

THE EFFECT OF MAHKOTA DEWA (*PHALERIA MACROCARPA*) (SCHEFF.) FRUIT PERICARP EXTRACT ON INOS IN MICE COLON INTERMITTENTLY-INDUCED BY DEXTRAN SODIUM SULFATE

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ABSTRACT

Objective: The objective of this research was to investigate the anti-inflammatory effect of Mahkota Dewa fruit pericarp extract (*Phaleria macrocarpa*) on inducible nitric oxide synthase (iNOS) in mice colon induced by dextran sodium sulfate (DSS).

Method: The simplisia of *P. macrocarpa* pericarp was weighed (1000 g) and extracted by maceration process. The total yield of the ethanolic extract was 26.43%. Phytochemical screening was carried out for the detection of the phytoconstituents by simple qualitative methods. The anti-inflammatory activity was performed by DSS-induced colitis model through assessment of hematoxylin-eosin staining and expression of iNOS by immunohistochemistry assay at four different doses, i.e., 650, 1250, 2500, and 5000 mg/kg. Swiss Webster male mice weighing 25-30 g were used for the study.

Results: Inflammation score in dose 625, 1250, 2500, and 5000 mg/kg were 1.63, 1.43, 1.32, and 2.20, respectively. This result is significantly different ($p=0.008$) with DSS group that was 4.37. The results of iNOS optical density score in dose 625, 1250, 2500, and 5000 mg/kg were 1.21, 1.119, 1.22, and 1.37, respectively. This result was significantly different ($p=0.000$) with DSS group that was 2.24.

Conclusion: Pericarp extract of *P. macrocarpa* fruit exhibited anti-inflammatory activity in the experimental model shown by suppressing the expression of inflammatory cell and iNOS.

Keywords: Pericarp, *Phaleria macrocarpa*, Inflammatory cells, Inducible nitric oxide synthase, Dextran sodium sulfate, Colon.

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INTRODUCTION

The risk of colorectal cancer in ulcerative colitis patients was increase with time of exposure to the disease, i.e., 1.6% after 10 years, 8.3% after 20 years, and 18.4% after 30 years [1]. The etiology of ulcerative colitis is not yet known, but there were several factors that play a role in the development of the disease, among others, genetic disorders that cause T-cell responses become aggressive and excessive to subset of commensal enteric bacteria, environmental factors that can damage the mucosal barrier rapidly and stimulate immune response, and enteric bacterial equilibrium disorders. Excessive immune responses to the colon will cause impairment of epithelial function and epithelial response to pathogens [2-4]. Besides, oxidative stress also associated with ulcerative colitis as revealed by Geetha *et al.* [5].

The current anti-inflammatory therapy aims to control the cardinal signs of inflammation, antagonizing or blocking key proinflammatory mediators that are released at the beginning of an acute inflammatory response. However, prolonged use of many anti-inflammatory agents has serious adverse reactions such as gastric intolerance and bone marrow depression [6]. Hence, it is important to search for substances that can promote resolution of inflammation, homeostatic, and modulators efficient and which are tolerated by the body [7].

The development of standardized herbal medicines with proven efficacy and safety can be considered as an important source for increasing the access of people toward medicine and offers new therapeutic options [8]. *Phaleria macrocarpa* known as Mahkota Dewa is a traditional medicinal plant in Indonesia. Literature indicates

in vitro anti-inflammatory activity of *P. macrocarpa* fruit pericarp extract [9]. *In vivo* study conducted by Tjandrawinata *et al.* showed anti-inflammatory activities of bioactive fraction obtained from a combination of *P. macrocarpa* and *Nigella sativa* [10].

Flavonoid content of *P. macrocarpa* has proven to decrease expression of inducible nitric oxide synthase (iNOS), an enzyme that plays a role in the synthesis of NO which then can influence the production of proinflammatory cytokines and cause the reactive oxygen state and then will cause mucosal damage [11].

