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ANTI-INFLAMMATORY EFFECT OF ETHANOL EXTRACT OF MAHKOTA DEWA [*PHALERIA MACROCARPA* (SCHEFF. BOERL)] LEAVES IN MOUSE COLITIS INDUCED BY AZOXYMETHANE (AOM) AND DEXTRAN SODIUM SULFATE (DSS): FOCUS ON RECTAL TISSUE

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ABSTRACT

Objective: In this study, we aim to analyze the effect of this ethanol extract on the average number of goblet cells per crypt, number of inflammatory foci and number of angiogenesis in the rectal tissues of mice colitis-model that induced by azoxymethane (AOM) and dextran sodium sulfate (DSS).

Methods: This study was carried out by experimental *in vivo* using Balb/c mice. The mice were divided into five groups of treatment: normal, negative control (AOM/DSS), positive control (AOM/DSS+aspirin), EMD25 (AOM/DSS+25% ethanol extract) and EMD12.5 (AOM/DSS+12.5% ethanol extract). The mice were euthanized and their rectal tissues were placed on glass slides for histopathological observation using haematoxylin–eosin staining.

Results: The administration of the ethanol extract of mahkota dewa (*Phaleria macrocarpa*) leaves cannot inhibit the decrease in the average number of goblet cells per crypt (p=0.450) and cannot reduce the number of inflammation foci (p=0.146) and the number of angiogenesis (p=0.728). The ethanol extract of mahkota dewa (*Phaleria macrocarpa*) leaves could not inhibit the inflammation induced by AOM/DSS in the rectal tissues of mice. However, the extract has a tendency to maintain the average number of goblet cells per crypt.

Conclusion: Ethanol extract of Mahkota Dewa leaves can inhibit inflammation in mice's rectal tissue.

Keywords: Ethanol extract, Goblet cells, Inflammation, Mahkota dewa *(Phaleria macrocarpa)*, Rectum

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INTRODUCTION

Inflammation in the rectum of mice is a risk factor for colorectal cancer. The most frequent inflammation in the gastrointestinal system is inflammatory bowel disease (IBD) [1, 2]. Two types of IBD is ulcerative colitis (UC) and Crohn's disease (CD) [3]. The world UC incidence in 2014 reached 1.2 to 20.3 cases per 100,000 population per year [4]. UC incidence in America and Europe reached 12 cases per 100,000 population per year [5, 6]. Meanwhile, in 2002, endoscopic units from several hospitals in Jakarta, Indonesia (Jakarta Hospital, Tebet Hospital, Siloam Gleneagles Hospital and Cipto Mangunkusumo Hospital) reported that about 12.2% of their patients had IBD accompanied by chronic diarrhea. Moreover, CD and UC were found in 5.2% of all patients examined by colonoscopy in Cipto Mangunkusomo Hospital [7].

The most common treatment currently used are corticosteroids and acetylsalicylic acids (aspirin), which are still effective as anti-inflammatory agents in the left (distal) colon. Corticosteroids are widely used because they are cheap and easy to obtain, such as methylprednisolone, prednisone and steroid enemas [7]. However, aspirin administration alone cannot treat severe UC or CD cases, and corticosteroids have side effects, such as bone depletion, skin thickening, development of moon face, suppression of the hypothalamus–pituitary–adrenal axis, cataract development and osteoporosis if taken for long periods [8, 9].

At the same time, research on herbs extracts as anti-inflammatory agents have been carried out. Herbs contain many secondary metabolites, such as tannins, terpenoids, flavonoids and alkaloids that have been tested *in vitro*. These compounds act as antioxidants and antimicrobial and anti-inflammatory agents. One of them is ethanol extract obtained from mahkota dewa (*Phaleria macrocarpa*) leaves [10].

MATERIALS AND METHODS

Materials

Inflammation-inducing compounds are dextran sodium sulfate (DSS) and azoxymethane (AOM) manufactured by Sigma Chemical Company. Aspirin is produced by PT. Brataco. The ethanol extract of mahkota dewa (*Phaleria macrocarpa*) leaves is produced by Biopharmaca Study Centre, Bogor Agricultural University (IPB).

