

# Antiinflammatory Effect of Mahkota Dewa (*Phaleria macrocarpa* (Scheff.) Boerl.) Leaves Extract on Colon Carcinogenesis Induced by Azoxymethane and Dextran Sodium Sulphate: Focus on the iNOS, $\beta$ -catenin and COX-2 Expression

Tati Suprapti,\* Melva Louisa\*\*, Aryo Tedjo\*\*\*, Kusmardi\*\*\*\*, Fadilah\*\*\*, Diah Rini Handjari\*\*\*\*\*, Yulhasri\*\*\*\*\*

\* Department of Pharmacy, Polytechnic of Health, Ministry of Health Republic of Indonesia JakartaII

\*\* Department of Pharmacology and Therapeutic, Medical Faculty, University of Indonesia

\*\*\* Department of Medical Chemistry, Medical Faculty, University of Indonesia

\*\*\*\* Department of Phatological Anathomy, Medical Faculty, University of Indonesia

\*\*\*\*\* Department of Biochemistry, Medical Faculty, University of Indonesia

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**ABSTRACT**--- *Colorectal cancer is a malignant tumor caused by excessive growth derived from the colon wall. Chemical carcinogens, azoxymethane (AOM)+dextran sodium sulphate (DSS) are known to cause the formation of adenoma in the colon characterized by increased expressions of iNOS,  $\beta$ -catenin and COX-2. Ethanol extract of mahkota dewa leaves are known to have anticancer effects, but until now the mechanism has not been fully understood. This study was aimed to determine the effect of Ethanol extracts of mahkota dewa leaves (MD) on colon carcinogenesis induced by AOM-DSS. Balb/c mice were randomly divided into 6 groups of untreated control; AOM + mahkota dewa 25 mg + DSS; AOM + mahkota dewa 50 mg + DSS; AOM + aspirin 0,21 mg + DSS (positive control 1); AOM+ aspirin 0,84 mg + DSS (positive control 2). MD/aspirin was administered daily per oral for 7 days, starting 1 week after AOM induction, while DSS 1% was given through drinking water daily for 7 days ad libitum. Twenty weeks post AOM administration, mice were sacrificed, colons were taken and analyzed histopathologically. Expression of iNOS,  $\beta$ -catenin and COX-2 were analyzed using immunohistochemistry. Tissue damage induced by AOM + DSS goblet cells were in the form of inflammation (inflammation score 192). Ethanol extracts of mahkota dewa dose 25 mg and 50 mg were shown significant to reduce tissue damage induced by chemical carcinogens AOM+DSS (inflammation score decreased to 45 and 58;  $p < 0,05$ ). Ethanol extracts of mahkota dewa leaves suppressed the expression of iNOS,  $\beta$ -catenin and COX-2 significantly vs AOM+DSS at all doses, except for Ethanol extracts of mahkota dewa dose 50 mg, which was unable to suppress the expression of COX-2. Ethanol extracts of mahkota dewa leaves can reduce inflammatory process induced by chemical carcinogens AOM+DSS. This effect is due to the inhibitory effect of mahkota dewa leaves extract to the iNOS,  $\beta$ -catenin and COX-2 expressions.*

**Keywords**---- mahkota dewa, AOM+DSS, COX-2,  $\beta$ -catenin and iNOS

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## 1. INTRODUCTION

Until now the cancer is still has been a health problem in the world. Cancer is the second leading cause of death or the cause of one in every four deaths in the United States . In a report issued by the American Cancer Society in 2013 , there were an estimated 1,660,290 new cases and 580,350 deaths of people or 1,600 people die every day due to cancer[1].

Cancer incidence data in Indonesia based on the 2007 Household Survey in Indonesia ratio of tumor or cancer is 4.3 per 1,000 population, and cancer is the seventh leading cause of death (5.7%) after stroke, tuberculosis, hypertension, injury, perinatal and Diabetes Mellitus. [2]

Among cancers, colorectal cancers are the third leading cause of cancer death in men and women in the United States, in the year 2013 there were approximately 102 480 new cases and an estimated 50.830 deaths caused by colorectal cancer, or 9 % of all deaths from caused by cancer. [1] Colorectal cancer in Indonesia based on the observation of Anatomic Pathology section Cipto Mangunkusumo Hospital, Jakarta showed that in 1986-1990, totaling 275 colorectal cancer patients, an increase to 368 by the year 1991 to 1995, and between 1999-

2003, reaching 584 patients. [3] Colorectal cancer patients in Indonesia under the age of 40 were more than 30 % different from the incidence in developed countries, the incidence of colon cancer increased at the age group above 50 tahun. [4].

Until now the main modality of cancer treatment is chemotherapy, surgery and radiation, which has not been fully effective for healing pasien [5]. Therefore, studies for the search of effective therapies for cancer is still something that needs to be prioritized.

Plant mahkota dewa has been widely used by people in Indonesia for the treatment of various diseases including cancer[6]. These plants contain active substances falerin, mangiferin, kaempferol, gallic acid, ikarisida, and other phenolic compounds[7,8,9]. Study the effects of anticancer plant mahkota dewa has done such a cytotoxic effect of extracts Phaleria against Hela cell line [10], anticancer effects of gallic acid isolated from the plant mahkota dewa in human cancer cell lines[11], anticancer activity of ethanol extract [Phaleria macrocarpa (Scheff.) Boerl] in C3H breast mouse tumors[12] and inhibition activity benzophenone glucoside isolated of mahkota dewa extracts on L1210 leukemia cell line[13].

Giving azoxymethane chemical carcinogens as initiators can cause DNA mutations that would increase the expression of  $\beta$ -catenin and dextran sodium sulfate as the promoter that causes inflammation of the colonic mucosa is accompanied by increased expression of iNOS and COX-2. Therefore in this study were selected proteins iNOS and COX-2 as a marker of inflammation and  $\beta$ -catenin as a proliferation marker whose expression increases in carcinogenesis.

The purpose of this study is to prove whether the ethanol extract of mahkota dewa leaves can inhibit inflammation in mice colon carcinogenesis induced by AOM and DSS and anti-inflammatory effects of ethanol extract of mahkota dewa leaves marked by decreased expression of iNOS,  $\beta$ -catenin and COX-2 in colon tissue mice induced by AOM and DSS.

#### Research Hypothesis

1. Mahkota dewa ethanol extract can inhibit inflammation in mice colon carcinogenesis induced by AOM + DSS.
2. Mahkota dewa ethanol extract has anti-inflammatory effects that are characterized by a decreased expression of iNOS,  $\beta$ -catenin and COX-2, in colonic tissue of mice induced by AOM and DSS.

## 2. METHODS

### Research Design

This research is experimental in vivo, using experimental animals Balb/c .

Balb/c males as much as 30 were divided into 6 groups (each group consisted of 5 animals), namely:

1. K-0 is the group of mice that did not receive treatment.
2. AD is a group of mice that received one injection of 0.2 mL azoxymethane 0.1% w/v ip, 7 days and then given DSS solution of 1% w/v through drinking water ad libitum every day for 7 days.
3. AD+MD 25 is a group of mice that received one injection of 0.2 mL azoxymethane 0.1% w/v ip, 7 days and then given orally mahkota dewa extract solution 12.5% w/v by 0.2 mL (equivalent to 25 mg extract) and DSS solution of 1% w/v through drinking water ad libitum every day for 7 days.
4. AD+ MD 50 is a group of mice that received one injection of 0.2 mL azoxymethane 0.1% w/v ip, 7 days then orally administered mahkota dewa extract solution 25% w/v by 0.2 mL (equivalent to 50 mg extract) and DSS solution of 1% w/v through drinking water ad libitum every day for 7 days.
5. AD + Asp 0.21 is the group of mice that received one injection of 0.2 mL azoxymethane 0.1% w/v ip, 7 days then orally administered aspirin suspension 0.21 mL 0.1% w/v (equivalent to 0.21 mg aspirin) and DSS solution 1% w/v through drinking water ad libitum every day for 7 days.
6. AD+ Asp 0.84 is the group of mice that received one injection of 0.2 mL azoxymethane 0.1% w/v ip, 7 days then orally administered aspirin suspension 0.21 mL 0.4% w/v (equivalent to 0.84 mg aspirin) and DSS solution 1% w/v through drinking water ad libitum every day for 7 days.

