 400x; D: treatment group 2, magnification 400x.

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FULL PAPER

Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. *Batu* bananas and *kepok* bananas contain resistant starch which can inhibit inflammation and COX-2 in mice colon that are induced by Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of banana flour on inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice in 4 groups, negative control group, positive control group, *batu* banana treatment group, and *kepok* banana treatment group. The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of *batu* and *kepok* banana flour had a significantly lower level of inflammation when compared to the positive group ($p = 0.035$). The COX-2 score in the treatment group resistant starch of *batu* and *kepok* banana was significantly lower than the positive control group ($p<0.001$). The COX-2 intensity in both groups was lower than the positive group but not significant ($p<0.001$). The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups was also had lower than the positive control group ($p<0.001$). Resistant starch of *batu* and *kepok* banana can inhibit inflammation and suppressed the expression of COX-2.

1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and was expected to increase by 60% in 2030 (Birt and Phillips, 2014). The incidence of colon cancer would increase, especially in developing countries, including Indonesia (Tiranda and Safitriana, 2018). It was estimated that Indonesians have a colon cancer risk by 5% or 1 in 10 Indonesians was estimated to have colon cancer (Kemenkes, 2017). Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the aetiology of colon cancer (Le Leu *et al*., 2007) and one study determined that about 80% of colon cancer cases were related to diet (Le Leu *et al.*, 2002). The results indicate that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fibre that is beneficial to human health including preventing colon cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan *et al.*, 2009; Purwanti and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu *et al*., 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht *et al*., 2002). Butyrate also has chemoprotective properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis (Augenlicht *et al*., 2002; Wong *et al*., 2006).

The source of RS is contained in the Indonesian local bananas where the largest component of its starch was found in the pulp. *Batu* banana (*Musa balbisiana* Colla) and *Kepok* banana (*Musa paradisiaca* formatypica) were types of banana that had high resistant starch content of 39.5% and 27.7%, respectively (Musita, 2009; Afifah *et al.*, 2020). The method used to increase the RS content in

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banana flour was through autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) (Fuentes-Zaragoza *et al*., 2010; Ratnasari *et al*., 2018; Afifah *et al*., 2018). Current studies had revealed that *kepok* banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase the RS in *batu* banana and *kepok* banana up to 52.95% and 55.8% respectively (Afifah *et al*., 2021a).

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers (Chandrasekharan and Simmons, 2004). COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane production.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS (Hu *et al*., 2016). Butyrate produced by RS may directly influence the reduction of COX-2 (Jahns *et al*., 2011; Afifah *et al*., 2021b) by inhibiting COX-2 transcription elongation (Tong *et al*., 2005). In addition, a mechanism that might occur was the effect of inflammatory mediators that play a role in the transcriptional activation of COX-2 (Jung *et al*., 2005; Usami *et al*., 2008).

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas can protect the colon in the cancer induction effect. This study aimed to prove whether the resistant starch of *batu* banana flour and *kepok* banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Animal food was made in the Diponegoro University Integrated Laboratory. Experimental animals were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was done for 11 weeks. This research had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval for Ethical Clearance under number 33/ EC/H/FK-UNDIP/IV/2019.

2.1 Experimental animals

This study used male BALB/c mice aged 5 weeks weighing about 20 to 25 g. The animals were acclimatized beforehand for 7 days, then divided into 4 groups randomly, the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 were groups given AOM and DSS induction and feed that had been modified with banana flour *batu* and *kepok* respectively. The composition of feed for each group can be seen within the Table 1.

Table 1. Composition of experimental animal feed g/100 g

Component (g)	Mice Group			
	K-	K +	P1	P2
Batu banana flour	-	-	19	-
Kepok banana flour	-	-	-	18
Corn starch	46.57	46.57	46.57	46.57
Protein (casein)	14	14	14	14
Dectrin	15.5	15.5	5.5	5.5
Sucrose	10	10	10	10
Soybean oil	4	4	4	4
Alpacel (fiber)	5	5	5	5
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1
L-cystine	0.18	0.18	0.18	0.18
Choline	0.25	0.25	0.25	0.25

Animals were caged in a room that had air conditioning and had a naturally dark setting. They were injected by AOM induction as much as 10 mg/kg intraperitoneally after acclimatization. DSS 2% was given the following day for 7 days.

Batu and *kepok* bananas were peeled and washed with water. The bananas were sliced to about 2 mm thin then dried in the sun for 3 days. Dried banana pieces were crushed and sieved with an 80 mesh sieve. Banana flour was treated by autoclaving it at 121°C for 15 mins and were cooled at 4°C for 24 hrs. The pH of banana flour was adjusted with 0.2 M acetate buffer then 2% of pullulanase enzyme was added (v/w of banana flour). The banana flour was incubated at 40°C for 12 hrs at 150 rpm. The reaction was stopped by heating the suspension at 85°C for 5 mins. Banana flour was wrapped in aluminium foil then were autoclaved at 121°C for 15 mins and were cooled at 4°C for 24 hrs.

2.2 Immunohistochemistry

Animals were quickly terminated by dislocation of the cervical and sterilized. The colons were fixed in 10% Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the paraffin block was serially sectioned using a rotary microtome. The colon sections were stained with hematoxylin-eosin (HE). Immunostaining COX-2 was performed according to the manufacturer's recommendations (Fine Test, Wuhan, China).

The observation of colon tissues was done using a light microscope with 400× magnification. Each colon tissue sample was captured with 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, the score was 0 or negative if there was no inflammation; the score was 1 or mild if inflammation infiltrates the mucosa; the score was 2 or moderate if inflammation infiltrates the mucosa and submucosa; the score was 3 or moderate if the inflammation infiltrates transmural (infiltrates to the tunica intima) (Erben *et al*., 2014).

Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 were divided into 5 scores, the score was 0 if the estimated percentage was 0% to 5%; the score was 1 if the estimated percentage was 6% to 25%, the score was 2 if the estimated percentage was 26% to 50%, the score was 3 if the estimated percentage was 51% to 75% and the score was 4 if the estimated percentage was 76% to 100%. The intensity of staining had 3 scores, score 1 if the tissue has a slightly yellow colour, score 2 when it has a brownish-yellow colour and score 3 when it is brown (Wu and Sun, 2015).