Experimental animal

Balb/c mice aged 2–3 mo with a weight of 20–25 grams were used in this *in vivo* experimental study [11]. The mice were raised and bred in the Laboratory of Experimental Pathology, Department of Pathologic Anatomy, Faculty of Medicine of our university.

Mice are placed in cages with room temperature was set at 23±1 °C, humidity at 55±5% and dark/light cycle every 12 h [12]. All surgical procedures and activities were carried out according to the protocol of the Animal Care and Use Committee (Guide for the Care and Use of Laboratory Animals). This study has passed the ethical review conducted by the Health Research Ethics Committee of our university (number 0891/UN2. FI/ETIK/2018).

Sample size

The number of animals needed was calculated using the Federer formula [13], (t−1) (n−1) ≥ 15 animals. The coefficient "t" indicates the number of treatment groups (5 groups), and "n" is the number of test animals needed for each treatment group [13].

$(5-1)(n-1) \geq 15$

$(n-1) \geq 15/4$

$(n-1) \geq 3.75$

$n \geq 4.75$ (rounded to $n \geq 5$)

The total number of experimental animals needed is 25 mice and distributed randomly into 5 groups.

Inflammation induction

1. The normal group (N) was the untreated mice.

2. The negative group (K−) was mice given one injection of 0.1 ml of 0.1% w/v AOM intraperitoneally (IP), and then drinking water mixed with 1% w/v DSS was given daily *ad libitum* for 7 d.

3. The positive group (K+) was mice given one injection of 0.2 ml of 0.1% w/v AOM IP. Then, as much as 0.21 ml of a 0.4% w/v aspirin suspension (equivalent to 0.84 mg of aspirin) was administered orally, and drinking water mixed with 1% w/v DSS was given daily *ad libitum* for 7 d.

4. The high-dose group (EMD25) was mice given one injection of 0.2 ml of 0.1% w/v AOM IP. Then, as much as 0.2 ml of 25% w/v mahkota dewa (*Phaleria macrocarpa*) extract (equivalent to 50 mg of extract) was administered orally, and drinking water mixed with 1% w/v DSS was given daily *ad libitum* for 7 d.

5. The low-dose group (EMD12.5) was mice given one injection of 0.2 ml of 0.1% w/v AOM IP. Then, as much as 0.2 ml of 12.5% w/v mahkota dewa (*Phaleria macrocarpa*) extract (equivalent to 25 mg of extract) was administered orally, and drinking water mixed with 1% w/v DSS was given daily *ad libitum* for 7 d.

Histopathology preparation

The mice were euthanized and their rectal tissues taken. The tissues were cleaned with water and fixed with 10% formalin buffer. They were then processed in paraffin blocks and placed on glass slides for histopathological observation using hematoxylin-eosin staining.

Histopathology observation

Researchers observed the histopathological preparations from the rectal tissue and assessed the anti-inflammatory effect without knowing the treatment group. After that, researchers were informed of the treatment group's distribution and the code number of slides (double-blind). Observations were carried out under 400x magnification.

Statistical analysis

All ImageJ calculation data were compiled in a Microsoft Excel spreadsheet. Data were processed using SPSS version 24. The type of data used was numerical. Since the amount of data is 22 (<50), a Shapiro–Wilk normality test was performed, and normal data distribution was obtained. Then, inferential statistical tests were carried out with a comparison of>2 variables (one-way ANOVA) to determine the differences between groups [13].

RESULTS

Histological preparations of rectal tissue from three variables showed in fig. 1-3. On the basis of statistical tests, we found that there was no significant difference in the average number of goblet cells per crypt between groups. Fig. 4A shows that the value of the EMD25 group is close to and even higher than that of the normal group. In addition, the value of the EMD25 group is 1.6x higher than that of the K− group. The statistical tests also did not reveal any significant differences (p>0.05) in the number of inflammatory foci and the number of angiogenesis between groups.