### Euthanized mice

Mice were sacrificed after 20 weeks of tumor post induction with AOM, mice were euthanized done by anesthesia with ether, until the mice die, and then dissected and taken the colon from the proximal to distal.

### Preparation of tissue samples

Colon of mice fed into a buffer solution formalin 10%, the colon of mice that had been soaked

in buffered formalin, taken part of the colon that bulged there, to do the paraffin block. Subsequently made preparations with hematoxylin-eosin staining to observe histopathologic changes.

### **Histopathologic assessment results**

Histopathological examination was performed on mice colon. Analysis of histopathological changes made by reading the outward appearance in a blind way, meaning that the preparations are not aware of which group, and performed by 2 technicians, then the results were averaged. Readings were taken at 200 times magnification. Preparations were taken every 3 representative field of view, then an assessment in accordance with the criteria of Cooper et al. [14]. Assessment by the method of Cooper et al, [14] as follow: any preparations predetermined intensity of the inflammatory network, which is given a value between 1 to 3. Then calculated the area is inflamed, whose value ranges between 0 - 100. For example, one group of mice colon tissue preparations mahkota dewa has inflammatory intensity = 2, with extensive areas of inflammation 25% of the field of view, then the score of inflammation of the tissue preparations is =  $2 \times 25 = 50$ . Readings preparations done at 200 times magnification.

### **INOS, $\beta$ -catenin, COX-2 staining**

#### **a. Antibody optimization anti-COX-2/iNOS/ $\beta$ -catenin**

Before staining, optimize anti-COX-2/iNOS/ $\beta$ -catenin antibody. From the results of the optimization used anti-iNOS antibody with 1: 300 dilution (anti-iNOS antibody 1mL + 299 mL diluent), anti-  $\beta$ -catenin 1: 400 (1 mL antibody anti- $\beta$ -catenin + 399 mL diluent), and antibody anti-COX-2 with a 1: 400 dilution (1 mL antibody anti-COX-2 + 399 mL of diluent).

#### **b. INOS staining procedure, $\beta$ -catenin, COX-2**

Colonic tissue samples were fixed with phosphate-buffered formalin 10% for 10 hours at 4°C, dehydrated in graded concentrations of ethanol, once passed in xylol, made paraffin blocks. Network cut to a thickness of 4  $\mu$ m for pewarnaan immunohistochemistry. Once done deparaffinize and rehydration, preparations dyed in 0.01 M citrate buffer (pH 6.0) in a microwave for 5 minutes. Apply the preparation with 3% hydrogen peroxide to eliminate endogenous peroxide for 5 min at room temperature. Preparations were incubated with antibodies anti iNOS/ $\beta$ -catenin/COX-2 in phosphate buffer solution (PBS) for 2 hours at room temperature in a humidity chamber incubation continued overnight at 4°C. Used as a negative control N-Universal negative control (Dako). Preparations were then incubated with the appropriate secondary antibody for 1 hour at room temperature, followed by incubation for 30 min with HRP - conjugated streptavidin. Proteins visualized using 3,3'-diaminobenzidine (DAB) for 10 min at room temperature. The preparation was added counterstain with Harris Hematoxylin hydrate and do mounting[15].

### **Interpretation of the results of immunohistochemical**

Tumor cells that express each protein was calculated from 1000 on colonic epithelial cells. The percentage of cells expressing these proteins are presented in tabular form for data analysis. Calculating many colonic epithelial cells that express the protein iNOS/ $\beta$ -catenin/COX-2 of 3 representative field of view, done blind (not knowing the preparations from which the group), done by 2 technicians, then the results are averaged. Readings were taken at 200 times magnification.

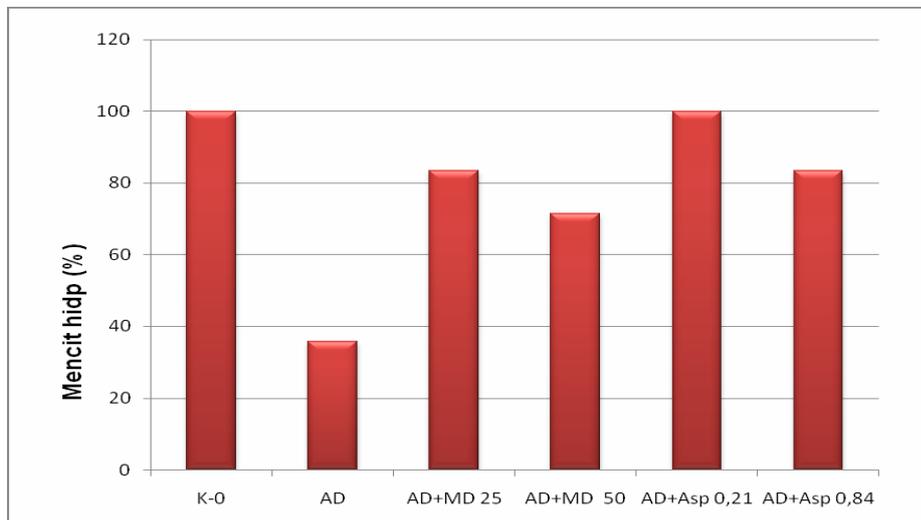
## **7. ANALYSIS OF THE DATA**

To test the hypothesis of differences with the control group on a test group of each extract was 1-way ANOVA followed by Tukey test. Prior to the distribution of the test data is determined by Kolmogorov - Smirnov test and homogeneity variants carried by Levene test. When the data are not homogeneous analyzed by analysis of variance using the analysis of non - parametric Kruskal - Wallis. All data were analyzed using SPSS version 16.

## 8. RESULTS

### 1. Induction results with Azoksimetan and Dextran Sulfate Sodium

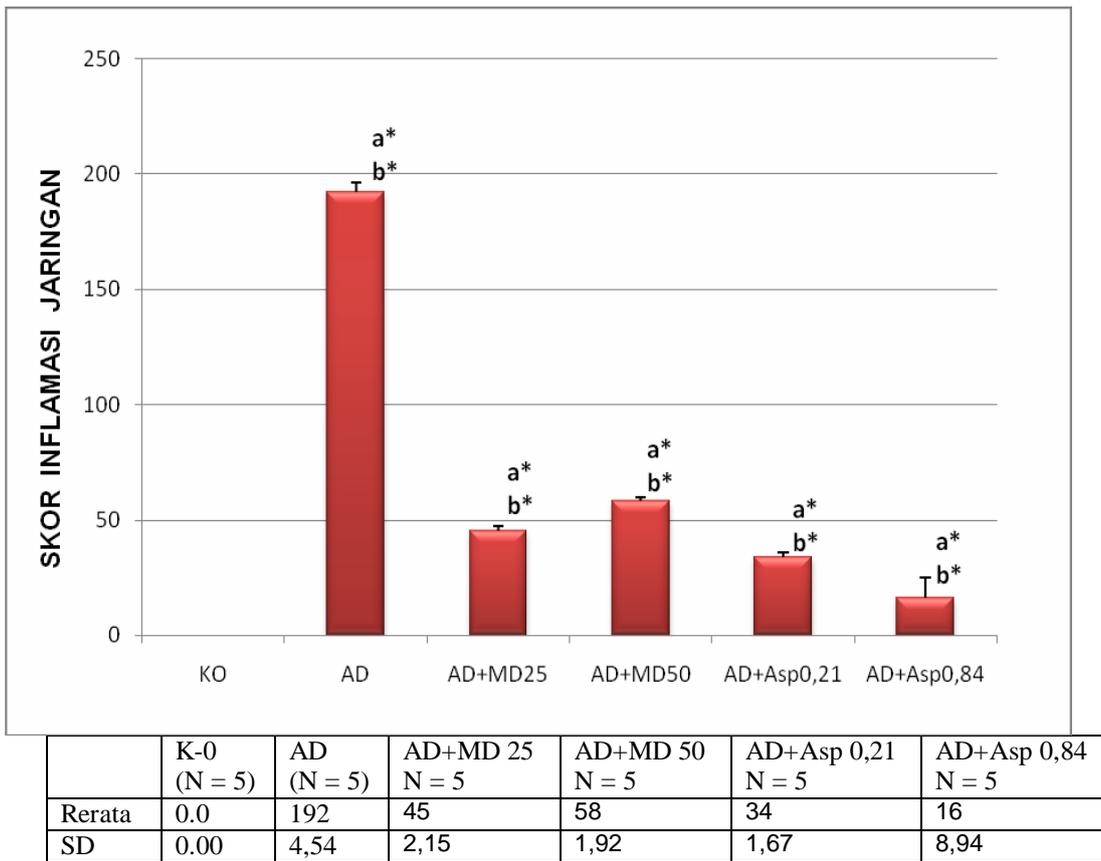
In Figure 4.1. showed that in the aspirin group was 0.21 mg survival rate of the control group equaled 100%. The second highest survival rate occurred at 0.84 mg aspirin group, the mahkota dewa extract dose of 25 mg, followed by a group of mahkota dewa extract 50 mg. While the lowest survival occurred in the AOM + DSS group.



