Annona muricata Leaves Extract Inhibit Carcinogenesis and Regulates Inflammatory Responses in AOM/DSS-Induced Colitis-Associated Carcinogenesis Mice

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ABSTRACT

Background: *Colon cancer is a major public health problem. The present study was conducted to determine the inhibitory effect of Annona muricata leaves on carcinogenesis and inflammation in the colorectal preneoplasia development of mice.*

Methods*: The study was experimental research that conducted in Faculty of Medicine Universitas Indonesia. The effect of Ethanol Insoluble Fraction of Annona muricata water extract (EIFAM) was examined on the preneoplastic lesions. EIFAM was administered orally in three different doses (200 mg/kgBW (low dose), 400 mg/kgBW (medium dose), and 800 mg/kgBW (high dose)) in each group of mice per day until the mice were terminated. The expression of Caspase, COX-2, and β-catenin were observed in the epithelial cells of colon mucosa crypts. Analysis of the data was done using the ANOVA method.*

Results: *Histological assessment using Hematoxylin Eosin Staining was done in a blinded fashion to avoid bias. Our results indicate that multiple organ dysplasia was induced with azoxymethane (AOM) in male Swiss Webster mice. The effect of A. muricata extract administration showed significant result only on heart. The activity of caspase showed no significant effect on all organs when treated with the extracts as compared to untreated group. The effects of the extract on various organs' inflammatory markers varies. COX-2 lowering effect of high doses is significant in the liver. The medium dose has significant catenin lowering effect on jejenum, colon, and brain. While the medium and high dose have significant catenin lowering effect on duodenum, heart, and kidney respectively.*

Conclusions: *Ethanol-insoluble fraction of Annona muricata (EIFAM) leaves water extract has a potential inhibitory effect of Annona muricata leaves on carcinogenesis and inflammation in the colorectal preneoplasia development of mice.*

Keywords: *Apoptosis, Dysplasia, β-Catenin, COX-2, Colorectal cancer, caspase*

ABSTRAK

Latar Belakang: *Kanker usus besar merupakan masalah kesehatan masyarakat yang utama. Penelitian ini dilakukan untuk mengetahui efek penghambatan daun Annona muricata terhadap karsinogenesis dan inflamasi pada mencit.*

Metode: *Penelitian ini menggunakan desain eksperimental yang menguji efek Ethanol Insoluble Fraction of Annona muricata water extract (EIFAM) terhadap lesi preneoplastik. EIFAM diberikan secara oral dalam tiga dosis berbeda (200 mg/kgBB (dosis rendah), 400 mg/kgBB (dosis sedang), dan 800 mg/kgBB (dosis tinggi)) pada masing-masing kelompok mencit per hari hingga mencit tersebut dihentikan. Ekspresi Caspase, COX-2, dan β-catenin diamati pada sel epitel kripta mukosa usus besar. Analisis data mengunakan metode ANOVA.*

Hasil: *Penilaian histologis menggunakan pewarnaan Hematoksilin-Eosin dilakukan secara buta untuk menghindari bias. Hasil penelitian menunjukkan bahwa displasia terjadi pada beberapa organ dengan diinduksi azoxymethane (AOM) pada tikus Swiss Webster jantan. Pengaruh pemberian ekstrak A. muricata menunjukkan hasil yang signifikan hanya pada jantung. Sementara aktivitas caspase tidak menunjukkan efek yang signifikan pada semua organ ketika diberi ekstrak dibandingkan dengan kelompok kontrol. Efek ekstrak pada penanda inflamasi berbagai organ bervariasi. Efek penurunan COX-2 dosis tinggi signifikan pada hati. Dosis sedang mempunyai efek penurunan catenin yang signifikan pada jejenum, usus besar, dan otak. Sedangkan dosis sedang dan tinggi masing-masing memiliki efek penurunan catenin yang signifikan terhadap duodenum, jantung, dan ginjal.* ***Kesimpulan:*** *EIFAM berpotensi menghambat karsinogenesis dan inflamasi hewan model.*