Since *in vivo* anti-inflammatory activity of single *P. macrocarpa* fruit pericarp extract has never been published, meanwhile *in vitro* activity has been proven, we undertaken this researched to investigate *in vivo* anti-inflammatory activity of *P. macrocarpa* fruit pericarp extract.

METHODS

Materials

All the solvents and chemicals required were of analytical grade. Dextran sodium sulfate was obtained from Regent Science Industry Ltd (RSC), Hong Kong, iNOS antibody was obtained from Abcam Inc., Cambridge, MA, Aspirin was obtained from a local drug store, Jakarta.

Experimental animals

Healthy male Swiss Webster mice about (25-30 g) were procured from Veterinary Laboratory of Research and Development Centre, Ministry of Health, Jakarta. The animals were maintained under standard conditions of relative humidity and temperature and acclimatized under

laboratory conditions before carrying out the experiments. Ethical approval was obtained from The Health Research Ethics Committee, Faculty of Medicine Universities Indonesia, number 24/UN2. FI/ETIK/1/2017.

Phytochemical screening

Phytochemical screening was carried out for the detection of alkaloids, flavonoids, tannin, saponin, quinoa, and steroid by simple qualitative methods [12].

Colitis induction and tissue preparation

Dextran sodium sulfate 2% is given through drinking water for 7 days and then punctuated with 7 days without administration of DSS. This cycle is then repeated up to 3 cycles. The dosage of the test drug was designed based on previous publication research [13,14]. 42 experimental animals were divided into seven groups with six animals in each group. Mice were sacrificed at the end of week 7 by dislocation of the neck, subsequently, colon tissue put into 10% formalin buffer solution. Then paraffin blocks are made and afterward hematoxylin-eosin and immunohistochemical staining was then performed in accordance with standard laboratory procedures (Fig. 1).

Fig. 1: Research flow. ASP = Aspirin, EPMD 1 = *Phaleria macrocarpa* pericarp extract doses 625 mg/kg, EPMD II = *P. macrocarpa* pericarp extract doses 1250 mg/kg, EPMD III = *P. macrocarpa* pericarp extract doses 2500 mg/kg, and EPMD IV = *P. macrocarpa* pericarp extract doses 5000 mg/kg

Hematoxylin eosin staining

The procedures for hematoxylin-eosin staining were in accordance with previous studies [15], in brief, deparaffinized section using xylol then re-hydrated using alcohol and washed in running water. The preparation inserted into hematoxylin (Meyer solution), rinsed in running water and dipped into a saturated lithium carbonate, after that rinsed with running water for 5 minutes.

Histopathology assessment result

Analysis of histopathological changes made by reading the outward appearance in a blind way, and performed by two technicians, then the results were averaged. Readings were taken at 400 times magnification. Preparations were taken every five representative fields of view, then assessed the score of severity, extent and level of inflammation [16].

iNOS immunohistochemical staining

After deparaffinization and rehydration, specimens were soaked by 3% of hydrogen peroxide to eliminate endogenous peroxide and then with 0.01 M of citrate buffer (pH 6.0) in microwave to return the structure of the antigen and then spilled with 5 % normal serum to block unspecific protein. The next step was incubated the specimens with rabbit

polyclonal antibodies of inducible nitric oxide synthase (1:100 dilution) followed by appropriate secondary antibody, HRP-conjugated streptavidin and 3,3'-diaminobenzidine (DAB) subsequently. Next counterstained with Harris hematoxylin, dehydrated and mounting.

Reading was taken at 400 times magnification and every five representative fields of view. The interpretation of results was using image J profiler software that will assess the percentage of color intensity as high positive, positive, low positive, and negative, and then optical density score calculated [17,18] using the formula:

$$\frac{\left\{ \begin{array}{l} (\% \text{ high positive} \times 4) + (\% \text{ positive} \times 3) + (\% \text{ low positive} \times 2) \\ + (\% \text{ negative} \times 1) \end{array} \right\}}{100}$$

Statistical analysis

The values were expressed as mean standard deviation. $P < 0.05$ was considered significant, denoted by symbol (*). The data were analyzed by one-way analysis of variance followed by Tukey multiple comparisons using SPSS 20 Software. Inhomogeneous data analyzed non-parametrically by Kruskal-Wallis.