**Figure 1. the graph of induced survival mice with AOM / DSS or without medication.** Description: The percentage of live mice K-0 = a group of mice, no treatment (100%); AD = a group of mice AOM + DSS (35.7%); AD+MD25 = a group of mice AOM+ 25 mg mahkota dewa extract + DSS (83.3%); AD+MD50 = a group of mice AOM+ 50 mg mahkota dewa extract + DSS (71.4%); AD+Asp0,21 = a group of mice AOM + 0.21 mg aspirin + DSS (100.0%); AD+Asp0,84 = a group of mice AOM + 0.84 mg aspirin + DSS (83.3%).

### 2. Histopathology Observation

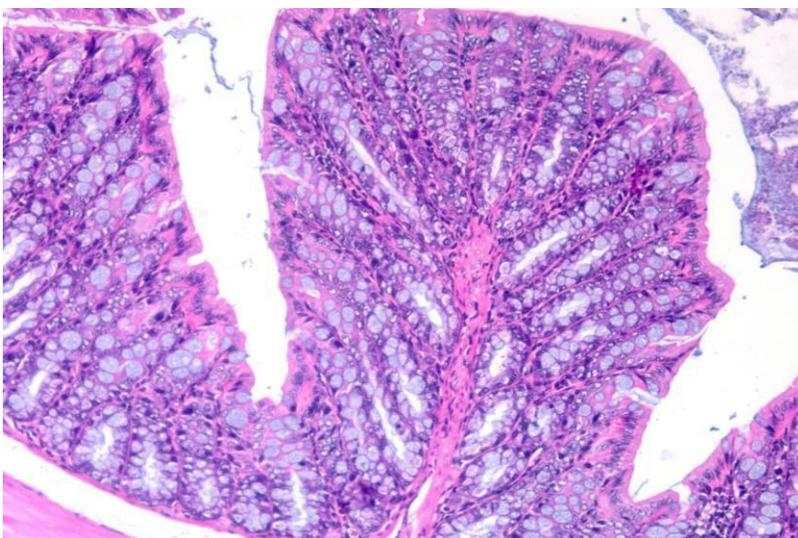
Figure 2. showed that in the control of inflammation is not indicated by a score of = 0.0. Colonic damage is greatest in the AOM + DSS group with score = 192. Ethanol extract of mahkota dewa and giving aspirin can reduce inflammation score caused by OM + DSS significantly. Decreasing in colonic tissue inflammation scores were also significant vs. controls, but did not approach the control value.



**Figure 2. Graph score of mice colon tissue inflammation was assessed using scores colonic inflammation by Cooper et al. [14].**

Description: K-0 group = is a group without treatment, inflammatory score = 0; AD group = a group AOM + DSS, inflammatory score = 192; AD+MD25 group = a group of AOM + 25 mg Mahkota dewa extract + DSS, inflammatory score = 45; AD+MD50 group = is a group of AOM+ 50 mg Mahkota dewa extract + DSS, inflammatory score = 58; AD+ Asp 0,21 group = a group of AOM + 0,21 mg Aspirin + DSS, inflammatory score = 34; AD+ Asp 0,84 group = a group of AOM + 0,84 mg Aspirin + DSS, inflammatory score = 16.

**A. Normal tissue**



B.

Degree of inflammation 1

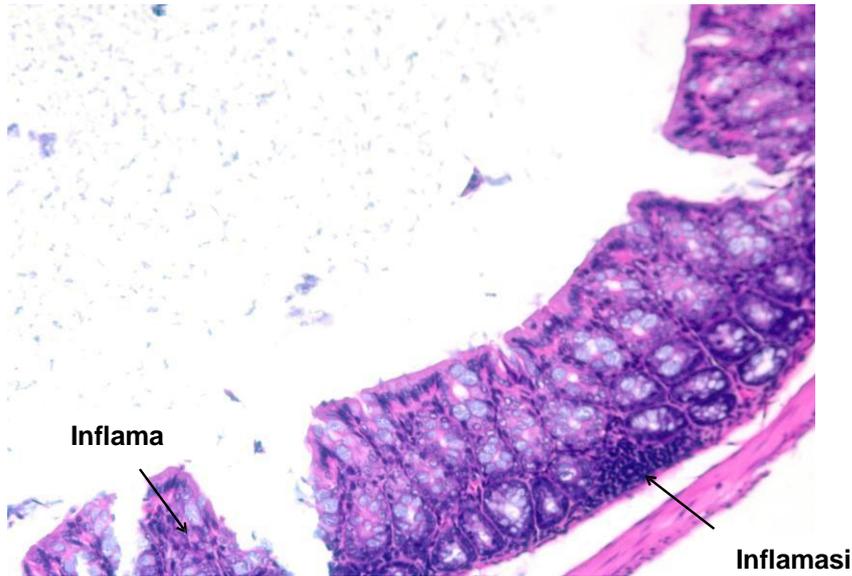
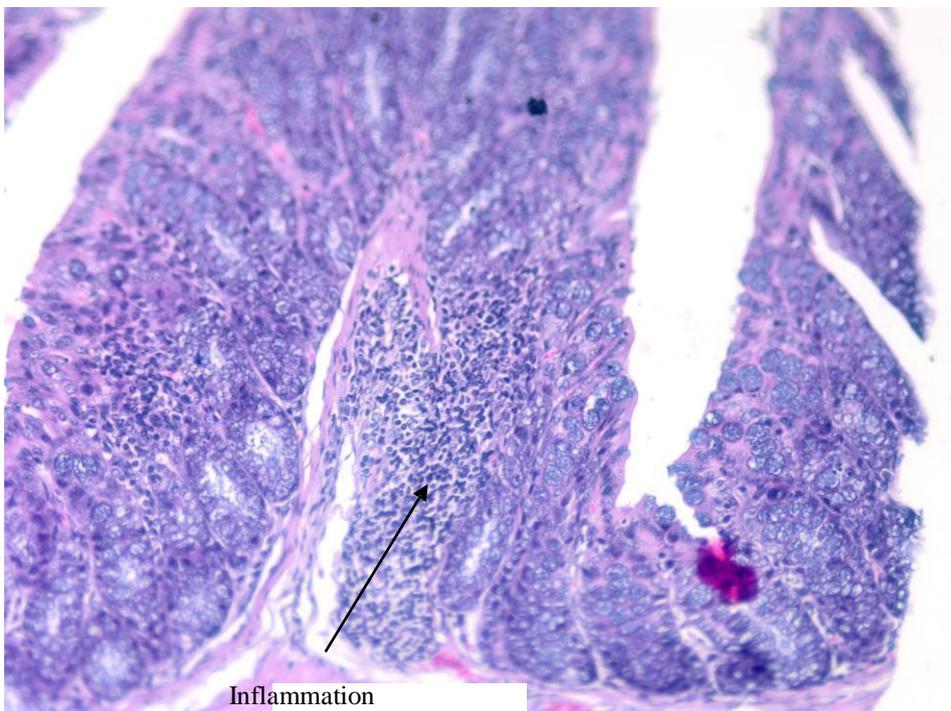


Figure 3. A. Normal colon tissue; B. Inflammatory colon tissue with intensity of inflammation 2, and 10% extensive inflammation with inflammatory scores 20.