The final scores were the sum of the staining intensity score and the cell staining percentage score. The combination of the score would have 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8) (Wu and Sun, 2015).

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by using the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results

Figure 1 shows that image B (positive control) was the most inflamed among other images. Tissue in treatment 1 and treatment 2 was more inflamed than the positive control but the negative control had the least amount of inflammation. Table 2 reveals that each group had a significant difference in inflammatory values (p-Value = 0.035). The positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

Table 2. Inflammation after intervention

Group	Mean±Standard Deviation	*p*-Value
K-	1.32±0.33[a]	
K +	2.48±0.50[b]	
P1	1.56±0.38[c]	0.035 *
P2	1.40±0.47[c]	

*Statistically significant difference (p<0.05). Different superscripts within the same column are significantly different.

Furthermore, the inflammation value of the positive control group was significantly different from the negative control group, both treatment groups displayed the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group did not have a significant difference in both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow colour in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 3 where the positive control group had the highest severity percentage which had the expression of COX-2 with the category of strongly positive as much as 84%. Both treatment groups had lower severity with a positive category percentage of 48% and 50%, respectively.

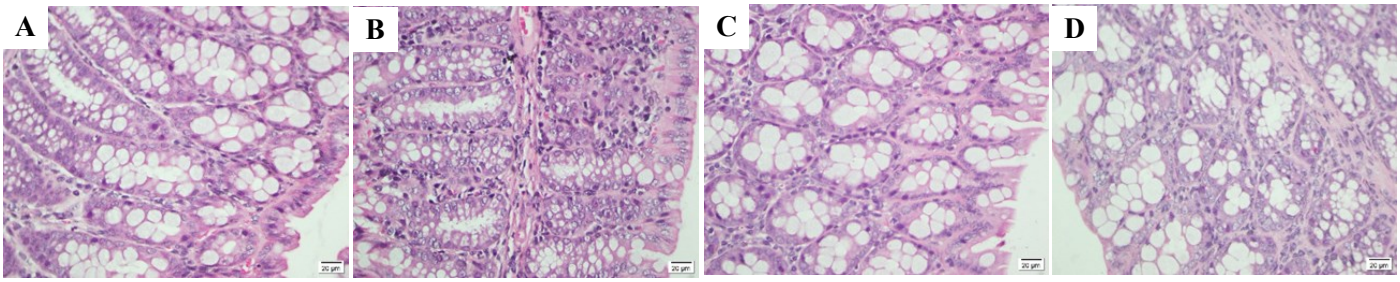

Figure 1. Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification 400×; B: positive control group, magnification 400×; C: treatment group 1, magnification 400×; D: treatment group 2, magnification 400×.

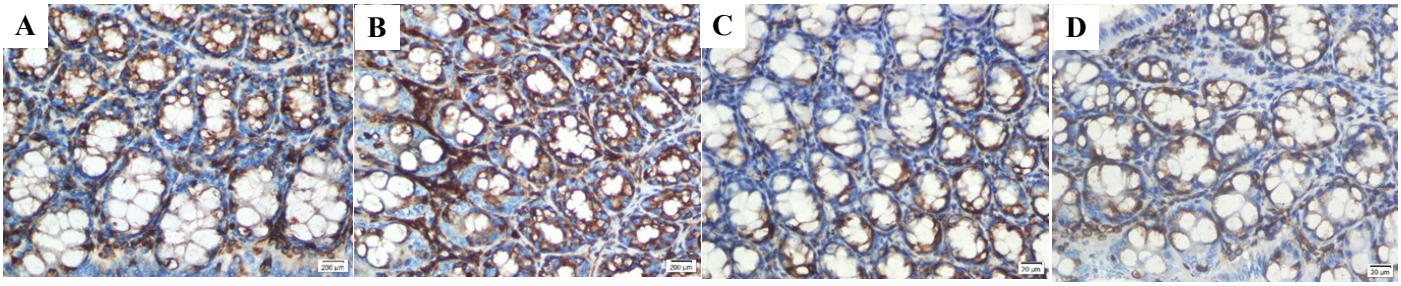

Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification 400×; B: positive control group, magnification 400×; C: treatment group 1, magnification 400×; D: treatment group 2, magnification 400×.

Table 3. The relationship between COX-2 expression with various treatments

Variable	COX-2				
	n	Positive	Moderately positive	Strongly positive	*p*-value
Intervention					<0.001
Control-negative	25	4 (16)	10 (40)	11 (44)	
AOM positive control	25	0 (0)	4 (16)	21 (84)	
AOM *batu* treatment	25	12 (48)	7 (28)	6 (24)	
AOM *kepok* treatment	20	10 (50)	9 (45)	1 (1)	

Table 4 reveals that the positive control group had a different COX-2 percentage score and intensity for both treatment groups where the positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences, thus, it could be said that the administration of *batu* and *kepok* banana resistant starch had the same effect on the COX-2.

Table 4. Expression of COX-2 after intervention

Group	COX-2 percentage score	COX-2 intensity	Score and COX-2 intensity
K-	2.52±0.92^{a}	2.48±0.51^{a}	5.00±1.32^{a}
K +	3.52±0.59^{b}	2.76±0.44^{b}	6.28±0.84^{b}
P1	1.80±1.32^{c}	2.04±0.74^{c}	3.84±1.86^{c}
P2	1.40±0.88^{c}	2.35±0.49^{ac}	3.75±1.12^{c}
p	<0.001*	<0.001*	<0.001*

*Statistically significant difference ($p<0.05$). Different superscripts within the same column are significantly different.

The results of the combined value between percentages and intensity of COX-2 between groups showed differences. The positive control group had the highest combined value when compared to other groups and treatment group 1 had the lowest combined value among the other groups. Both treatment groups did not have any differences in combined value means the administration of *kepok* banana and *batu* banana resistant starch had the same effect.

4. Discussion

The above results indicate that resistant starch in *batu* and *kepok* banana flours may have a protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support Hu *et al.* (2016) where the inflammation score decreased in experimental animals that were induced by AOM and DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the inflammation may induce chronic immune response resulting in cellular proliferation (Mariani *et al*., 2014). If the immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation (Mariani *et al*., 2014).