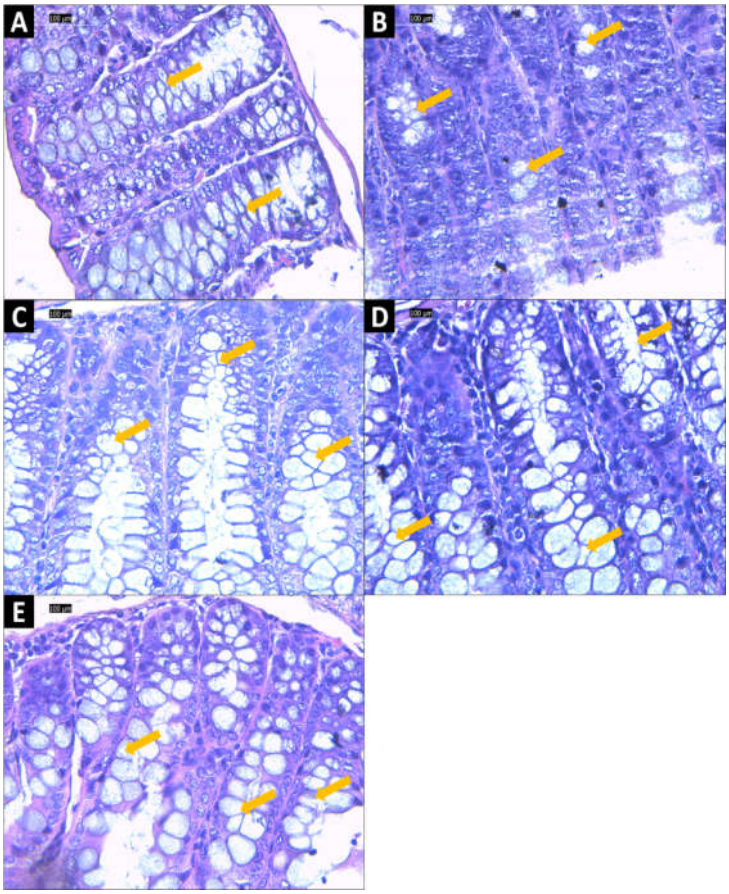

Fig. 1: Comparison of the average number of goblet cells per crypt (yellow arrow) in histopathological preparations of rectal tissues of mice. In the K− group, there seems to be a decrease in the number of goblet cells. (A) Normal, (B) K−, (C) K+, (D) EMD25 and (E) EMD12.5

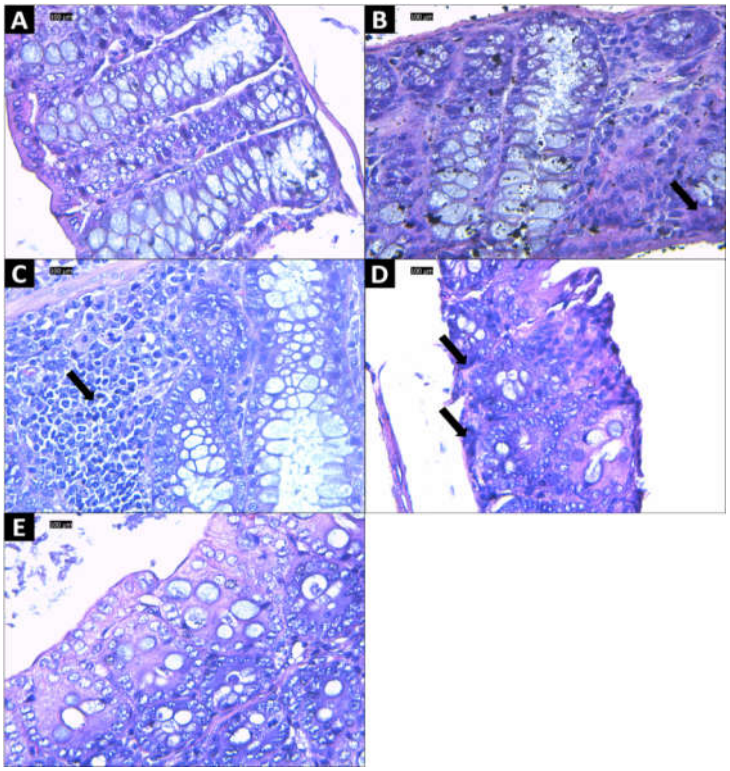

Fig. 2: Comparison of the number of inflammatory foci (black arrow) in histopathological preparations of rectal tissues of mice. Inflammatory foci were found in the K−, K+and EMD25 groups. (A) Normal, (B) K−, (C) K+, (D) EMD25 and (E) EMD12.5