Kata kunci: *Apoptosis, Dysplasia, β-Catenin, COX-2, Colorectal cancer, caspase*

INTRODUCTION

Several decades ago, colorectal cancer was infrequently diagnosed. Nowadays, it is the world's fourth most deadly cancer with almost 900,000 deaths annually1. Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths in the world with an estimated number of 1.8 million new cases and about 881,000 deaths worldwide in 20182. Cancer is one of the world's biggest healthcare challenges, with colorectal cancer (CRC) being one of the three most frequently encountered malignancies worldwide3. Colorectal cancer is the third common cancer in this world, accounting for more than 1 million cases each year4.

Pathogenesis of CRC is a multistep process, during which different molecular pathways come into play. The cardinal genomic alteration that has been found universally present in CRC is a mutation in the adenomatous polyposis coli gene (APC). APC mutation causes unrestricted action of the Wnt signaling pathway which subsequently enhances the intracellular accumulation of a protein called beta-catenin, responsible for cell proliferation, differentiation, and enhanced survival of colorectal epithelial cells. This property of beta-catenin can determine the malignant potential of various premalignant neoplasms of the large intestine3. Wnt/β-catenin signaling contributes to carcinogenesis and tumor progression of several cancers, including colon cancer, hepatocellular carcinoma, pancreatic cancer, lung cancer and ovarian cancer5. However, detailed etiology and mechanism of colorectal cancer have not been fully understood. For example, cyclooxygenase-2 (COX-2) and its product prostaglandin E2 (PGE2) have been closely linked to its occurrence, progression and prognosis. However, the mechanisms on how COX-2 and PGE2-mediate the pathogenesis of colorectal cancer are obscure. In this review, we have summarized recent advances in studies of pathogenesis and control in colorectal cancer to assist further advances in the research for the cure of the cancer. In addition, the knowledge gained may also guide the audiences in the reduction of the risk and control of this deadly disease4.

Cyclooxygenase-2 (COX-2, PTGS2) is an enzyme involved in the synthesis of prostaglandins and thromboxanes, which are regulators of biologic processes such as inflammation, cell proliferation, and angiogenesis. Levels of COX-2 mRNA are found over-expressed in almost 80% of the colorectal tumors, compared to paired adjacent normal colorectal mucosa, suggesting an inhibitor of COX-2 could be of value in chemoprevention of colon cancer6. COX-2 is activated in response to inflammatory stimuli and is one of the major molecules that is involved in the development and progression of colorectal cancer (CRC). Consistent with such a conceptual framework, it has been shown that COX-2 inhibitors prevent the carcinogenesis of

CRC and aid in the treatment of sporadic or familial cases of CRC as shown by an overall increase in the survival rate7. Findings from research that explore the association between body composition profiles and 5-year colon cancer outcomes suggest that low skeletal muscle area and high visceral to total fat ratio were associated with worse colon cancer outcomes and with increased expression of proinflammatory cytokines8.

A study that evaluated the apoptosis-inducing effect of soursop (Annona muricata) leaf extract on the colorectal cancer cell line COLO-205 suggested that Annona muricata leaf extract had anticancer properties by enhancing caspase-3 activity which is a proapoptotic marker9.

Annona muricata and other plants have been shown to possess promising compounds that can be used in cancer treatment. Native to the tropical and subtropical parts of the world, A. muricata plant extracts contain compounds that have the potential to be integrated as one of the treatment options against cancer10. Annona muricata Linn has been reported to contain valuable bioactive compounds known as Annonaceous acetogenins. These long-chain fatty acids were widely discussed for their potential to promote anticancer and antiproliferative activity in various cancer cell lines11.