The results of this study are reinforced by previous studies where the positive control group that was given only a standard diet had a high inflammatory score and the number of bacteria from the genus *Fusobacterium*, *Escherichia* and *Enterococcus* were also associated with CRC in humans (Feng *et al*., 2015). Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumour formation (Zackular *et al*., 2013). The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumour formation emerged (Zackular *et al*., 2013). The crucial role of microbiota dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer (Vannucci *et al*., 2008).

Hospital administration can increase bacteria associated with RS fermentation such as *Parabacteroides*, *Ruminococcus* and *Bifidobacterium* (Hu *et al*., 2016). In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa (Nava and Stappenbeck, 2011; Wong, *et al*., 2013).

SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-protein coupled receptors (GPRs) or histone deacetylation inhibition (Sebastián and Mostoslavsky, 2014). GPR43 is GPR that was expressed at a lower concentration in the intestinal epithelial cells and certain immune cells when an individual has CRC and colitis condition (Maslowski, *et al*., 2009; Tang *et al*., 2011). Previous studies had shown that a high-fibre diet has a positive effect that can increase the activation of GPR43 (Macia *et al.*, 2015). Another study showed that RS can increase the activation of GPR43 expression significantly. Those studies indicate that GPR43 activation may have a role in intestinal homeostasis (Hu *et al*., 2016). Furthermore, acetate and propionate have a significant inverse correlation with tumour occurrence by modulating Treg cell and immune function. Those indicate an anti-inflammatory effect of SCFA produced by RS fermentation by colonic microbiota (Fukuda, *et al*., 2011; Smith, *et al*., 2013).

High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This is because COX -2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumour cells, increasing cell proliferation, regulating the cell cycle, increasing tumour angiogenesis, increasing the expression of metalloproteinases in tumour cells and stimulating the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 was reinforced by previous studies where most colon cancers have high COX-2 expression and result in tumour angiogenesis, immune system damage and tumour invasion (Brown and DuBois, 2005).

COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia (Hu *et al*., 2016). RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses (Haenen *et al*., 2013).

Conflict of interest

The authors declare no conflict of interest.

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FOOD RESEARCH

FULL PAPER

Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. *Batu* bananas and *kepok* bananas contain resistant starch which can inhibit inflammation and COX-2 in mice colon that are induced by Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of banana flour on inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice in 4 groups, negative control group, positive control group, *batu* banana treatment group, and *kepok* banana treatment group. The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of *batu* and *kepok* banana flour had a significantly lower level of inflammation when compared to the positive group ($p = 0.035$). The COX-2 score in the treatment group resistant starch of *batu* and *kepok* banana was significantly lower than the positive control group ($p<0.001$). The COX-2 intensity in both groups was lower than the positive group but not significant ($p<0.001$). The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups was also had lower than the positive control group ($p<0.001$). Resistant starch of *batu* and *kepok* banana can inhibit inflammation and suppressed the expression of COX-2.

1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and was expected to increase by 60% in 2030 (Birt and Phillips, 2014). The incidence of colon cancer would increase, especially in developing countries, including Indonesia (Tiranda and Safitriana, 2018). It was estimated that Indonesians have a colon cancer risk by 5% or 1 in 10 Indonesians was estimated to have colon cancer (Kemenkes, 2017). Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the aetiology of colon cancer (Le Leu *et al*., 2007) and one study determined that about 80% of colon cancer cases were related to diet (Le Leu *et al.*, 2002). The results indicate that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fibre that is beneficial to human health including preventing colon cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan *et al.*, 2009; Purwanti and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu *et al*., 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht *et al*., 2002). Butyrate also has chemoprotective properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis (Augenlicht *et al*., 2002; Wong *et al*., 2006).

The source of RS is contained in the Indonesian local bananas where the largest component of its starch was found in the pulp. *Batu* banana (*Musa balbisiana* Colla) and *Kepok* banana (*Musa paradisiaca* formatypica) were types of banana that had high resistant starch content of 39.5% and 27.7%, respectively (Musita, 2009; Afifah *et al.*, 2020). The method used to increase the RS content in

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banana flour was through autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) (Fuentes-Zaragoza *et al*., 2010; Ratnasari *et al*., 2018; Afifah *et al*., 2018). Current studies had revealed that *kepok* banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase the RS in *batu* banana and *kepok* banana up to 52.95% and 55.8% respectively (Afifah *et al*., 2021a).

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers (Chandrasekharan and Simmons, 2004). COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane production.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS (Hu *et al*., 2016). Butyrate produced by RS may directly influence the reduction of COX-2 (Jahns *et al*., 2011; Afifah *et al*., 2021b) by inhibiting COX-2 transcription elongation (Tong *et al*., 2005). In addition, a mechanism that might occur was the effect of inflammatory mediators that play a role in the transcriptional activation of COX-2 (Jung *et al*., 2005; Usami *et al*., 2008).

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas can protect the colon in the cancer induction effect. This study aimed to prove whether the resistant starch of *batu* banana flour and *kepok* banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Animal food was made in the Diponegoro University Integrated Laboratory. Experimental animals were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was done for 11 weeks. This research had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval for Ethical Clearance under number 33/ EC/H/FK-UNDIP/IV/2019.

2.1 Experimental animals

This study used male BALB/c mice aged 5 weeks weighing about 20 to 25 g. The animals were acclimatized beforehand for 7 days, then divided into 4 groups randomly, the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 were groups given AOM and DSS induction and feed that had been modified with banana flour *batu* and *kepok* respectively. The composition of feed for each group can be seen within the Table 1.

Table 1. Composition of experimental animal feed g/100 g

Component (g)	Mice Group			
	K-	K +	P1	P2
Batu banana flour	-	-	19	-
Kepok banana flour	-	-	-	18
Corn starch	46.57	46.57	46.57	46.57
Protein (casein)	14	14	14	14
Dectrin	15.5	15.5	5.5	5.5
Sucrose	10	10	10	10
Soybean oil	4	4	4	4
Alpacel (fiber)	5	5	5	5
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1
L-cystine	0.18	0.18	0.18	0.18
Choline	0.25	0.25	0.25	0.25

Animals were caged in a room that had air conditioning and had a naturally dark setting. They were injected by AOM induction as much as 10 mg/kg intraperitoneally after acclimatization. DSS 2% was given the following day for 7 days.