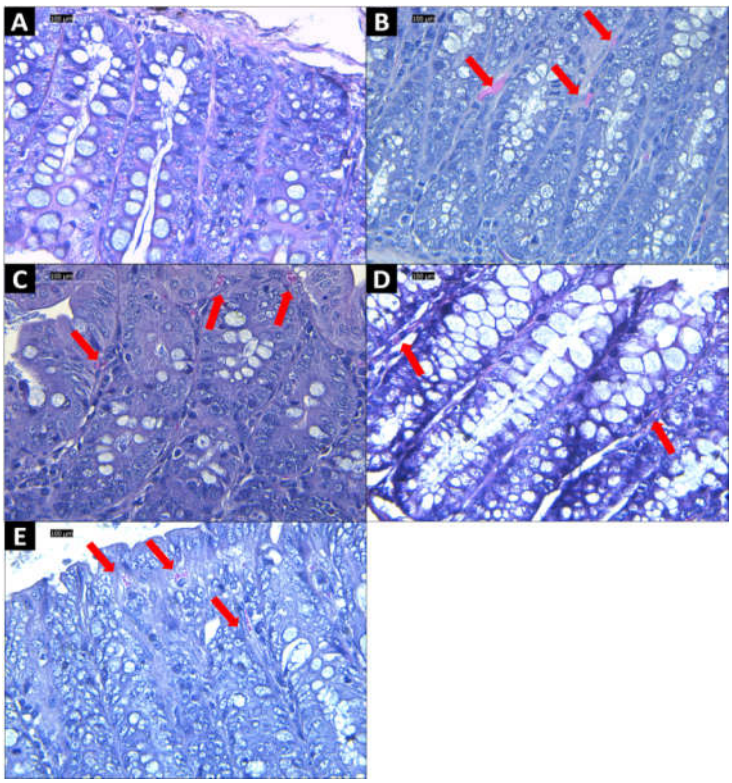

Fig. 4: Comparison of the number of angiogenesis (red arrows) in histopathological preparations of rectal tissues of mice. An increase in the number of angiogenesis was found in four intervention groups, except the normal group. (A) Normal, (B) K−, (C) K+, (D) EMD25 and (E) EMD12.5.

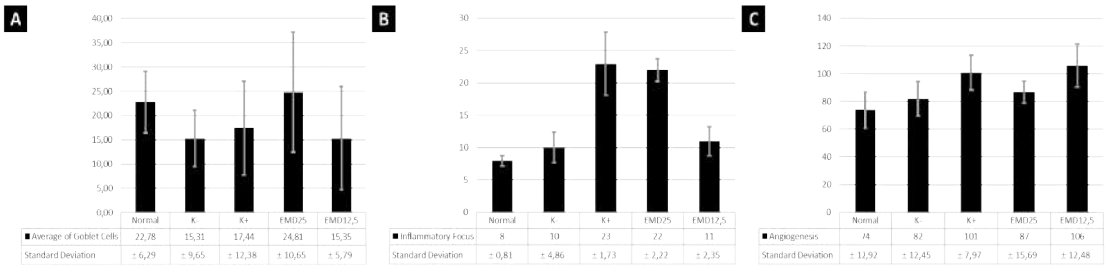

Fig. 4: (A) Average number of goblet cells per crypt, (B) number of inflammatory foci and (C) number of angiogenesis in rectal tissues of mice colitis-model induced by AOM/DSS, Normal: No intervention, K–: AOM/DSS, K+: AOM/DSS+Aspirin, EMD25: AOM/DSS+Mahkota Dewa Extract 25%, EMD12.5: AOM/DSS+Mahkota Dewa Extract 12.5%

DISCUSSION

Some previous studies have shown that extracts from mahkota dewa (*Phaleria macrocarpa*) leaves can inhibit DSS-induced inflammation in colon epithelial cells of mice [14], which is characterized by the suppression of mitosis and hyperplasia in cryptic epithelial cells [15].

Effects on the average number of goblet cells per crypt

Normally, the percentage of goblet cells among epithelial cells increases along the small intestine, from 4% in the duodenum to 16% in the distal colon. Under the UC condition, the number and size of goblet cells will decrease. The reduced number of goblet cells, which is a marker of the severity of inflammation [16], is observed by counting the number of goblet cells per crypt.