An experiment was performed by isolating the cytotoxic compound of EEAML (Annomuricin E) and its apoptosis-inducing effect was investigated against HT-29 colon cancer cell line using a bioassay-guided approach. Immunohistochemistry analysis showed the down-regulation of PCNA and Bcl-2 proteins and the up-regulation of Bax protein after administration of ethyl acetate extract of A. muricata leaves (EEAML) compared with the cancer control group. In addition, an increase in the levels of enzymatic antioxidants and a decrease in the malondialdehyde level of the colon tissue homogenates were observed, suggesting the suppression of lipid peroxidation. These findings highlight annomuricin E as one of the contributing compounds in the anticancer activity of A. muricata leaves.12

This study was motivated by empirically usage of A. muricata leaves as traditional tea drink that is currently being studied on the developing effort of treating some types of cancers. In vitro studies and cytotoxicity test in animals, also clinical trial have been conducted using the extract of these leaves13-15

METHODS

The study was experimental research that conducted in the Faculty of Medicine Universitas Indonesia in 2019 for about one year.

Annona muricata L. extract

The A. muricata extract used in this study is the water extract and its fractions. They are ethanol-insoluble fraction (EIFAM) and ethanol-soluble fraction of A. muricata leaves water extract (ESFAM)14.

The procedure of ethanolic fractionation from water extract was as follows:

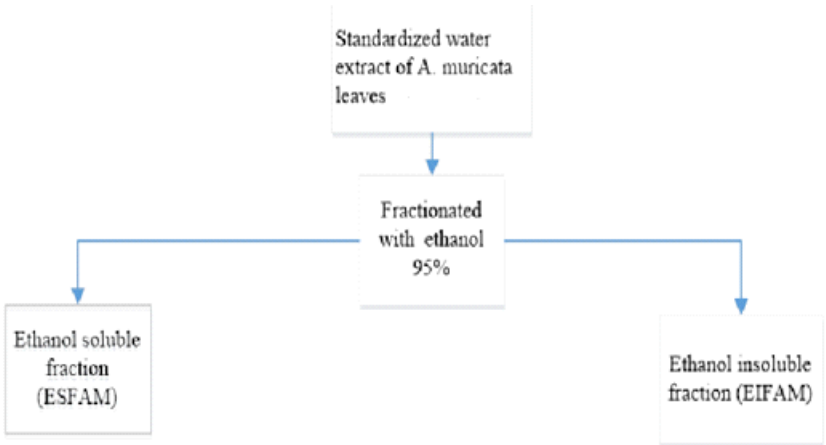

Figure 1. The procedure of Fractionation on Standardized Water Extract of A. Muricata Leaves

Animals

Experimental animals used were male Swiss Webster mice, aged 2-3 months, weighing 20-25 g. Mice were bred and reared in the Laboratory of Experimental Pathology, Department of Anatomic Pathology, Faculty of Medicine, Universitas Indonesia. Mice were kept in a room with a temperature regulator from 22±2°C in 12 h light and 12 h dark cycle. Mice were fed with standard pellets and ad libitum drinking water was provided. Before being used in the experiment, the mice were confirmed free from parasitic diseases. Animals were reared and treated in accordance with the Guide for the Care and Use of Laboratory Animals of the Animal Care and Use Committee and had gained approval from the Research Ethics Committee of the Faculty of Medicine, University of Indonesia (No. 0256/UN2. F1/ETIK/2018). Animal rearing and treatment were carried out by Felasa-certified researchers.

Induction of Colon Carcinogenesis

Induction of colon carcinogenesis in mice was performed according to the method developed by Tanaka, et al. (2003) and Suzuki, et al. (2006). Mice were injected intraperitoneally with azoxymethane (AOM/Sigma) dissolved in 0.9% NaCl at a dose of 10 mg/kg of body weight for one administration. Post-AOM induction the mice were given standard feed and mineral water for one week. For the next week, the water was replaced with aquadest containing 1% dextran sodium sulfate (DSS/ Sigma). The mice were reared for the first, second, third, and fourth months until the time of sacrifice to get the colon tissue. The colonic tissue collected is the distal portion.