Batu and *kepok* bananas were peeled and washed with water. The bananas were sliced to about 2 mm thin then dried in the sun for 3 days. Dried banana pieces were crushed and sieved with an 80 mesh sieve. Banana flour was treated by autoclaving it at 121°C for 15 mins and were cooled at 4°C for 24 hrs. The pH of banana flour was adjusted with 0.2 M acetate buffer then 2% of pullulanase enzyme was added (v/w of banana flour). The banana flour was incubated at 40°C for 12 hrs at 150 rpm. The reaction was stopped by heating the suspension at 85°C for 5 mins. Banana flour was wrapped in aluminium foil then were autoclaved at 121°C for 15 mins and were cooled at 4°C for 24 hrs.

2.2 Immunohistochemistry

Animals were quickly terminated by dislocation of the cervical and sterilized. The colons were fixed in 10% Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the paraffin block was serially sectioned using a rotary microtome. The colon sections were stained with hematoxylin-eosin (HE). Immunostaining COX-2 was performed according to the manufacturer's recommendations (Fine Test, Wuhan, China).

The observation of colon tissues was done using a light microscope with 400× magnification. Each colon tissue sample was captured with 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, the score was 0 or negative if there was no inflammation; the score was 1 or mild if inflammation infiltrates the mucosa; the score was 2 or moderate if inflammation infiltrates the mucosa and submucosa; the score was 3 or moderate if the inflammation infiltrates transmural (infiltrates to the tunica intima) (Erben *et al*., 2014).

Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 were divided into 5 scores, the score was 0 if the estimated percentage was 0% to 5%; the score was 1 if the estimated percentage was 6% to 25%, the score was 2 if the estimated percentage was 26% to 50%, the score was 3 if the estimated percentage was 51% to 75% and the score was 4 if the estimated percentage was 76% to 100%. The intensity of staining had 3 scores, score 1 if the tissue has a slightly yellow colour, score 2 when it has a brownish-yellow colour and score 3 when it is brown (Wu and Sun, 2015).

The final scores were the sum of the staining intensity score and the cell staining percentage score. The combination of the score would have 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8) (Wu and Sun, 2015).

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by using the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results

Figure 1 shows that image B (positive control) was the most inflamed among other images. Tissue in treatment 1 and treatment 2 was more inflamed than the positive control but the negative control had the least amount of inflammation. Table 2 reveals that each group had a significant difference in inflammatory values (p-Value = 0.035). The positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

Table 2. Inflammation after intervention

Group	Mean±Standard Deviation	*p*-Value
K-	1.32±0.33[a]	
K +	2.48±0.50[b]	
P1	1.56±0.38[c]	0.035 *
P2	1.40±0.47[c]	

*Statistically significant difference (p<0.05). Different superscripts within the same column are significantly different.

Furthermore, the inflammation value of the positive control group was significantly different from the negative control group, both treatment groups displayed the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group did not have a significant difference in both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow colour in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 3 where the positive control group had the highest severity percentage which had the expression of COX-2 with the category of strongly positive as much as 84%. Both treatment groups had lower severity with a positive category percentage of 48% and 50%, respectively.

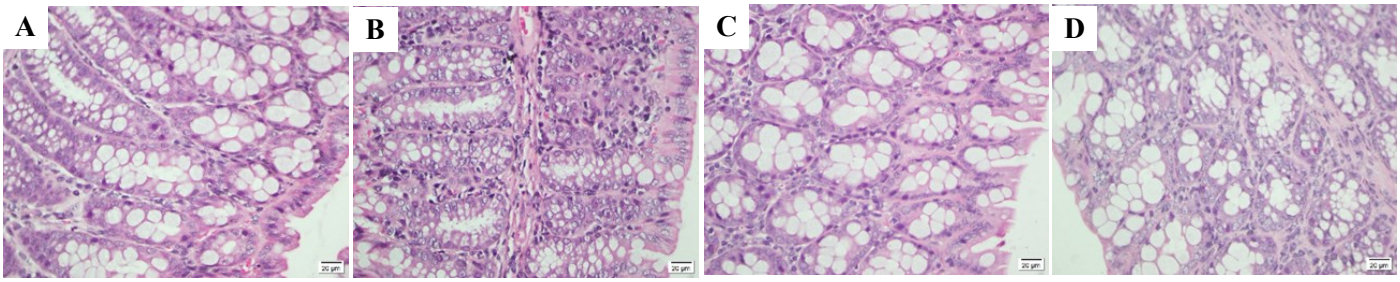

Figure 1. Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification 400×; B: positive control group, magnification 400×; C: treatment group 1, magnification 400×; D: treatment group 2, magnification 400×.

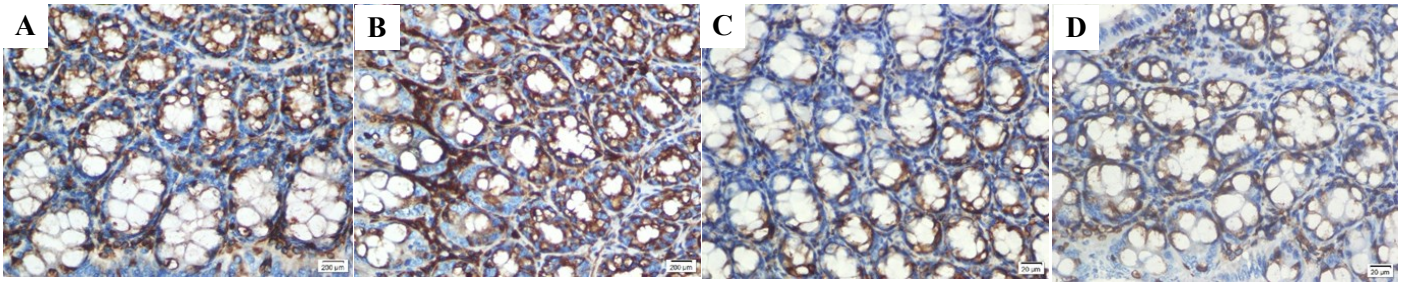

Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification 400×; B: positive control group, magnification 400×; C: treatment group 1, magnification 400×; D: treatment group 2, magnification 400×.