C. Degree of inflammation 2



**D. Degree of inflammation 3**

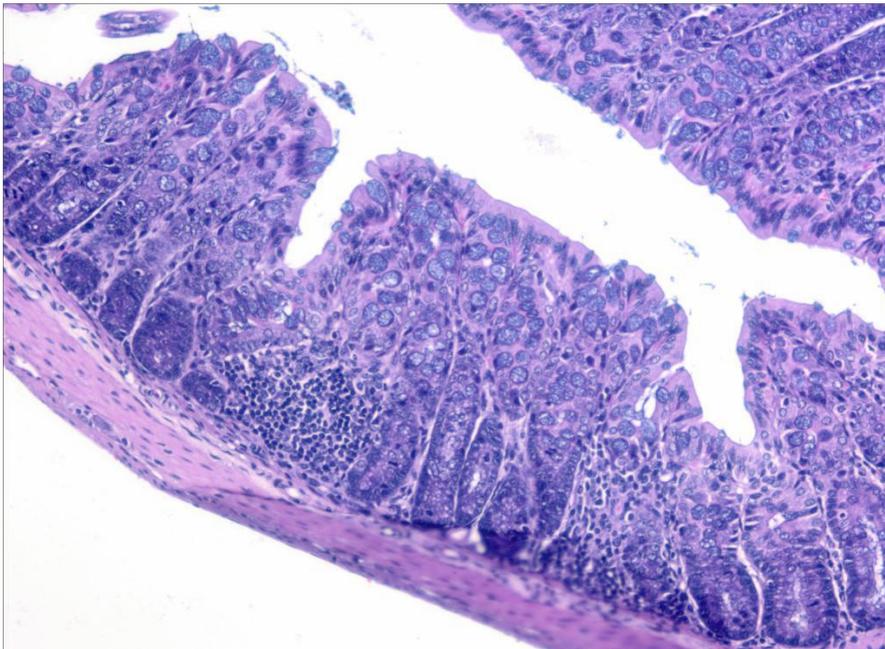
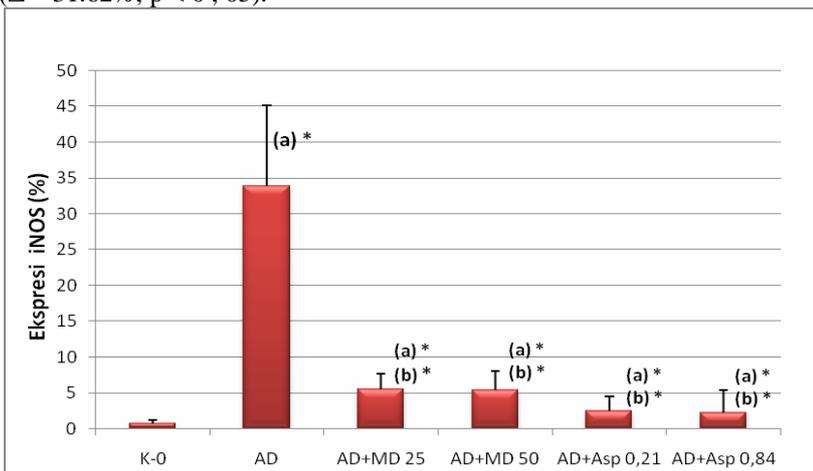


Figure 4. C. Inflammatory colon tissue with intensity 3, and 10% broad-inflammatory, inflammatory scores 75; D. Inflammatory colon tissue with intensity 3, and 10% broad- inflammatory, inflammatory scores 60. 200X magnification.

**3. Percentage expression of inducible Nitric Oxide Synthase (iNOS) after reading by immunohistochemistry.**

Figure 5. showed that there was a significant increase in iNOS expression in the AOM + DSS group at 33.82% vs. controls ( $\Delta = 33.1\%$ ;  $p < 0,05$ ). Increased expression of iNOS by AOM + DSS can be significantly reduced by ethanol extract of mahkota dewa 25 mg ( $\Delta = 28.3\%$ ;  $P < 0.05$ ) and ethanol extract of mahkota dewa 50 mg ( $\Delta = 28; 4\%$ ;  $p < 0.05$ ), as well as the 0.21 mg aspirin group ( $\Delta = 31.42\%$ ;  $p < 0.05$ ) and 0.84 mg aspirin ( $\Delta = 31.62\%$ ;  $p < 0, 05$ ).



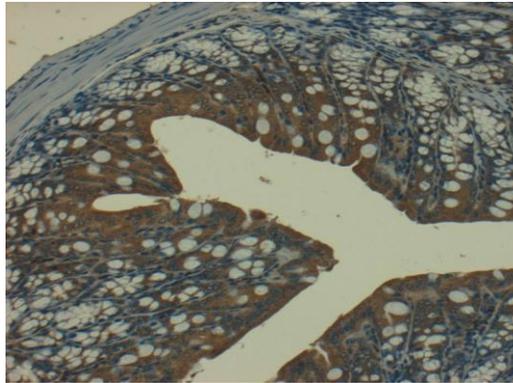
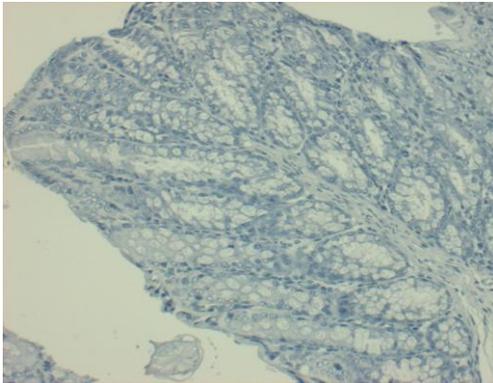
	K-0 (N = 5)	AD (N = 5)	AD+MD 25 (N = 5)	AD+MD 50 (N = 5)	AD+Asp 0,21 (N = 5)	AD+Asp 0,84 (N = 5)
Average	0.7	33.8	5.5	5.4	2.4	2.2
SD	0.49	11.27	2.14	2.66	2.03	3.22

**Figure 5. Graph mice colon tissue iNOS expression, after reading the immunohistochemistry.**

Description: a =  $p < 0.05$  compared to group K-0; b =  $p < 0.05$  compared to the AD group; \* = Indicates significance at  $p < 0.05$ , after statistical test with Kruskal-Wallis method; NS = Not significant after statistical tests, with the Kruskal-Wallis method ( $p > 0.05$ ); K-0 = a group of mice, no treatment; AD = a group of mice AOM + DSS; AD + MD25 = a group of mice AOM + 25 mg extract of mahkota dewa + DSS; AD + MD50 = a group of mice AOM+ 50 mg extract of mahkota dewa + DSS; AD + Asp0.21 = a group of mice AOM+0.21 mg aspirin+ DSS; AD+Asp0,84 = a group of mice AOM+0.84 mg aspirin+ DSS.

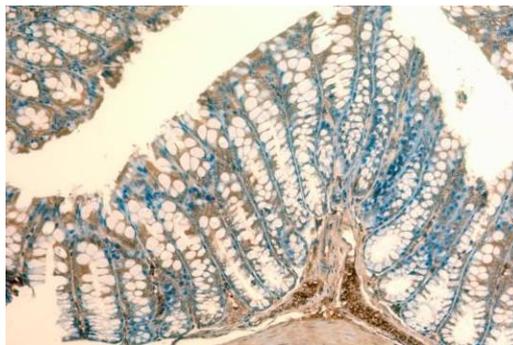
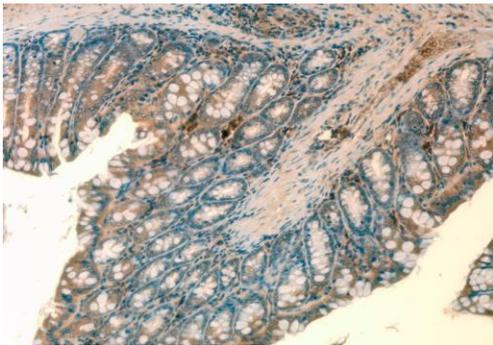
**K-0**