RESULTS

Phytochemical screening

The phytochemical screening of ethanolic extract of *P. macrocarpa* pericarp showed the presence of flavonoids and saponin.

Histopathological outcome

All doses of *P. macrocarpa* pericarp extractable to reduce infiltration inflammation significantly different from the DSS group ($p = 0.008$) (Figs. 2 and 3).

Fig. 2: Graph score of mice colon tissue inflammation. All values are shown as mean \pm standard of error and $n = 6$. $*p < 0.05$ compared to the DSS group. DSS = Dextran sodium sulfate, ASP = Aspirin, EPMD = *Phaleria macrocarpa* pericarp extract

Fig. 3: An overview of histopathological results of colon tissue of mice, (a) Healthy tissue from normal mice, (b) inflammatory tissue of DSS group, (c) inflammatory tissue of aspirin group, (d) inflammatory tissue of *Phaleria macrocarpa* pericarp extract group dose 652, 1250, 2500, and 5000 mg/kg, yellow arrow shows inflammation in epithelial cell crypt, HE 400 \times , iNOS expression after immunohistochemical readings

Normal group showed weak expression of iNOS in the cytoplasmic of epithelial cell crypt, in contrast to the strong expression in DSS group. While iNOS expression in *P. macrocarpa* extract doses 625 and

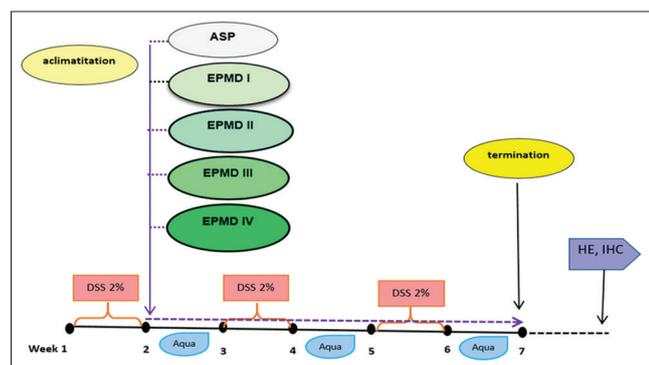


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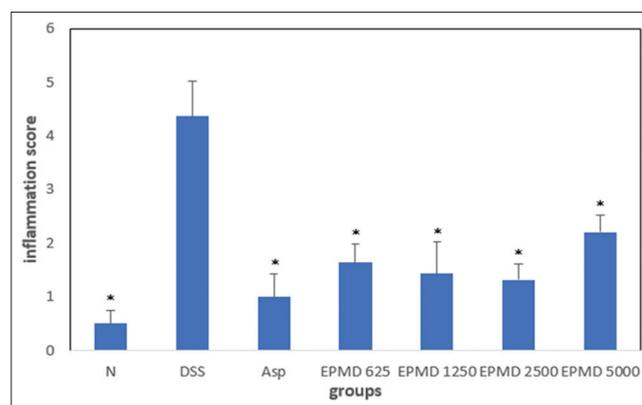


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1250 mg/kg weaker than doses 2500 and 5000 mg/kg (Fig. 4).

Fig. 4: INOS expression on the mice colon tissue. (a) Healthy tissue from normal mice, (b) iNOS expression of DSS group, (c) aspirin group, (d-g) iNOS expression of *Phaleria macrocarpa* pericarp extract group dose 652, 1250, 2500, and 5000 mg/kg. Yellow arrow is showing iNOS expression in the cytoplasm of epithelial cell crypt (IHC ×400)

P. macrocarpa pericarp extract can significantly decrease iNOS expression in cytoplasmic of epithelial crypt compared with DSS groups (p=0.000) as shown in Fig. 5.