Table 3. The relationship between COX-2 expression with various treatments

Variable	COX-2				
	n	Positive	Moderately positive	Strongly positive	*p*-value
Intervention					<0.001
Control-negative	25	4 (16)	10 (40)	11 (44)	
AOM positive control	25	0 (0)	4 (16)	21 (84)	
AOM *batu* treatment	25	12 (48)	7 (28)	6 (24)	
AOM *kepok* treatment	20	10 (50)	9 (45)	1 (1)	

Table 4 reveals that the positive control group had a different COX-2 percentage score and intensity for both treatment groups where the positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences, thus, it could be said that the administration of *batu* and *kepok* banana resistant starch had the same effect on the COX-2.

Table 4. Expression of COX-2 after intervention

Group	COX-2 percentage score	COX-2 intensity	Score and COX-2 intensity
K-	2.52±0.92^{a}	2.48±0.51^{a}	5.00±1.32^{a}
K +	3.52±0.59^{b}	2.76±0.44^{b}	6.28±0.84^{b}
P1	1.80±1.32^{c}	2.04±0.74^{c}	3.84±1.86^{c}
P2	1.40±0.88^{c}	2.35±0.49^{ac}	3.75±1.12^{c}
p	<0.001*	<0.001*	<0.001*

*Statistically significant difference ($p<0.05$). Different superscripts within the same column are significantly different.

The results of the combined value between percentages and intensity of COX-2 between groups showed differences. The positive control group had the highest combined value when compared to other groups and treatment group 1 had the lowest combined value among the other groups. Both treatment groups did not have any differences in combined value means the administration of *kepok* banana and *batu* banana resistant starch had the same effect.

4. Discussion

The above results indicate that resistant starch in *batu* and *kepok* banana flours may have a protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support Hu *et al.* (2016) where the inflammation score decreased in experimental animals that were induced by AOM and DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the inflammation may induce chronic immune response resulting in cellular proliferation (Mariani *et al*., 2014). If the immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation (Mariani *et al*., 2014).

The results of this study are reinforced by previous studies where the positive control group that was given only a standard diet had a high inflammatory score and the number of bacteria from the genus *Fusobacterium*, *Escherichia* and *Enterococcus* were also associated with CRC in humans (Feng *et al*., 2015). Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumour formation (Zackular *et al*., 2013). The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumour formation emerged (Zackular *et al*., 2013). The crucial role of microbiota dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer (Vannucci *et al*., 2008).

Hospital administration can increase bacteria associated with RS fermentation such as *Parabacteroides*, *Ruminococcus* and *Bifidobacterium* (Hu *et al*., 2016). In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa (Nava and Stappenbeck, 2011; Wong, *et al*., 2013).

SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-protein coupled receptors (GPRs) or histone deacetylation inhibition (Sebastián and Mostoslavsky, 2014). GPR43 is GPR that was expressed at a lower concentration in the intestinal epithelial cells and certain immune cells when an individual has CRC and colitis condition (Maslowski, *et al*., 2009; Tang *et al*., 2011). Previous studies had shown that a high-fibre diet has a positive effect that can increase the activation of GPR43 (Macia *et al.*, 2015). Another study showed that RS can increase the activation of GPR43 expression significantly. Those studies indicate that GPR43 activation may have a role in intestinal homeostasis (Hu *et al*., 2016). Furthermore, acetate and propionate have a significant inverse correlation with tumour occurrence by modulating Treg cell and immune function. Those indicate an anti-inflammatory effect of SCFA produced by RS fermentation by colonic microbiota (Fukuda, *et al*., 2011; Smith, *et al*., 2013).

High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This is because COX -2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumour cells, increasing cell proliferation, regulating the cell cycle, increasing tumour angiogenesis, increasing the expression of metalloproteinases in tumour cells and stimulating the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 was reinforced by previous studies where most colon cancers have high COX-2 expression and result in tumour angiogenesis, immune system damage and tumour invasion (Brown and DuBois, 2005).

COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia (Hu *et al*., 2016). RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses (Haenen *et al*., 2013).

Conflict of interest

The authors declare no conflict of interest.

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FULL PAPER

Banana resistant starch inhibitory inflammation and cyclooxygenase-2 in BALB/c mice induced by azoxymethane and dextran sodium sulfate

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Abstract

Inflammation in colonic tissue is one of the earliest developmental pathways in colorectal cancer and proteins such as cyclooxygenase are crucial to this inflammation. *Batu* bananas and *kepok* bananas contain resistant starch which can inhibit inflammation and COX-2 in mice colon that are induced by Azoxymethane and Dextran Sodium Sulfate. This study was to observe the effect of banana flour on inflammation and cyclooxygenase enzyme in colonic tissue. This study involved 20 BALB/c mice in 4 groups, negative control group, positive control group, *batu* banana treatment group, and *kepok* banana treatment group. The level of inflammation was seen from the colon tissue treated with hematoxylin eosin while Cyclooxigenase-2 (COX-2) with COX-2 immunohistochemical staining. The specimens were viewed under a light microscope at a magnification of 400 times. The treatment groups with resistant starch of *batu* and *kepok* banana flour had a significantly lower level of inflammation when compared to the positive group ($p = 0.035$). The COX-2 score in the treatment group resistant starch of *batu* and *kepok* banana was significantly lower than the positive control group ($p<0.001$). The COX-2 intensity in both groups was lower than the positive group but not significant ($p<0.001$). The combined score between the percentage and the intensity of COX-2 expression in the two treatment groups was also had lower than the positive control group ($p<0.001$). Resistant starch of *batu* and *kepok* banana can inhibit inflammation and suppressed the expression of COX-2.