**A. AD**



**B. AD+MD 25**

**C. AD+MD 50**



**D. AD+Asp 0,21 E. AD+ Asp 0,84**

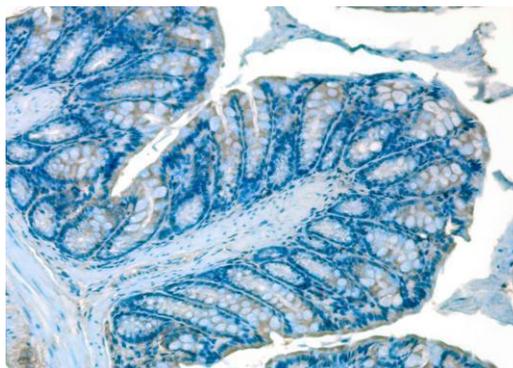
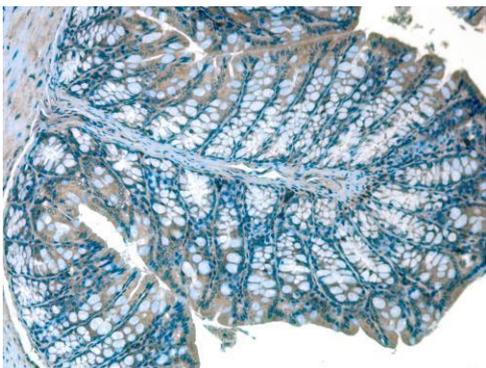


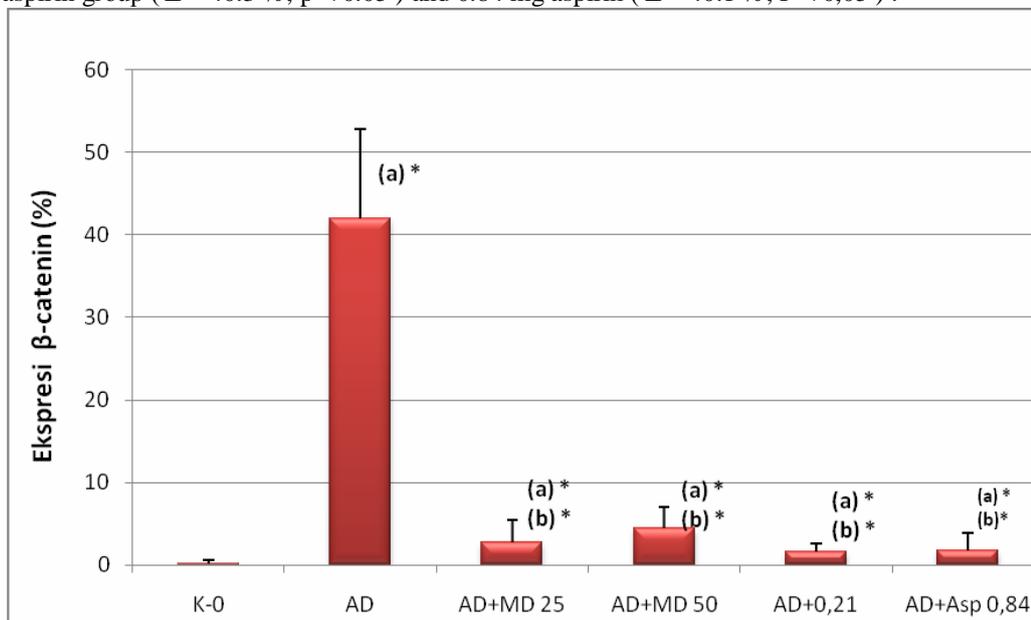
Figure 6 . Overview iNOS immunohistochemistry . Colon of mice given the treatment as follows A. K - 0 ; B. AD ; C. MD 25 ; D. MD 50 ; E. Asp 0.21 ; F. Asp 0.84 . 200X magnification .

Description: K-0 = tissue of mice colon, without treatment with iNOS expression an average 0.7%; AD = = tissue of mice colon with AOM + DSS, with iNOS expression averaged 33.8%; AD+MD25 = = tissue of mice colon with AOM+ 25 mg extract of mahkota dewa + DSS, with iNOS expression averaged 5.5%; AD+MD50 = = tissue of mice colon with AOM+50 mg extract of mahkota dewa + DSS,

with iNOS expression averaged 5.4%; AD+Asp0,21 = tissue of mice colon AOM + Aspirin 0.21 mg + DSS with iNOS expression an average of 2.4%; AD +Asp0,84 = tissue of mice colon AOM + 0.84 mg Aspirin +DSS with iNOS expression an average of 2.2%.

**4 . The percentage of  $\beta$ -catenin expression by immunohistochemistry after reading**

Figure 4.6 . showed that the increased expression of  $\beta$  - catenin in AOM + DSS group was 41.9 % vs control group ( $\Delta = 41.7$  %  $p < 0,05$  ). Increased expression of  $\beta$  - catenin by AOM + DSS can be significantly reduced by extract of mahkota dewa 25 mg ( $\Delta = 39.2$  % .  $P < 0.05$  ) and extract of mahkota dewa 50 mg ( $\Delta = 37.5$  % .  $p < 0.05$  ), as well as the 0.21 mg aspirin group ( $\Delta = 40.3$  % ;  $p < 0.05$  ) and 0.84 mg aspirin ( $\Delta = 40.1$  % ;  $P < 0,05$  ) .



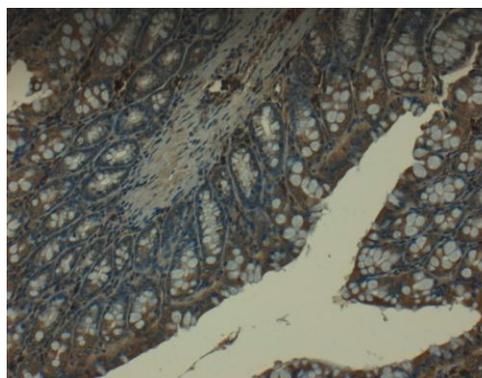
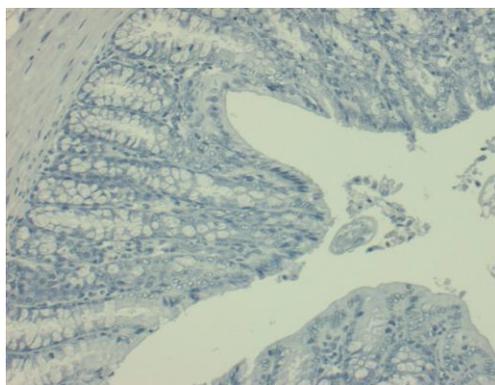
	K-0 (N =5)	AD (N = 5)	AD+MD 25 (N = 5)	AD+MD 50 (N = 5)	AD+Asp 0,21 (N = 5)	AD+Asp 0,84 (N = 5)
Average	0.2	41.9	2.7	4.4	1.6	1.8
SD	0.38	10.88	2.70	2.68	1.02	2.07

**Figure 7.  $\beta$ -catenin expression colonic tissue of mice, after reading the immunohistochemistry.**

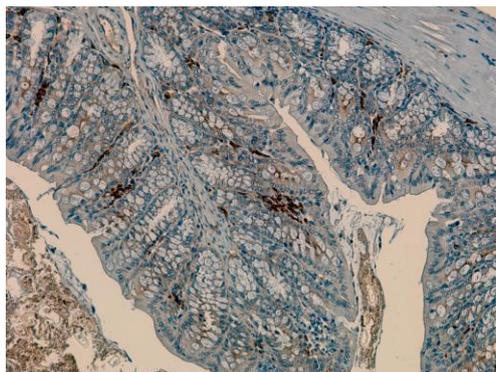
Description: a =  $p < 0.05$  compared to group K-0; b =  $p < 0.05$  compared to the AD group; \* = Indicates significance at  $p < 0.05$ , after statistical test with Kruskal-Wallis method; NS = Not significant after statistical tests, with the Kruskal-Wallis method ( $p > 0.05$ ); K-0 = a group of mice, no treatment; AD = a group of mice AOM + DSS; AD +MD25 = a group of mice AOM + 25 mg extract of mahkota dewa + DSS; AD + MD50 = a group of mice AOM+ 50 mg extract of mahkota dewa + DSS; AD + Asp0.21 = a group of mice AOM+0.21 mg aspirin+ DSS; AD+Asp0,84 = a group of mice AOM+0.84 mg aspirin+ DSS.