Extract Administration

The extract was administered orally to each mouse from the third week or after the completion of DSS administration. Mice were divided into 6 groups, each group consisting of 6 animals. The mice groupings are as follows: the first group or negative control group is the group of mice induced with AOM/DSS and only received distilled water during the treatment. The second group is mice induced with AOM/DSS and received 200 mg/kgBW (low dose) of EIFAM. Group 3 received 400 mg/kgBW (medium dose) of EIFAM, while group 4 received 800 mg/kgBW (high dose) of EIFAM per day. Group 5 received 40mg/kgBW of ESFAM and group 6 received 800 mg/kgBB of water extract per day. The treatment of extract administrations was carried out for six weeks and was sacrificed on the following day for pathological examination. All specimens taken from experimental animals were subjected to histopathological examination followed by immunohistochemistry for beta-catenin, COX-2, and caspase expression.

Hematoxylin Eosin Staining

A piece of colorectal tissue made paraffin blocks. Paraffin blocks made 4 μm thick slices and mounted on glass objects for HE staining stages as follows: Deparaffinizesection using xylol I, II and III, respectively 5 minutes. Then re-hydrated using absolute alcohol, 96% and 70% respectively for 5 minutes, and washed in running water for 5 minutes. The preparation inserted into hematoxylin (Meyer solution) for 7 minutes and rinsed in running water for 10 minutes. After that, the stocks dipped into a saturated lithium carbonate 1-2 minutes and rinsed with running water for 5 minutes.

Preparations controlled if the blue color is sufficient, if it is not put back into solution Meyer (hematoxylin) for 2 minutes, then rinsed in running water, soaked in eosin for 1-2 minutes, dehydrated in alcohol absolute 70%, 80%, 96% respectively for 3 minutes, clearing with xylol I - II - III, and the last drops with entelan and covered with a glass lid. Preparations viewed using a light microscope with ×400 magnification. Histological assessment was done in a blinded fashion to avoid bias. Based on H&E staining, histological alterations, such as mucosal ulceration, dysplasia, and carcinoma, were verified by a board-certified pathologist. Carcinoma was defined as a high-grade dysplasia of the colonic mucosa that had invaded beyond the muscularis mucosa and into the submucosa.

Immunohistochemistry

For cleaved-caspase 3 immuno-histochemistry analysis, colorectal tissue deriving from mice was fixed in buffered formalin, embedded in paraffin, and cut into 4 μm-thick serial sections. Sections were stained with the cleaved-caspase 3 (1:100 v/v) antibodies. After three 5-minute washes, the secondary antibody was added, and the samples were incubated at room temperature for 20 min. The streptavidin-HRP detection system was added, and samples were incubated at room temperature. After three 5-minute washes, 50 ml of chromogen was added, and the reaction stopped after 1 min in water. Histological analysis colorectal tissues were fixed in 10% formalin. Thin (0.5 mm) paraffined section were prepared and stained with toluidine blue as and then processed for light microscopy with ×400 magnification.

All specimens taken from experimental animals'

organs were subjected to histopathological examination followed by immunohistochemistry for beta-catenin, COX-2, and caspase expression. Catalog number and merk of the reagents used in this study are anti-CASP3 antibody Rabbit Caspase 3 Polyclonal Antibody, Catalog # MBS821357; anti-COX-2 antibody Rabbit COX-2 Monoclonal Antibody; Catalog # MBS370048 anti-CTNNB1 antibody Rabbit beta Catenin Polyclonal Antibody.Catalog # MBS175500.

The values of each marker are based on scoring the appearance of Ag-Ab reaction from the immunohistochemistry staining which is characterized by the appearance of a brown color in one area of view. Scores ranging from 1 = 0 - 25%; 2 = 26 - 50%, 3 = 51 - 75%, and 4 > 76%.

Data Analysis

Two-way analysis of variance (ANOVA) was used to determine the effect of Annona muricata leaf extract against colorectal carcinogenesis based on the assessment of the expression of COX-2, β–catenin, and caspase activity. The Tukey multiple comparison test is used to determine the differences between groups. Prior to doing ANOVA, the normality of data distribution was tested using Levene's test, whereas the homogeneity of variance was tested using the Kolmogorov–Smirnov test.