Even if the statistical test results are not significant, the graphical data tends to show that the high-dose (EMD25) administration of ethanol extracts of mahkota dewa (*Phaleria macrocarpa*) leaves can reduce the inflammatory effect and maintain the average number of goblet cells per crypt.

This result is in agreement with those of Estuningtyas *et al.* [17]. Results of their histopathological analyses of the UC mouse model showed that the ethanol extract of mahkota dewa (*Phaleria macrocarpa*) leaves (25 and 12.5 mg) can maintain the number of goblet cells per crypt in comparison with that of the normal group. The ethanol extract of mahkota dewa (*Phaleria macrocarpa*) leaves can suppress the inflammation that occurs in the tissue [17].

However, it was seen that the average number of goblet cells per crypt decreased in the K+ group. Aspirin administration is considered to trigger an inflammatory response in rectal tissue, as described by Chan *et al.* [18].

Effects on inflammatory foci

Fig. 4B reveals that the EMD25 and K+groups show an increase in the number of inflammatory foci. Instead of suppressing the inflammatory effect, the administration of aspirin and ethanol extract of mahkota dewa (*Phaleria macrocarpa*) leaves enhances the inflammatory effect. The numbers of inflammatory foci in the EMD12.5, K– and normal groups were also observed to be similar. This condition shows that there is no difference in the number of inflammatory foci in the inflamed tissue compared with that in the normal tissue. It is expected that the number of inflammatory foci is higher in the K– group than in the other groups.

This result is not in agreement with that of Kusmardi *et al.*, who showed that the skin of the mahkota dewa (*Phaleria macrocarpa*) fruits can reduce the number of inflammatory cells in the epithelial crypts of DSS-induced mouse colon (p=0008) [12]. It is hypothesized that the increased number of inflammatory foci in the K+group was due to aspirin administration. Aspirin has a detrimental effect; that is, it causes injury to the intestinal mucosa. Damage to the mucosal lining triggers the release of inflammatory mediators and a microvascular response, which then leads to the pathogenesis of UC and CD. This was confirmed by Chan *et al.*, who showed that aspirin administration is closely related to the incidence of CD [18]. Although the results of their study did not show a strong association between aspirin and UC, several events indicate that regular administration of aspirin is considered to cause UC [18, 19].

Effects on angiogenesis

The ability of tissues to induce angiogenesis characterizes the progression of inflammation into cancer. The condition occurs owing to genomic instability that causes random mutations in chromosomes. These mutations trigger premalignant lesions in the body's immune cells under inflammatory conditions. Angiogenesis can be positive because it is a sign of the body's efforts to repair tissue. However, it can also be negative if the growth is excessive [20].

So far, the authors have not found a journal article that discusses the effects of ethanol extracts on the number of angiogenesis in an inflammatory model. However, there are similar articles that discuss the effect of theaflavin, a proantocyanidin class of flavonoid polyphenols, on AOM-induced colon cancer. It is stated that theaflavin can inhibit angiogenesis through the suppression of VEGF and Ang1 [20].

Fig. 4C shows that the numbers of angiogenesis in the K+, EMD25, EMD12.5 and K– groups are higher than that in the normal group. The K+ and EMD12.5 groups showed the highest increase in the number of angiogenesis.

It is expected that aspirin can inhibit inflammation that occurs in the rectal tissues of mice. Four RCT studies showed that aspirin can reduce the risk of colorectal adenomas in both high-risk and low-risk populations. However, in this study, aspirin administration induces excess angiogenesis [21].

CONCLUSION

The ethanol extract of mahkota dewa (*Phaleria macrocarpa*) leaves in the high-dose group (EMD25) can maintain the average number of goblet cells per crypt close to that of the normal group. This research was not able to prove that the ethanol extract of mahkota dewa (*Phaleria macrocarpa*) leaves can reduce the number of inflammatory foci and the number of angiogenesis in rectal tissues of mice. Nevertheless, it is seen that the ethanol extract of mahkota dewa (*Phaleria macrocarpa*) leaves has a potential role as an inhibitor of rectal tissue inflammation induced by AOM/DSS.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors declare no competing interest.

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