1. Introduction

Colon cancer was the third leading cause of cancer-related death with 130,000 cases every year and was expected to increase by 60% in 2030 (Birt and Phillips, 2014). The incidence of colon cancer would increase, especially in developing countries, including Indonesia (Tiranda and Safitriana, 2018). It was estimated that Indonesians have a colon cancer risk by 5% or 1 in 10 Indonesians was estimated to have colon cancer (Kemenkes, 2017). Evidence from epidemiological and experimental studies suggested diet was a crucial factor in the aetiology of colon cancer (Le Leu *et al*., 2007) and one study determined that about 80% of colon cancer cases were related to diet (Le Leu *et al.*, 2002). The results indicate that colon cancer could be prevented.

Resistant starch (RS) is an insoluble fibre that is beneficial to human health including preventing colon cancer (Tharanathan and Mahadevamma, 2003; Hovhannisyan *et al.*, 2009; Purwanti and Suhartono, 2014). In the human body, RS will not be digested but will be fermented by bacteria in the colon and will produce one of the substrates in the form of Short Chain Fatty Acids (SCFA) is butyrate (Hu *et al*., 2016). Butyrate is the main source of energy for normal colon cells thereby the integrity and function of the colonic epithelium can be maintained (Augenlicht *et al*., 2002). Butyrate also has chemoprotective properties by inhibiting the proliferation of cancer cells, increasing differentiation and increasing apoptosis (Augenlicht *et al*., 2002; Wong *et al*., 2006).

The source of RS is contained in the Indonesian local bananas where the largest component of its starch was found in the pulp. *Batu* banana (*Musa balbisiana* Colla) and *Kepok* banana (*Musa paradisiaca* formatypica) were types of banana that had high resistant starch content of 39.5% and 27.7%, respectively (Musita, 2009; Afifah *et al.*, 2020). The method used to increase the RS content in

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banana flour was through autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) (Fuentes-Zaragoza *et al*., 2010; Ratnasari *et al*., 2018; Afifah *et al*., 2018). Current studies had revealed that *kepok* banana flour obtained by the autoclaving-cooling enzymatic autoclaving-cooling (AC-E-AC) method could increase the RS in *batu* banana and *kepok* banana up to 52.95% and 55.8% respectively (Afifah *et al*., 2021a).

The onset of colon cancer can be preceded by inflammation that occurs in the colonic tissue. This inflammation will lead to increased Cyclooxygenase-2 (COX-2) in which the expression of COX-2 overload associated with various cancers (Chandrasekharan and Simmons, 2004). COX-2 can increase the progression of colon cancer by affecting cell proliferation, platelet aggregation and thromboxane production.

Results of previous studies indicated that the expression of several genes related to inflammation such as COX-2 decreased significantly in the provision of RS (Hu *et al*., 2016). Butyrate produced by RS may directly influence the reduction of COX-2 (Jahns *et al*., 2011; Afifah *et al*., 2021b) by inhibiting COX-2 transcription elongation (Tong *et al*., 2005). In addition, a mechanism that might occur was the effect of inflammatory mediators that play a role in the transcriptional activation of COX-2 (Jung *et al*., 2005; Usami *et al*., 2008).

Past research had shown that resistant starch could prevent colon cancer. Our hypothesis against the effects of resistant starch which is found in bananas can protect the colon in the cancer induction effect. This study aimed to prove whether the resistant starch of *batu* banana flour and *kepok* banana might inhibit inflammation and COX-2 in mice induced colon cancer-forming compounds.

2. Materials and methods

This research was a quasi-experimental research design post only with the control group design. Animal food was made in the Diponegoro University Integrated Laboratory. Experimental animals were kept at the Inter-University Central Food and Nutrition Laboratory (PAU) Yogyakarta, Gadjah Mada University. The study was done for 11 weeks. This research had ethical approval from the Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University and obtain a letter of approval for Ethical Clearance under number 33/ EC/H/FK-UNDIP/IV/2019.

2.1 Experimental animals

This study used male BALB/c mice aged 5 weeks weighing about 20 to 25 g. The animals were acclimatized beforehand for 7 days, then divided into 4 groups randomly, the negative control (C-), positive control (C+), treatment 1 (T1), and treatment 2 (T2). C- will be given AIN-93 feed without induction and any treatment. C+ was given the standard feed AIN-93 and by the induction of AOM and DSS. T1 and T2 were groups given AOM and DSS induction and feed that had been modified with banana flour *batu* and *kepok* respectively. The composition of feed for each group can be seen within the Table 1.

Table 1. Composition of experimental animal feed g/100 g

Component (g)	Mice Group			
	C-	C +	T1	T2
Batu banana flour	-	-	19	-
Kepok banana flour	-	-	-	18
Corn starch	46.57	46.57	46.57	46.57
Protein (casein)	14	14	14	14
Dectrin	15.5	15.5	5.5	5.5
Sucrose	10	10	10	10
Soybean oil	4	4	4	4
Alpacel (fiber)	5	5	5	5
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1
L-cystine	0.18	0.18	0.18	0.18
Choline	0.25	0.25	0.25	0.25

Animals were caged in a room that had air conditioning and had a naturally dark setting. They were injected by AOM induction as much as 10 mg/kg intraperitoneally after acclimatization. DSS 2% was given the following day for 7 days.

Batu and *kepok* bananas were peeled and washed with water. The bananas were sliced to about 2 mm thin then dried in the sun for 3 days. Dried banana pieces were crushed and sieved with an 80 mesh sieve. Banana flour was treated by autoclaving it at 121°C for 15 mins and were cooled at 4°C for 24 hrs. The pH of banana flour was adjusted with 0.2 M acetate buffer then 2% of pullulanase enzyme was added (v/w of banana flour). The banana flour was incubated at 40°C for 12 hrs at 150 rpm. The reaction was stopped by heating the suspension at 85°C for 5 mins. Banana flour was wrapped in aluminium foil then were autoclaved at 121°C for 15 mins and were cooled at 4°C for 24 hrs.

2.2 Immunohistochemistry

Animals were quickly terminated by dislocation of the cervical and sterilized. The colons were fixed in 10% Neutral Buffer Formalin and embedded in paraffin blocks. The specimen colon on the paraffin block was serially sectioned using a rotary microtome. The colon sections were stained with hematoxylin-eosin (HE). Immunostaining COX-2 was performed according to the manufacturer's recommendations (Fine Test, Wuhan, China).