RESULT

The animals' body weight was comparable in all groups at baseline, and all those variables were normally distributed ($p > 0.05$; Shapiro Wilk test). The between-group comparability analysis found that randomization ensured equal distribution of all variables. All groups had an increase body weight throughout the eight-week study, but there was no significant difference in the elevation between groups16.

Inflammatory responses

The effects of the extract on various organs' inflammatory markers vary. COX lowering effect of a high dose is significant in the liver, compared to negative control ($p = .036$). The medium dose has a significant catenin-lowering effect on jejunum ($p= .027$), colon($p=.024$), and brain (0.036), compared to negative control. While the medium and high doses have significant catenin-lowering effects on duodenum ($p=.047$), heart ($p=0.015$), and kidney ($p=.005$) respectively, compared to low doses. In summary medium and high doses significantly lowered inflammatory responses (Table 1).

Histological Alterations and Caspase Activity

Histological assessment was done in a blinded fashion to avoid bias. Our results indicate that multiple organ dysplasia was induced with azoxymethane (AOM) in male Swiss Webster mice. The effect of A. muricata extract administration showed significant results only on heart ($p= .041$), which indicated that the medium and high dose have significant effect compared to low doses. The activity of caspase showed no significant effect on all organs when treated with the extracts as compared to the untreated group (Table 2).

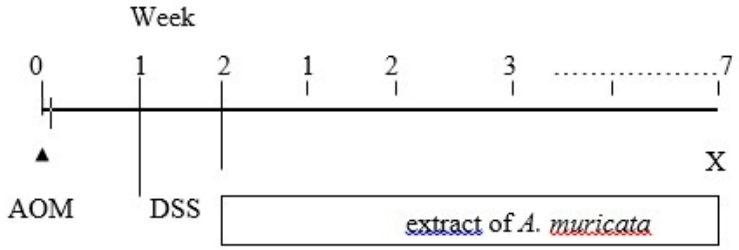

Figure 2. Research protocol (X, animals were sacrificed)

Table 1. COX and β-Catenin expression on several organs after the administration of Annona muricata extracts

	COX			β-Catenin		
Organ	Group (Extract dose)	Mean±SD/ Median(Min-Max)	p	Group	Mean±SD/ Median(Min-Max	p
Stomach	negative control low dose medium dose	1.00 (1.00-2.00) 1.750±1.258 1.500 (1.00-2.00)	.479	negative control low dose medium dose high dose	2.500±1.290 1.50 (1.00-2.00) 1.75±0.957 1.00 (1.00-2.00)	.317
Duodenum	negative control low dose medium dose	1.00±0.632 1.67±0.817 1.00 (1.00-2.00)	.272	negative control low dose medium dose	2.667±0.817 2.00±0.632 2.00 (2.00-3.00)	.047
Jejenum	negative control low dose medium dose	1.00±0.632 1.200±0.817 1.00 (0.00-1.00)	.667	negative control low dose medium dose	2.667±1.211 2.00 (1.00-2.00) 2.00±1.00	.027
Ileum	negative control low dose medium dose high dose	1.00 (0.00-1.00) 1.00±0.63246 1.00 (0.00-1.00) 1.00 (0.00-1.00)	0.639	negative control low dose medium dose	2.600±1.34164 1.667±0.8165 2.000 (1.00-2.00)	0.524
Colon	negative control low dose medium dose	1.000 (0.00-1.00) 1.000 (1.00-2.00) 1.000 (1.00-2.00)	.198	negative control low dose medium dose	3.1667±0.75277 2.00 (2.00-3.00) 1.500 (1.00-3.00)	.024
Liver	negative control low dose medium dose high dose	2.0000 ±.63246 1.000 (1.00-2.00) 1.000 (1.00-2.00) 1.000 (1.00-2.00)	.036	negative control low dose medium dose high dose	2.6667±1.21106 2.0000 (2.00-3.00) 1.6667±.81650 1.5000 (1.00-2.00)	.067
Kidney	negative control low dose medium dose	1.1667±.75277 1.000 (.00-1.00) .8571±.69007	.690	negative control low dose medium dose	3.0000±.89443 3.00(2.00-3.00) 1.00 (1.00-3.00)	.005
Lung	negative control low dose medium dose high dose	1.000 (.00-1.00) .500 (.00-1.00) .000 (.00-1.00) .500 (.00-1.00) 1.000 (.00-1.00)	.724	negative control low dose medium dose high dose	1.00 (1.00-2.00) 2.00 (1.00-3.00) 1.50 (1.00-2.00) 1.50 (1.00-2.00)	.573
Heart	negative control low dose high dose	1.000 (.00-1.00) 1.000 (.00-1.00) 1.000 (.00-2.00)	.072	negative control low dose medium dose	2.50±1.22474 2.50 (2.00-3.00) 1.00 (1.00-2.00)	.015
Brain	negative control low dose medium dose high dose	1.000±.70711 1.000 (.00-1.00) .500 (.00-1.00) .667±.81650	076	negative control low dose medium dose high dose	3.00±1.00000 2.00 (2.00-3.00) 2.00 (1.00-2.00) 2.00 (1.00-2.00)	.036

