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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

[1]Pratiwi, S.N., [1,2,]*Afifah, D.N., [3]Widyastiti, N.S., [4]Karlowee, V., [1,2]Anjani, G. and [4]Istiadi, H.

[1]*Department of Nutrition Science, Universitas Diponegoro, Semarang, Indonesia*
[2]*Centre of Nutrition Research (CENURE), Universitas Diponegoro, Semarang, Indonesia*
[3]*Department of Clinical Pathology, Universitas Diponegoro, Semarang, Indonesia*
[4]*Department of Anatomical Pathology, Universitas Diponegoro, Semarang, Indonesia*

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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

*Corresponding author.
Email: *d.nurafifah.dna@fk.undip.ac.id*

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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.

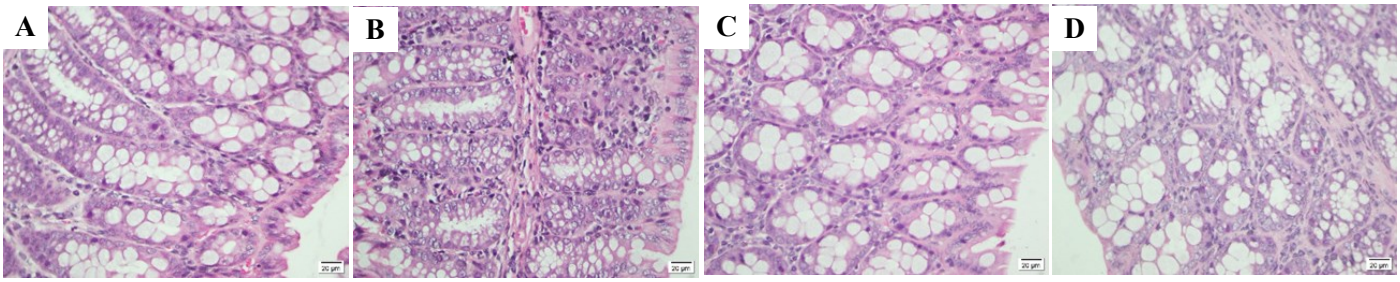

Figure 1. Hematoxylin Eosin staining on the colonic cell tissue of mice. A: negative control group, magnification 400×; B: positive control group, magnification 400×; C: treatment group 1, magnification 400×; D: treatment group 2, magnification 400×.

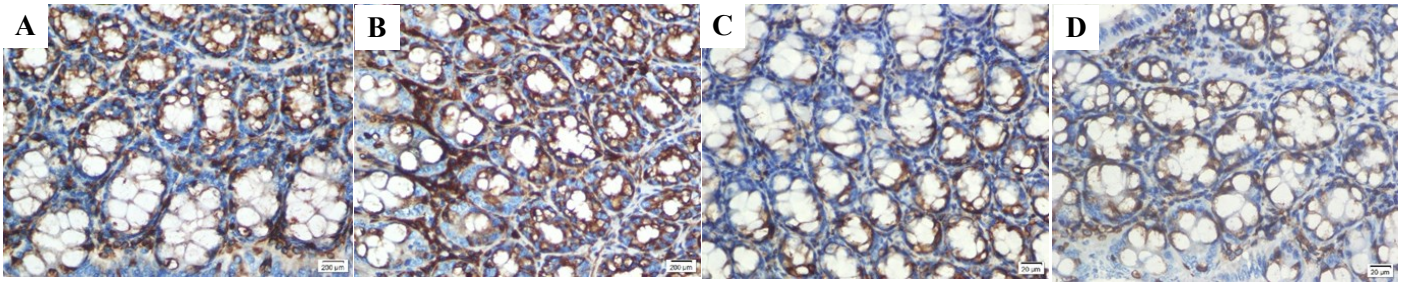

Figure 2. Immunohistochemical stain of COX-2 on mouse colon cells. A: negative control group, magnification 400×; B: positive control group, magnification 400×; C: treatment group 1, magnification 400×; D: treatment group 2, magnification 400×.

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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