**A. K-0**

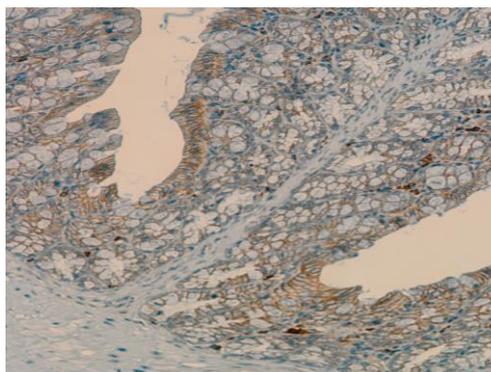
**B. AD**



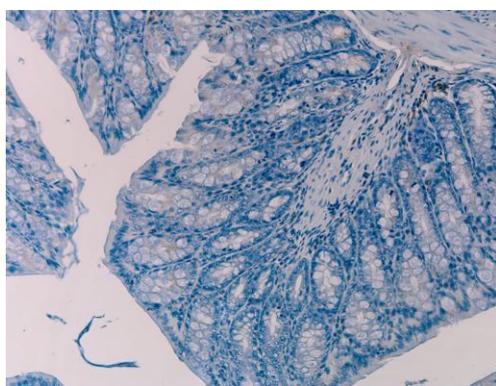
C. AD+MD 25



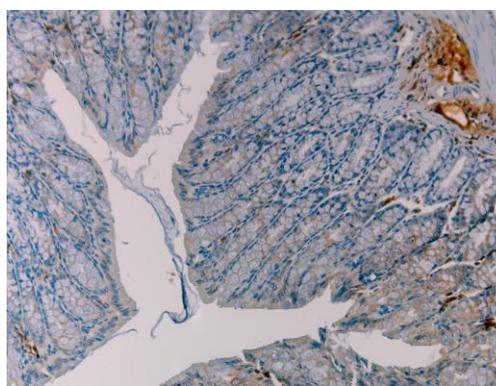
D. AD+MD 50



E. AD+Asp 0,21



F. AD+Asp 0,84

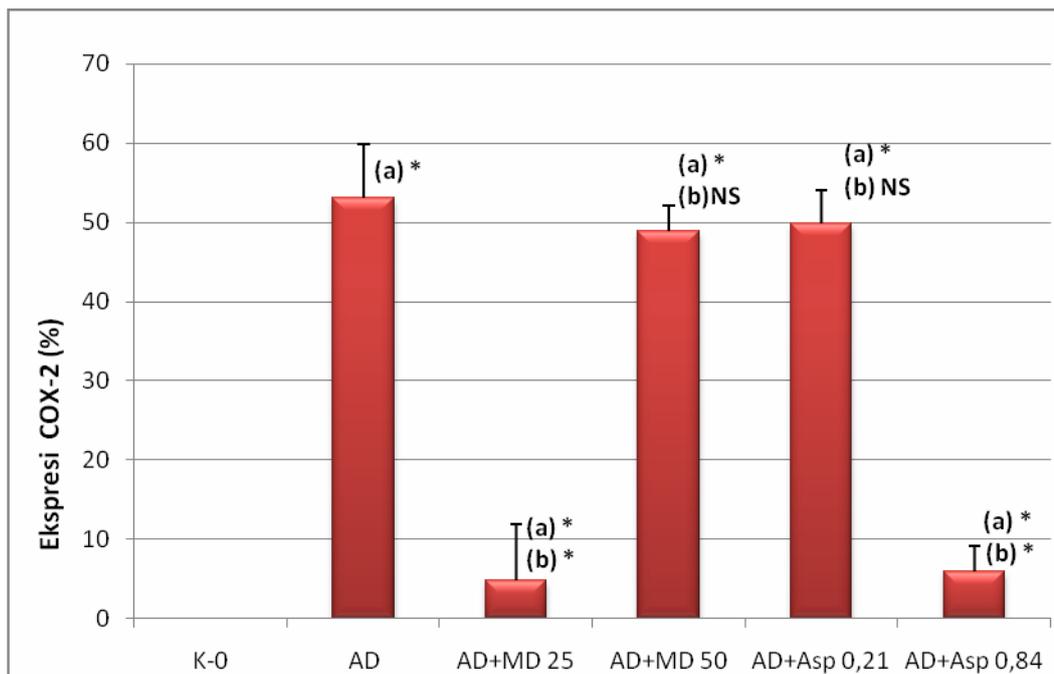


**Figure 8. Picture of  $\beta$ -catenin immunohistochemistry.**

Overview: K-0 = tissue of mice colon without treatment, with  $\beta$ -catenin expression an average 0.2%; AD = tissue of mice colon AOM + DSS, with  $\beta$ -catenin expression averaged 41,9 %; AD + MD25 = tissue of mice colon with AOM+ 25 mg extract of mahkota dewa + DSS, with  $\beta$ -catenin expression averaged 2.7%; AD +MD50 = tissue of mice colon with AOM+ 50 mg extract of mahkota dewa + DSS, with  $\beta$ -catenin expression averaged 4.4%; AD+Asp0,21 = tissue of mice colon AOM + Aspirin 0.21 mg + DSS, with  $\beta$ -catenin expression an average of 1.6%; AD +Asp0,84 = tissue of mice colon AOM + 0.84 mg Aspirin + DSS, with  $\beta$ -catenin expressio averag of 1.8%. 200Xmagnification.

##### 5. Percentage expression of cyclooxygenase-2 (COX-2) after reading by immunohistochemistry.

Figure 4.8. showed that the increased expression of COX-2 in the AOM + DSS group was 53.1% vs control group ( $\Delta = 53.1\%$ ;  $p < 0,05$ ). Increased expression of COX-2 by AOM + DSS can be significantly reduced by extract of mahkota dewa 25 mg ( $\Delta = 48.3\%$ ;  $P < 0.05$ ), and 0.84 mg aspirin group ( $\Delta = 47.2\%$  .  $P < 0.05$ ), but not in MD50 group ( $\Delta = 4.3\%$ ;  $p > 0.05$ ) and Asp 0.21 mg ( $\Delta = 3.3\%$ ;  $p > 0.05$ )



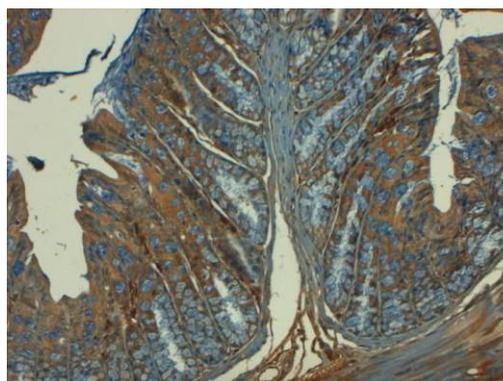
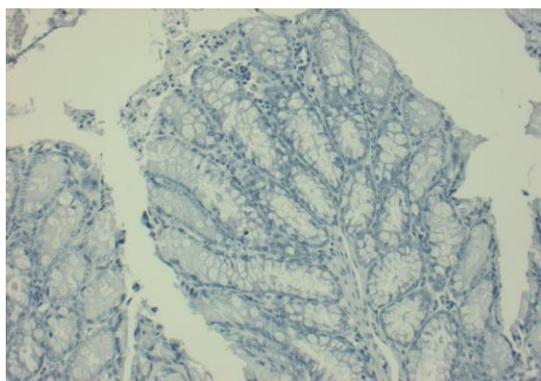
	K-0	AD	AD+MD 25	AD+MD 50	AD+Asp 0,21	AD+Asp 0,84
	(N = 5)	(N = 5)	(N = 5)	(N = 5)	(N = 5)	(N = 5)
Average	0.0	53.1	4.8	48,8	49.8	5.9
SD	0.00	6.84	7.03	3,34	4.17	3.34

Figure 9. Expression of COX-2 colon tissue of mice, after reading by immunohistochemistry.