Kruskal-Wallis Test was performed to analyze between groups difference, Mann-Whitney Test was performed to determine which group contributed to the differences

Tabel 2. Histological Alterations and Caspase activity on several organs after the administration of Annona muricata extracts

Hematoxylin Eosin Staining				Caspase		
Organ	Group (extract dose)	Mean±SD/ Median(Min-Max)	p	Group	Mean±SD/ Median(Min-Max)	p
Stomach	negative control low dose medium dose high dose	0.000 (0.00-2.00) 0.000 (0.00-1.00)	.501	negative control low dose medium dose high dose	1.00 (1.00-2.00) 1.25±0.957 0.500 (0.00-1.00) 1.000 (0.00-1.00)	.191
Duodenum	negative control low dose medium dose high dose	0.667 (±0.817) 0.000 (0.00-2.00) 0.000 (0.00-2.00) 0.000 (0.00-2.00)	.239	negative control low dose medium dose high dose	1.00 (0.00-1.00) 0.00 (0.00-1.00) 0.00 (0.00-1.00) 0.00 (0.00-1.00)	.546
Jejenum	negative control low dose medium dose	0.00 (0.00-1.00) 0.00 (0.00-1.00) 0.00 (0.00-1.00)	0.565	negative control low dose medium dose high dose	0.500 (0.00-1.00) 0.000 (0.00-1.00) 0.000 (0.00-1.00) 0.000 (0.00-1.00)	.955
Ileum	negative control low dose medium dose high dose	0.00 (0.00-2.00) 0.00 (0.00-2.00) 0.00 (0.00-1.00) 0.00 (0.00-2.00)	0.730	negative control low dose medium dose high dose	1.000 (0.00-1.00) 0.6667±0.81650 0.000 (0.00-2.00) 0.000 (0.00-1.00)	0.931
Colon	negative control low dose medium dose high dose	3.00 (0.00-3.00) 2.50 (2.00-4.00) 1.5714 ±1.13389 1.6667±1.36626	.069	negative control low dose medium dose high dose	1.000 (0.00-1.00) 1.000 (1.00-2.00) 1.000 (1.00-2.00) 1.000 (0.00-1.00)	.121
Liver	negative control low dose medium dose high dose	1.0000±.89443 .8000±.83666 1.0000±.89443 .0000 (.00-1.00)	.325	negative control low dose medium dose high dose	1.0000 (1.00-2.00) 1.0000 (1.00-2.00) .5000 (.00-1.00) 1.0000 (.00-1.00)	
Kidney	negative control low dose medium dose high dose	.8333±.75277 .0000 (.00-2.00) 1.1429±.89974 .0000(.00-1.00)	.295	negative control low dose medium dose high dose	1.1667±.75277 1.0000±1.00000 .8571±.89974 1.0000 (.00-1.00)	.860
Heart	negative control low dose medium dose high dose	.0000 (.00-1.00) 1.0000 ±.89443 1.1429±.69007 1.0000(.00-1.00	.041	negative control low dose medium dose high dose	1.0000 (.00-1.00) 1.0000 (.00-1.00) 1.0000 (.00-1.00)	.337