Fig. 5: Graph score of iNOS expression in mice colon tissue. All values are shown as mean±standard of error and n=6. *p<0.05. DSS = Dextran sodium sulfate group, ASP = Aspirin group, EPMD = *Phaleria macrocarpa* pericarp extract group, *p=0.001 compared to the DSS group

DISCUSSION

All dose of *P. macrocarpa* pericarp extract can decreased inflammation; this result strengthens the evidence of anti-inflammatory activity from previous *in vitro* studies in which flavonoid content inhibit 63.4 % NO production and classified as potentially moderate anti-inflammation [10,19]. Flavonoid apparently working through several mechanisms includes antioxidative effect, direct free radicals scavenger, immobilization leukocyte, and interaction with enzyme system. Flavonoid of *P. macrocarpa* pericarp extract containing kaempferol and myricetin grouped as flavone which working as an antioxidant through reaction with free radicals producing more stable and less reactive compound [20].

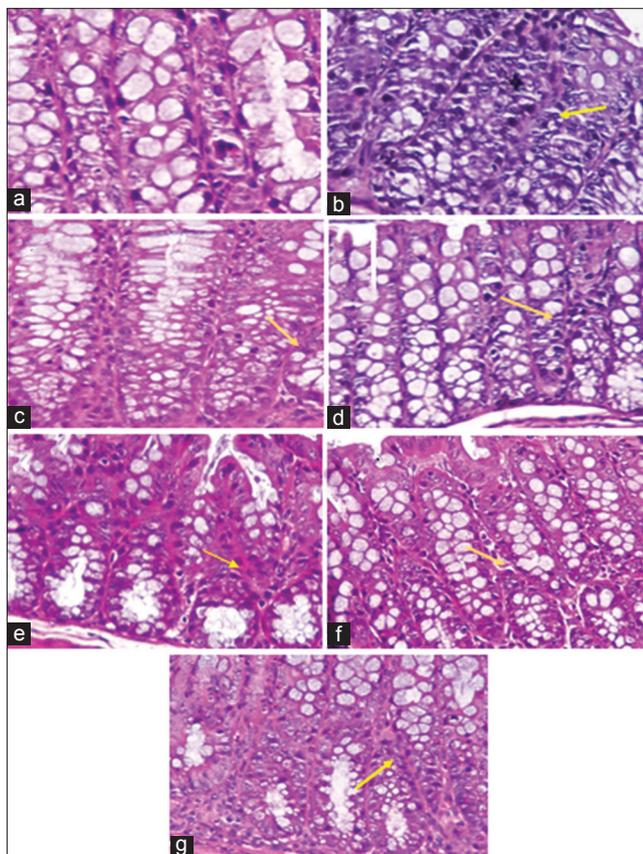


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Flavonoids of pericarp extract such kaempferol have anti-inflammatory effects *in vitro* and *in silico* through NF-κB activation barrier and have the binding energy and the same docking position with inhibitors NF-κB.