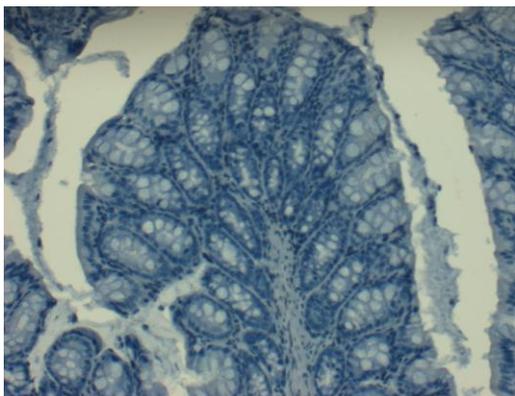
Description: a =  $p < 0.05$  compared to group K-0; b =  $p < 0.05$  compared to the AD group; \* = Indicates significance at  $p < 0.05$ , after statistical test with Kruskal-Wallis method; NS = Not significant after statistical tests, with the Kruskal-Wallis method ( $p > 0.05$ ); K-0 = a group of mice, no treatment; AD = a group of mice AOM + DSS; AD+MD25 = a group of mice AOM+ 25 mg extract of mahkota dewa + DSS; AD + MD50 = a group of mice AOM +50 mg extract of mahkota dewa + DSS; AD + Asp0.21 = a group of mice AOM + 0.21 mg aspirin + DSS; AD + Asp0,84 = a group of mice AOM + 0.84 mg aspirin + DSS.

A. K-0

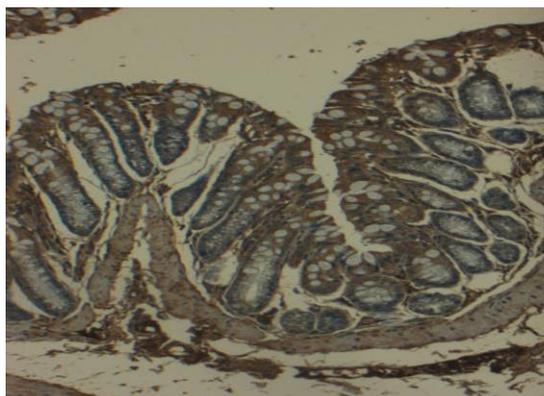
B. AD



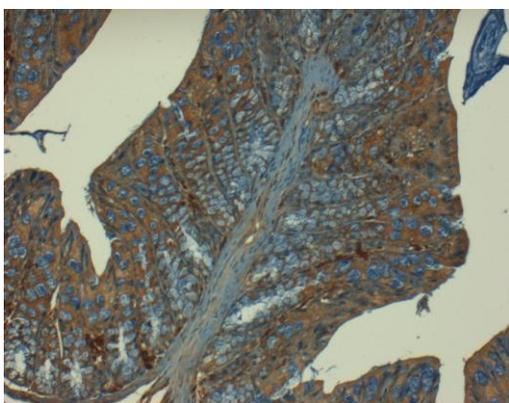
G. AD+MD 25



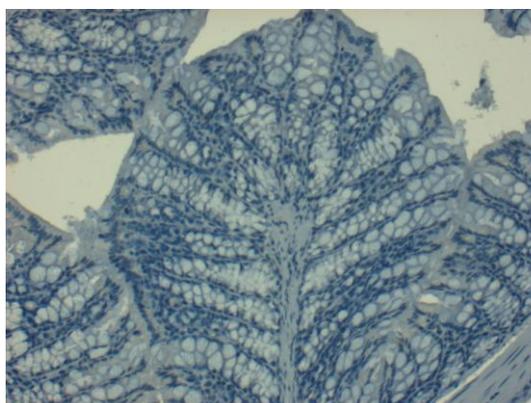
H. AD+MD 50



A. AD+Asp 0,21



B. AD+Asp 0,84



**Picture 10. Immunohistochemical results of COX-2.**

Overview: K-0 = tissue of mice colon, without treatment with COX-2 expression on average 0.0%; AD = tissue of mice colon AOM + DSS with expression of COX-2 on average 53.1%; AD+MD25 = tissue of mice colon with AOM + extracts of mahkota dewa 25 mg + DSS, with the expression of COX-2 on average 4.8%; AD + MD50 = tissue of mice colon + AOM mice colon tissue extracts of mahkota dewa 50 mg + DSS with expression of COX-2 average average 48.8%; Asp0 AD +, 21 = colon tissue of mice Aspirin AOM + DSS + 0.21 mg expression of COX-2 with an average of 49.8%; Asp0 AD +, 84 = colon tissue of mice AOM + DSS + 0.84 mg aspirin with COX - 2 expression averaged 5.9 %. 200x magnification.

## 9. DISCUSSION

Several previous studies have proved that the mahkota dewa antitumor effects. This study is aimed to determine the molecular mechanism of carcinogenesis by inhibition of the ethanol extract of mahkota dewa leaves. Mice induced by AOM and DSS in order to induce carcinogenesis. AOM is used as an initiator and as a tumor promoter[16] while the DSS is used as a promoter tumor[17,18].

These results indicate the high mortality of mice in the AOM + DSS group, it is likely due to the highly toxic nature of AOM, which was shown in a study conducted by Bisahoyo et al, [16] in acute toxicity tests using azoximethan dose of 20 mg/kg, all mice die shortly after azoximethan injected with a dose of 20 mg/kg. While the results of the study by Pears et al, [19] reported that metabolites azoximethan, metilazoximethanol (MAM) can memetilasi nucleic acids, MAM also binds to histones and form a bond with the target cells.

Likewise, the possibility of repeated DSS administration may increase the toxicity of AOM, causing inflammation resembling ulcerative colitis in human.[17,20] manifestations of colitis in mice include diarrhea, accompanied by blood in the stool, weight loss, appetite and decreased body movements, piloerection, anemia and even death[17,22]. DSS can also lead to an increase in spleen weight mencit [23].

From the research, Trivedi, et al,[24] DSS can significantly increase levels of various inflammatory markers such as myeloperoxidase (MPO), the expression of NF $\kappa$ B, iNOS and COX-2 levels of IL-6, TNF- $\alpha$  and PGE-2 in plasma, malonalaldehyd (MDA), release of oxidative stress, mice peripheral blood micronucleus formation. DSS also can cause liver damage, which is characterized by a significant increase in plasma transaminases, decreased liver and brain glutathione levels, and a decrease in the ratio of GSH/GSSG[24].

Ethanol extract of mahkota dewa leaves doses of 25 mg and 50 mg can lower score due to inflammatory colon tissue of mice induced by AOM and DSS significantly. This is likely due to the active substances contained in the extract which consists of various components such as polyphenols, flavonoids, tannins, gallic acid, kaempferol and mangiferin, which may be mutually reinforcing in reducing the degree of colon tissue of mice caused inflammation induced by AOM + DSS[12].

AOM is intraperitoneal induction followed by administration of DSS ad libitum via the drinking water for 7 days in this study does not lead to the occurrence of adenomas and adenocarcinomas in mice, but cause inflammation, goblet cells, dysplasia, and atypical epithelial cells is accompanied by increased expression of iNOS,  $\beta$ -catenin and COX-2.

Histopathological results of this research in the form of tissue damage such as inflammation, goblet cell dysplasia, and atypical epithelial cells, not in accordance with colon cancer research in general, using AOM and DSS. No occurrence of adenoma and adenocarcinoma in this study is likely due to the use of DSS is too large molecular weight (500,000), so it can not penetrate the colonic mucosa. Based on research conducted by Perse et al,[18] reported that the molecular weight of DSS is a very important factor in inducing colitis, a high level of severity experienced Balb/c mice were given DSS with BM 40,000 (40 kDa), mice were given DSS BM 5000 (5 kDa) had moderate colitis, whereas mice given DSS BM 500,000 (500 kD) had mild lesions in the colon tissue of mice.