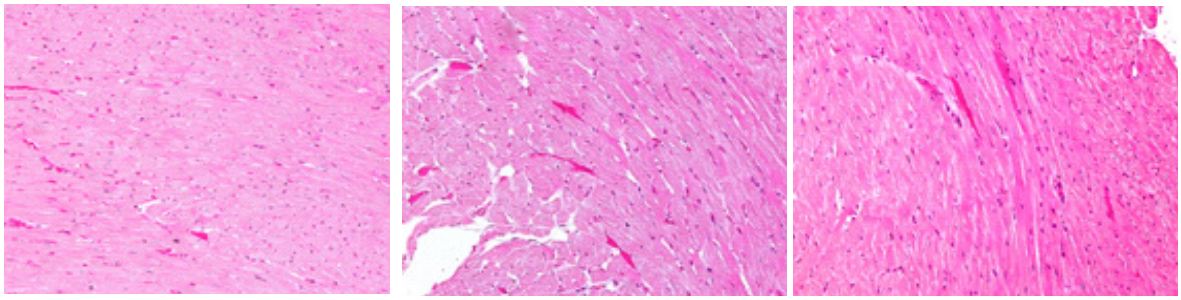

Figure 1. HE staining of heart tissue, medium, and high dose compared to the control group, 200 × magnification

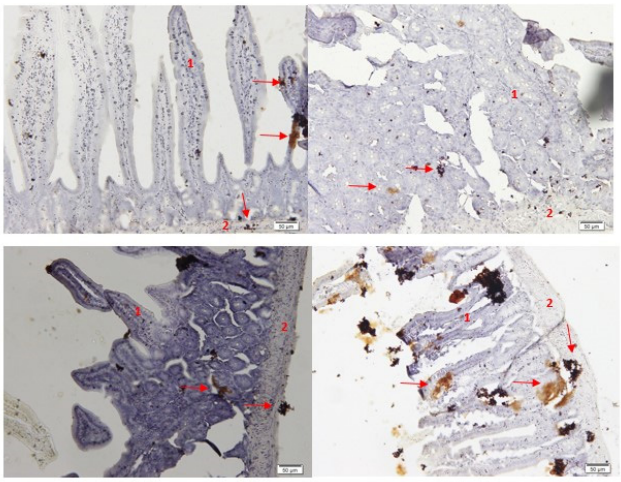

Figure 2. β-Catenin expression on duodenum after the administration of medium dose of Annona muricata extracts. x200. 1. Villi mucosa, and 2. Tunica muscularis. The markers (arrow, brownish) are scattered around the mucosal villi and tunica muscularis.

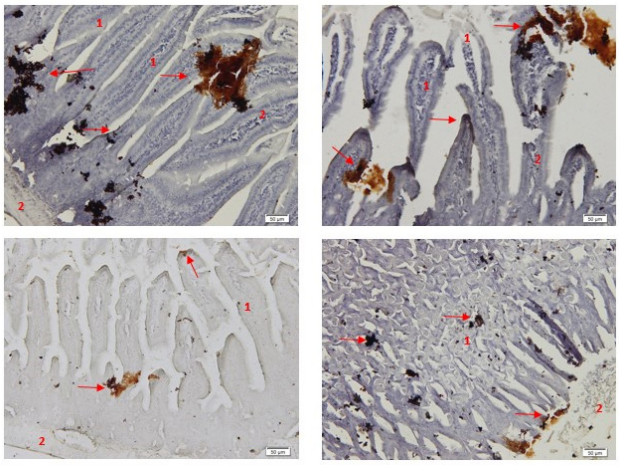

Figure 3. β-Catenin expression on duodenum after the administration of a high dose of Annona muricata extracts. x200. 1. Villi mucosa, and 2. Tunica muscularis. The markers (arrow, brownish) are scattered around the mucosal villi and tunica muscularis. The staining of tissues is faded.