The observation of colon tissues was done using a light microscope with 400× magnification. Each colon tissue sample was captured with 5 random images from 5 different fields. Inflammation scoring was performed within 4 scores, the score was 0 or negative if there was no inflammation; the score was 1 or mild if inflammation infiltrates the mucosa; the score was 2 or moderate if inflammation infiltrates the mucosa and submucosa; the score was 3 or moderate if the inflammation infiltrates transmural (infiltrates to the tunica intima) (Erben *et al*., 2014).

Immunohistochemical observations were carried out by estimating the percentage of cells stained and the staining intensity. Scores percentage stained of COX-2 were divided into 5 scores, the score was 0 if the estimated percentage was 0% to 5%; the score was 1 if the estimated percentage was 6% to 25%, the score was 2 if the estimated percentage was 26% to 50%, the score was 3 if the estimated percentage was 51% to 75% and the score was 4 if the estimated percentage was 76% to 100%. The intensity of staining had 3 scores, score 1 if the tissue has a slightly yellow colour, score 2 when it has a brownish-yellow colour and score 3 when it is brown (Wu and Sun, 2015).

The final scores were the sum of the staining intensity score and the cell staining percentage score. The combination of the score would have 4 meaning, negative (if the value of the combination is 1 to 2), positive (if the value of the combination is 3 to 4), positively moderate (if the value of the combination of 5 to 6), and strongly positive (if the value of the combination is 7 up to 8) (Wu and Sun, 2015).

2.3 Data analysis

Univariate analysis was performed to calculate the mean value and standard deviation. The normality test was carried out by using the Saphiro Wilk test. Bivariate analysis was performed using the Anova test for normally distributed data and using the Kruskal Wallis test if the data was not normal. Multivariate analysis was performed by performing a Post Hoc test to see which variables contributed to the differentiation value. The statistical value is significant if the p-value is less than 0.05.

3. Results

Figure 1 shows that image B (positive control) was the most inflamed among other images. Tissue in treatment 1 and treatment 2 was more inflamed than the positive control but the negative control had the least amount of inflammation. Table 2 reveals that each group had a significant difference in inflammatory values (p-Value = 0.035). The positive control group had levels of inflammation that was the highest among the other groups, while the negative control group had the lowest levels of inflammation.

Table 2. Inflammation after intervention

Group	Mean±Standard Deviation	*p*-Value
C-	1.32 ± 0.33^{a}	
C+	2.48 ± 0.50^{b}	
T1	1.56 ± 0.38^{c}	0.035 *
T2	1.40 ± 0.47^{c}	

*Statistically significant difference ($p<0.05$). Different superscripts within the same column are significantly different.

Furthermore, the inflammation value of the positive control group was significantly different from the negative control group, both treatment groups displayed the mean value of inflammation in the positive control group was higher than the other groups. The inflammation value in the negative group did not have a significant difference in both treatment groups. This suggests that treatment 1 and treatment 2 had a value equal to the inflammation value in the negative group.

Cells that had COX-2 showed a brownish yellow colour in the cytoplasm which could be seen in Figure 2. Categories COX-2 expression in all four groups could be seen in Table 3 where the positive control group had the highest severity percentage which had the expression of COX-2 with the category of strongly positive as much as 84%. Both treatment groups had lower severity with a positive category percentage of 48% and 50%, respectively.

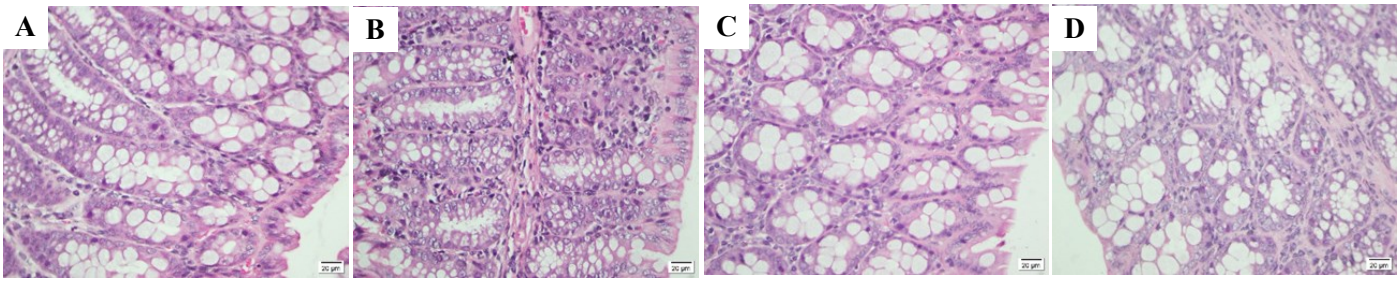

Figure 1. Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification 400×; B: positive control group, magnification 400×; C: treatment group 1, magnification 400×; D: treatment group 2, magnification 400×.

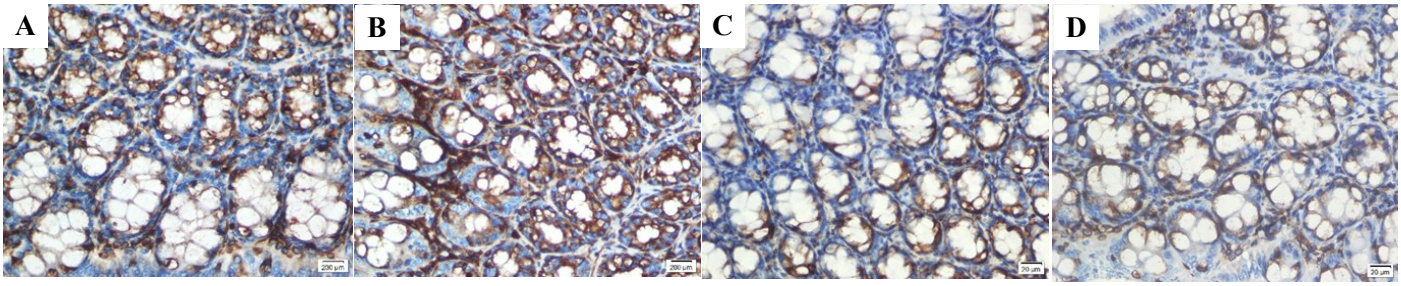

Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification 400×; B: positive control group, magnification 400×; C: treatment group 1, magnification 400×; D: treatment group 2, magnification 400×.