Results of immunohistochemistry using anti-iNOS antibody, an increase in the level of expression was highest in the AOM+DSS group. The high expression of iNOS in this group is likely due to the provision of a DSS, a polysaccharide sulfate can induce transcription iNOS.[17] DSS can cause damage to mucosal epithelial barrier function, which allows the entry of luminal antigens and microorganisms (commensal bacteria) into the colonic mucosa, which causes a severe inflammatory response.[17,18] Furthermore commensal bacteria, can induce chronic inflammation mediated by the immune system such as IL-1 $\beta$ , IL-6, which would induce transcription iNOS.[17,18]

From numerous studies have demonstrated that the induction in mice by using the AOM and DSS shown to increase the expression iNOS[17]. The high expression of iNOS was reported by Suzuki, et al, [17,21] that the results of immunohistochemistry nitrotyrosin greater in mice that received DSS 2% and 1% after the first induced by AOM. Nitrotyrosin is a marker of the reaction between peroxynitrite with tyrosine protein. Amount of nitric oxide (NO) will trigger the formation of peroxynitrite, which is a result of reaction of NO with superoxide (O<sub>2</sub>)[17,21]. NO number indicates the involvement of iNOS.

Ethanol extract of mahkota dewa leaves decrease iNOS expression was significantly vs. AOM + DSS but can not restore as normal conditions. This is probably due mahkota dewa plants contain active substances falerin, mangifer, kaempferol, gallic acid, ikarisida and other phenolic compounds [7-9], can reduce the expression of iNOS, although lower than aspirin.

This is reinforced by the results of research conducted by Crespo et al,[26] who compared the effects of kaempferol and quercetin in inducing proinflammatory cytokines in human vein endothelia cell umbilicae (HUMAC), that both these flavonoids can inhibit the expression of iNOS and COX-2, and from the second the active substance which lowers the expression of iNOS and COX-2 is more powerful is quercetin.

The results are reported by Oskoueian et al,[27] that the antioxidant activity of phenolic compounds and its ability to scavenge free radicals and NO scavenger, the radical forming compounds fenoksil. While the results of the study by Kazlowska et al,[28] barriers iNOS expression in RAW 264.7 cells caused by flavonoids and phenolic compounds contained in mahkota dewa, can suppress the action of nitric oxide. Results of other studies conducted by Choi et al,[25] reported that gallic acid contained in the plant, such as mahkota dewa inhibit the expression of IL-6, COX-2, IL-1 $\beta$  and iNOS in primary macrophages of mice, induced with Lipopolysaccharide (LPS).

Giving aspirin to the results of this study showed that aspirin can significantly reduce the expression of iNOS.

INOS decline by aspirin may be caused by the ability of aspirin to prevent I $\kappa$ B $\alpha$  phosphorylation and degradation of the complex signaling pathways IKK- I $\kappa$ B $\alpha$ -NF- $\kappa$ B, that NF- $\kappa$ B can not be separated and entered into nukleus[29]. Thus, aspirin may decrease the transcription of iNOS expression is upregulated by NF- $\kappa$ B.

$\beta$ -catenin protein expression is very high in mice indicated AOM+DSS group. Induction AOM intraperitoneally, may lead to  $\beta$ -catenin mutations at codons 33 and 41 on serine and threonine residues that are phosphorylation targets GSK-3 $\beta$ ,  $\beta$ -catenin consequently unable to form a complex and not degraded,  $\beta$ -catenin becomes stable which will accumulate in the cytoplasm so that the expression will meningkat[30,31]. Mahkota dewa group at all doses can decrease the expression of  $\beta$ -catenin, due to mice induced with AOM and DSS. The content of kaempferol in mahkota dewa is a flavonoid compound that can lead to upregulation of GSK-3 $\beta$ , resulting in increased GSK-3 $\beta$  binding to  $\beta$ -catenin, and  $\beta$ -catenin phosphorylation, allegedly flavonoid compounds may inactivate Wnt signaling pathway/ $\beta$ -catenin[32].

Small and large doses of aspirin can restore the increased expression of  $\beta$ -catenin caused by the induction of AOM-DSS kekonidisi normal. Mechanisms of aspirin in reducing the expression of  $\beta$ -catenin has been demonstrated from the results of the study Dihlmann et al.[33] that aspirin can stabilize the phosphorylation of  $\beta$ -catenin signaling pathway in order to reduce  $\beta$ -catenin/TCF, this phosphorylation response specific to colon cancer cells but not to non-cancerous cells of the colon.

Likewise, the results of research conducted by Greenspan et al.[34] that aspirin can increase the phosphorylation of  $\beta$ -catenin, which can reduce the accumulation of nuclear  $\beta$ -catenin and transcription of Wnt target gene/ $\beta$ -catenin such as cyclin D1, c-myc and PPAR $\delta$ , in addition to Aspirin can also degrade the bond NF $\kappa$ B-I $\kappa$ B $\alpha$ . The degradation caused translocation of NF $\kappa$ B - P65 into the nucleus, and inhibits NF $\kappa$ B targets genes that contribute to cell growth that is independent of COX-2 and induce apoptosis[34]. COX-2 expression occurs most great especially on AOM+DSS group.

Giving AOM + DSS can increase the expression of COX-2,[17] due to damage to mucosal epithelial barrier, commensal bacteria that enter, causing inflammatory responses such as IL-1 $\beta$ , IL-6[18]. Extract of mahkota dewa small doses can be lower levels of COX-2 expression induced by AOM-DSS, although not yet reached normal. Decreased expression of COX-2 may be caused by the content of gallic acid in the extract, according to research conducted by Choi, et al.[25] reported that gallic acid contained in plants such as the mahkota dewa can inhibit the expression of IL-6, COX-2, IL-1 $\beta$  and iNOS in primary murine macrophages induced by lipopolysaccharide (LPS). Similarly, research conducted by Tjandrawinata et al.[35] reported that the bioactive substances contained in mahkota dewa can decrease the expression of COX-2 and phospholipase A2 (cPLA2) regulation of gene expression by decreasing NF- $\kappa$ B.

However, at a large dose of ethanol extract of mahkota dewa is not able to suppress the expression of COX-2, it is probably due to the dose used is too large. From the research conducted, the high expression of COX-2 in the mahkota dewa group of mice large dose, small doses compared, likely due to ethanol extracts of mahkota dewa leaves in large doses contain polyphenolic compounds that are as pro-oxidant[36]. Polyphenolic compounds can act as a pro-oxidant with presence of copper ions (ion Cu<sup>2+</sup>) which will catalyze DNA breakage, induces apoptosis that is accompanied by the formation of ROS (reactive oxygen species)[37]. The formation of ROS activates IKK kinase to outline the bond between I $\kappa$ B with NF- $\kappa$ B in the cytoplasm, NF will be free -  $\kappa$ B translocation into the nucleus and experience, which will affect the genes that regulate transcription factor COX-2[38].

Aspirin dose of 0.21 mg or the equivalent of 81 mg aspirin dose in humans, can not suppress the expression of COX-2, it is likely due to a small dose of aspirin works by blocking the enzyme thromboxan A2, did not inhibit the enzyme COX-2.<sup>39</sup> contrast to the large doses of aspirin (0.84 mg or 325 mg equivalent to the human dose) decreased the expression of COX-2 were significantly vs AOM-DSS. This is probably due to aspirin dose 0.84 mg worked as anti-inflammatory by acetylating a serine residue of the enzyme COX-2, so that the COX-2 enzyme activity is inhibited, can not bind to arachidonic acid[39].

## 10. CONCLUSION

1. Ethanol extract of mahkota dewa leaves doses of 25 mg and 50 mg doses can inhibit the inflammatory process induced murine colonic by AOM + DSS.
6. Ethanol extracts of mahkota dewa leaves doses 25 mg and 50 mg can inhibit the process of carcinogenesis induced by

AOM+DSS through suppression of iNOS expression,  $\beta$ -catenin and COX-2, but the Ethanol extract of mahkota dewa leaves 50 mg not able to reduce the expression of COX-2 in the colon of mice after induction with AOM + DSS ( $p > 0,05$ ).

## 11. SUGGESTION

1. Necessary to study the dose range of Ethanol extract of mahkota dewa leaves, to be used in the treatment.
2. In the colon cancer study dinduksi with AOM - DSS DSS should be used with BM 40,000, in order to form adenomas and adenocarcinoma.
3. Need to do a thorough examination of the colon to determine the location of the tumor .

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