DISCUSSION

Discussion

This study uses the same water extract but a different fraction from the author's previous study, the previous used ethanolic soluble fraction (ESFAM) but currently used ethanolic insoluble fraction (EIFAM). Ex vivo and clinical studies showed higher cytotoxicity in the ESFAM-supplemented group compared with the placebo group14. A. muricata leaves water extract and its ESFAM exhibited cytotoxic activities in colorectal cancer cells in vitro. ESFAM significantly induced caspase-9 activity of DLD-1 colorectal cancer cell line ex vivo using serum of colorectal cancer patients after eight weeks of treatment with ESFAM13.

The reason why this study is using EIFAM is because in previous study EIFAM showed selective cytotoxic activities in vitro, while it contains the lowest concentration of flavonoid and annonacin15. However, in this study the effect of it only significant on one organ, it is the heart. This could be because the active ingredient tends to act on the heart. Many findings on phytochemicals are inconclusive partly due to our limited understanding of phytochemical bioavailability, on which health benefits depend. In addition, the transport mechanisms for delivering phytochemicals to target sites, the phytochemical metabolism of the human body, and biomarkers exerting health benefits are poorly understood. Therefore, an optimal dose should be determined for administration to provide the adequate amount required to reach the blood.

This study showed that the extracts have more prominent impact on inflammatory processes rather than on cancer development. The markers of inflammation measured in this study are COX and Catenin. The previous ex vivo study using ESFAM showed a strong significant correlation between IFN- γ and IL-10 (coefficient correlation = 0.47, p = 0.05) was found after 8 weeks Annona muricata

extract supplementation, as shown by increasing of IL-10 production, in response to TNF-α and IFN-γ in an inflammatory condition. While animal model studies using EIFAM showed that EIFAM had the potential to be an anti-colon cancer proven by the extract capability to reduce ICAM-1 and VCAM-1. The intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are two immunoglobulin superfamily adhesion molecules. Both are responsible for the endothelium adhesion of cancer cells and the immune responses of cancers16. All those results agree with the present study indicating that the extract can potentially be an anti-inflammatory agent.

Inflammation is emerging as one of the hallmarks of cancer, yet its role in most tumors remains unclear. Inflammation might have many as-yet-unrecognized facets, among which an indolent course might be far more prevalent than previously appreciated. The various inflammatory processes underlying the development and progression of colorectal cancer and discuss anti-inflammatory means for its prevention and treatment17. Studying the polarization and activation profiles of immune cells and stromal cells in the tumor microenvironment has been shown to be more informative, thus making CRC a prototypical example of the importance of an inflammatory microenvironment for tumorigenesis18.

Furthermore, this study does not show the effect on dysplasia but reveals an impact on inflammatory response. This could be because of the study period. Mice might be better investigated in one 10-week and one 20-week study to provide opportunity for the extracts to show further effects. In other studies using the colon carcinogenesis model, trends in the results appeared to be highly dependent on sacrifice time-point19. Finally, the amount of extract used in this study was not calculated in terms of the equivalent concentration of the active ingredient, because the active ingredient of the EIFAM has not been determined yet. Thus, the amount of the extract given to the mice is only based on a maximal tolerable dose that will not cause harm.

CONCLUSION

Ethanol-insoluble fraction of Annona muricata (EIFAM) leaves water extract has a potential inhibitory effect of Annona muricata leaves on carcinogenesis and inflammation in the colorectal preneoplasia development of mice. The limitation of this study could be the study period that limit the appearance of effect of the extract. This result also needs further investigation to ensure its effect and safety in human body, as well as to investigate the phytochemical content of EIFAM.

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