Table 3. The relationship between COX-2 expression with various treatments

Variable	COX-2				
	n	Positive	Moderately positive	Strongly positive	*p*-value
Intervention					<0.001
Control-negative	25	4 (16)	10 (40)	11 (44)	
AOM positive control	25	0 (0)	4 (16)	21 (84)	
AOM *batu* treatment	25	12 (48)	7 (28)	6 (24)	
AOM *kepok* treatment	20	10 (50)	9 (45)	1 (1)	

Table 4 reveals that the positive control group had a different COX-2 percentage score and intensity for both treatment groups where the positive control had the highest mean among the other groups. Negative control had a different value against other groups where the COX-2 score in treatment 1 and treatment 2 was lower. Both treatment groups did not have any differences, thus, it could be said that the administration of *batu* and *kepok* banana resistant starch had the same effect on the COX-2.

Table 4. Expression of COX-2 after intervention

Group	COX-2 percentage score	COX-2 intensity	Score and COX-2 intensity
C-	2.52±0.92^{a}	2.48±0.51^{a}	5.00±1.32^{a}
C+	3.52±0.59^{b}	2.76±0.44^{b}	6.28±0.84^{b}
T1	1.80±1.32^{c}	2.04±0.74^{c}	3.84±1.86^{c}
T2	1.40±0.88^{c}	2.35±0.49^{ac}	3.75±1.12^{c}
p	<0.001*	<0.001*	<0.001*

*Statistically significant difference ($p<0.05$). Different superscripts within the same column are significantly different.

The results of the combined value between percentages and intensity of COX-2 between groups showed differences. The positive control group had the highest combined value when compared to other groups and treatment group 1 had the lowest combined value among the other groups. Both treatment groups did not have any differences in combined value means the administration of *kepok* banana and *batu* banana resistant starch had the same effect.

4. Discussion

The above results indicate that resistant starch in *batu* and *kepok* banana flours may have a protective effect on AOM and DSS-induced colorectal cancer initiation. Resistant starch can inhibit inflammation due to the induction of AOM and DSS compounds where the level of inflammation is the same as the groups that are not induced by AOM and DSS compounds.

The results of this study support Hu *et al.* (2016) where the inflammation score decreased in experimental animals that were induced by AOM and DSS and given a diet containing RS. Inflammation and colorectal tissue injury caused by infection, mechanics or chemical compounds play an important role in the early stages of colorectal carcinogenesis in which the inflammation may induce chronic immune response resulting in cellular proliferation (Mariani *et al*., 2014). If the immune response fails, cytokines, growth factors and cellular respiration products will continue to proliferate to repair the wound. This can lead to the accumulation of genetic errors and improper proliferation (Mariani *et al*., 2014).

The results of this study are reinforced by previous studies where the positive control group that was given only a standard diet had a high inflammatory score and the number of bacteria from the genus *Fusobacterium*, *Escherichia* and *Enterococcus* were also associated with CRC in humans (Feng *et al*., 2015). Administration of AOM which induces DNA damage and DSS that triggers infection may contribute to dysbiosis in the microbiota community which contributes to tumour formation (Zackular *et al*., 2013). The study also indicated a dynamic change in the microbiota population in the initial response to AOM and DSS before signs of macroscopic tumour formation emerged (Zackular *et al*., 2013). The crucial role of microbiota dysbiosis is supported by research where experimental animals that do not experience dysbiosis do not experience inflammation and colon cancer (Vannucci *et al*., 2008).

Hospital administration can increase bacteria associated with RS fermentation such as *Parabacteroides*, *Ruminococcus* and *Bifidobacterium* (Hu *et al*., 2016). In addition, there is also an increase in bacteria which is not directly related to RS or CRC fermentation. However, these bacteria likely have a role in preventing inflammation or regeneration of the colonic mucosa (Nava and Stappenbeck, 2011; Wong, *et al*., 2013).

SCFAs produced by the colonic microbiota can stimulate cell function through activation of G-protein coupled receptors (GPRs) or histone deacetylation inhibition (Sebastián and Mostoslavsky, 2014). GPR43 is GPR that was expressed at a lower concentration in the intestinal epithelial cells and certain immune cells when an individual has CRC and colitis condition (Maslowski, *et al*., 2009; Tang *et al*., 2011). Previous studies had shown that a high-fibre diet has a positive effect that can increase the activation of GPR43 (Macia *et al.*, 2015). Another study showed that RS can increase the activation of GPR43 expression significantly. Those studies indicate that GPR43 activation may have a role in intestinal homeostasis (Hu *et al*., 2016). Furthermore, acetate and propionate have a significant inverse correlation with tumour occurrence by modulating Treg cell and immune function. Those indicate an anti-inflammatory effect of SCFA produced by RS fermentation by colonic microbiota (Fukuda, *et al*., 2011; Smith, *et al*., 2013).

High COX-2 expression is the beginning of tumorigenesis (Wu and Sun, 2015). This is because COX -2 has a role in increasing prostaglandins, inhibiting the body's immune response, inhibiting apoptosis of tumour cells, increasing cell proliferation, regulating the cell cycle, increasing tumour angiogenesis, increasing the expression of metalloproteinases in tumour cells and stimulating the activation of precursor substances that are carcinogenic (Wu and Sun, 2015). The role of COX-2 was reinforced by previous studies where most colon cancers have high COX-2 expression and result in tumour angiogenesis, immune system damage and tumour invasion (Brown and DuBois, 2005).

COX-2 expression in the positive control group was significantly higher than the COX-2 expression in the negative control group and the two treatment groups. The results of this study are supported by previous studies that showed the expression of genes associated with inflammation such as COX-2 decreased significantly in the group given RS. The increased expression of the inflammatory cytokine COX-2 is evidence that administration of AOM and DSS have an inflammatory microenvironment that can enhance tissue dysplasia (Hu *et al*., 2016). RS triggers major changes in colonic gene expression that inhibits inflammatory pathways and suppresses immune responses (Haenen *et al*., 2013).

Conflict of interest

The authors declare no conflict of interest.

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