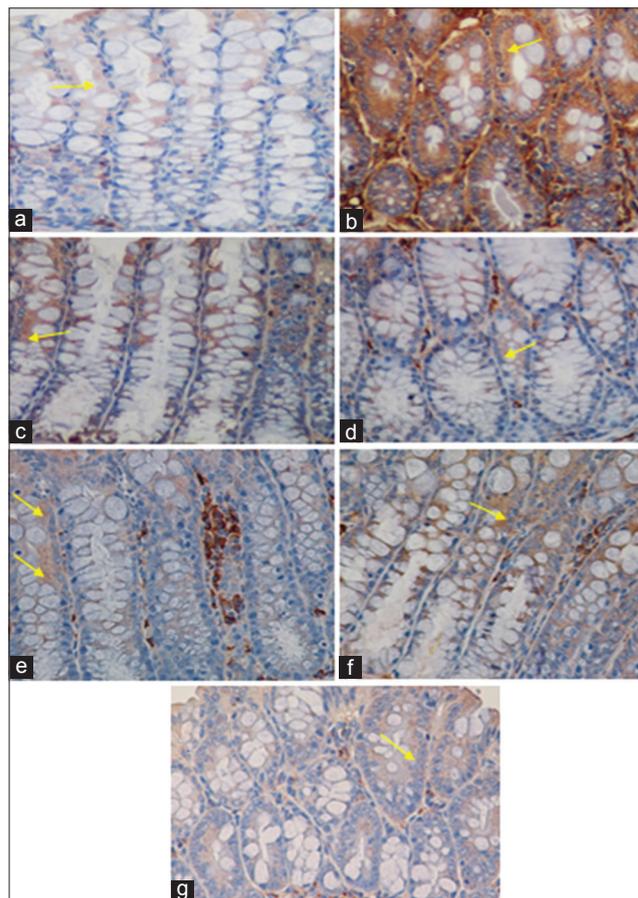


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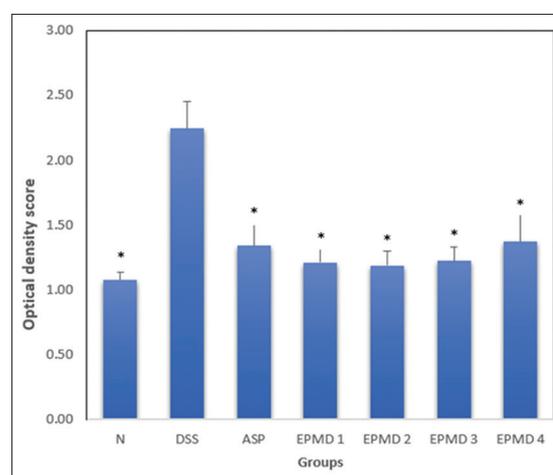


Fig. 5: Graph score of iNOS expression in mice colon tissue. All values are shown as mean±standard of error and n=6. *p<0.05. DSS = Dextran sodium sulfate group, ASP = Aspirin group, EPMD = *Phaleria macrocarpa* pericarp extract group, *p=0.001 compared to the DSS group

NF- κ B is activated by various stimuli including lipopolysaccharides from bacterial cell walls, proinflammatory cytokines, and viral DNA. This stimulus can induce intracellular signaling cascade that activates I κ B kinase complex (IKK) to phosphorylate specific serine residues in the molecule I κ B. Phosphorylation of I κ B bonds in NF- κ B will degrade NF- κ B I κ B so it can translocate into the nucleus [14]. Kaempferol can inhibit endothelial adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), which upregulate during inflammation and help recruitment leukocytes, migration and activation of T cell. Kaempferol inhibits the expression of mRNA and ICAM-1 proteins resulting in blockage of leukocyte uptake and migration and T cell activation [21].

Anti-inflammatory activity of *P. macrocarpa* pericarp extract also proven through decreasing of iNOS expression in mice colon, this results in line with Rostoka *et al.* [22] and Choi *et al.* [23] whom study flavonoid isolates, state that kaempferol, myricetin and rutin lowering iNOS protein expression in the livers of mice, and myricetin lowering nitric oxide production in rat liver. Besides kaempferol, myricetin and rutin, naringin also can inhibit the activation of iNOS enzyme and activation of NF- κ B through barriers to degrades I κ B and translocation of p65, a subunit of the protein NF- κ B which undergo translocation into the cell nucleus when NF- κ B regardless of I κ B [24].

CONCLUSION

The phytochemical screening of ethanolic extract of *P. macrocarpa* pericarp showed the presence of flavonoids and saponin. All doses of *P. macrocarpa* pericarp extract can suppress inflammatory cells in epithelial crypt induced by DSS as indicated by decreasing inflammatory score compare to DSS group ($p=0.008$). All doses of *P. macrocarpa* pericarp extract can decrease expression of iNOS on mice colon epithelial crypt induced by DSS compare to DSS group ($p=0.000$).

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