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Original Article

PHALERIA MACROCARPA LEAF EXTRACT-CHITOSAN NANOPARTICLES SUPRESS ANGIOGENESIS INDUCED BY DEXTRAN SODIUM SULFATE IN MICE COLON

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ABSTRACT

Objective: Although an anti-inflammatory effect of *Phaleria macrocarpa* (Mahkota Dewa in Indonesian) leaf extract has been reported, the extract shows toxicity as the dose increases. Chitosan nanoparticles are known to have transport properties that can enhance the targeting of active compounds to tissues at a lower dose. The present study sought to determine whether the extract in chitosan nanoparticles can suppress angiogenesis in the colon tissue of mice.

Methods: We determined the antiangiogenic effect in 6 groups Swiss Webster mice: normal (N) group, negative control (NC) administered drinking water containing DSS 2% w/v (7 d) and followed by water without DSS (7 d) in 3 cycles, 12.5 and 25 mg leaf extract of *P. macrocarpa*/mouse (EPM 12.5 and EPM 25) groups, and 6.25 and 12.5 mg leaf extract of *P. macrocarpa* in chitosan nanoparticles/mouse (NPPM 6.25 and NPPM 12.5) groups. Hematoxylin and eosin-stained samples was performed to determine the amount of angiogenesis in the colon tissue.

Results: The angiogenesis in the NPPM 12.5 ($p = 0.105$) and EPM 25 ($p = 0.07$) groups was not significantly different to that in the negative control group (administered DSS alone). By contrast, angiogenesis in the EPM 12.5 ($p = 0.03$) and NPPM 6.25 ($p = 0.02$) groups was significantly less than that in the DSS group.

Conclusion: Angiogenesis in the colon tissue of mice was reduced by the extract with or without chitosan nanoparticles. The greatest reduction was found for the 12.5 mg/mouse dose of *P. macrocarpa* leaf extract in chitosan nanoparticles.

Keywords: Angiogenesis, Anti-inflammation, Chitosan nanoparticles, *Phaleria macrocarpa* leaf extract

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammation of the digestive tract, which is induced by immunological reactions including Crohn's disease (CD) and ulcerative colitis (UC). Globally, there are 10 cases of IBD per 100,000 individuals, with a high incidence in Europe and North America. About 2.2–14.3 cases of UC per 100,000 individuals and 3.1–14.6 cases of CD per 100,000 individuals occur each year in North America [1]. In Indonesia, the incidence of UC is 0.55 cases per 100,000 individuals, while for CD the incidence is 0.33 cases per 100,000 individuals for period 2011–2012. The incidence is increasing because of the unclear etiology and pathophysiology of the disease [2].

Persistent proinflammatory cytokine exposure in IBD leads to chronic inflammation. This condition is supported by one of the components that plays a role in angiogenesis as part of the inflammatory process. Angiogenesis is the natural process of new vascular formation to supply oxygen, nutrition, and several growth factors, thus maintaining the inflammation. Over time, chronic inflammation will initiate an oncogenic response such as mutation and epigenetic changes in the digestive tract, especially in the colon, and triggers progenitor cancer cell formation (cancer-associated colitis) [3, 4].

Anti-angiogenesis-based IBD treatment using several types of drugs has been developed. Anti-angiogenesis will inhibit the inflammation process by suppressing cytokine secretion and proangiogenic factor. Bevacizumab is one of most commonly used anti-angiogenic agents used with a high success rate. However, bevacizumab is known to cause serious side effects, such as intestinal perforation, impaired wound healing, and bleeding. Moreover, its high cost is a consideration for researchers attempting to discover alternative treatments that are not only safe but also affordable [4, 5].

The use of naturally occurring ingredients as anti-angiogenesis drugs has been studied extensively. Suprapti *et al.* [6] tested the effectiveness of Mahkota Dewa (*Phaleria macrocarpa L.*) leaf extract as an anti-inflammatory agent. Active compounds from the extract include kaempferol, quercetin, and phalerin, which have anti-inflammatory and antiangiogenic effects, suggesting the extract as a potential candidate for IBD therapy. However, high doses of the extract were found to be toxic in animals [6]. Therefore, we sought to determine whether loading the extract in chitosan nanoparticles could facilitate better diffusion of the active compound to specific tissues to achieve antiangiogenic therapeutic effects with a lower extract dose than by using the extract alone [7].

MATERIALS AND METHODS

This experimental study *in vivo* used a randomized and parallel design and was conducted in the Pharmacokinetics Laboratory, Pharmacology and Therapeutic Department, Faculty of Medicine, Universitas Indonesia (FMUI) in 2018 with approval from the Health Research Ethics Committee of FMUI (approval no. 0747/UN2. F1/ETIK/2018).

Animal sample preparation

Male Swiss Webster mice aged 12 w with an average body weight of about 25 g were obtained from the Animal Laboratory of the Center for Health Research and Development, Ministry of Health, Jakarta, and acclimatized for 7 d before treatment. The mice were housed in temperatures below 30 °C under a 12-h light/dark cycle, fed with standard feed, and allowed water ad libitum. Based on a power calculation, 36 mice were used and were divided into 6 groups that received different treatments: no treatment (N), negative control (NC) administered drinking water containing DSS 2% w/v (7 d) and followed by water without DSS (7 d) in 3 cycles, EPM 25 (DSS 2% w/v as described before+25 mg/mouse *P. macrocarpa* leaf extract for 5 w orally, starting on week 2 (day 8), EPM 12.5 (DSS 2% w/v as described before+12.5 mg extract/mouse for 5 w orally, starting on week 2 (day 8), NPPM 12.5 (DSS 2% w/v as described before+12.5

mg extract in chitosan nanoparticles/mouse for 5 w orally, starting on week 2 (day 8), NPPM 6.25: DSS 2% w/v as described before+6.25 mg extract in chitosan nanoparticles/mouse for 5 w orally, starting on week 2 (day 8).

Tissue preparation

The mice were killed in week 7 by cervical dislocation. Then, colon tissues were harvested and cleaned with water. Colon tissues were fixed for 24–48 h with a 10% formalin buffer, dehydrated, cleared through a graduated series of increasing concentrations of xylol solutions, and infiltrated with paraffin using an automatic tissue processor. Subsequently, the colon tissues were embedded into paraffin medium in a box cassette and sliced at a thickness of 3–5 µm using a microtome. The slices were placed in a water bath (40–50 °C), fixed to glass slides, and dried for 1 h at 40 °C.

Hematoxylin and eosin staining

The colon tissue sections fixed on the slides were saturated with xylol I and xylol II for 5 min each and then submerged in a graduated series of ethanol solutions (90%, 75%, and 70%) for 5 min each. Then, the sections were stained by soaking them in a hematoxylin solution for 5–10 min, followed by rinsing them under water. Subsequently, the sections were dipped into a lithium carbonate solution, followed by another water rinse. Then, the sections were soaked in an eosin solution for 1-3 min, followed by 3 times dehydrating them through a graduated series of alcohol concentrations (70%, 75%, and 90%). After that, the sections were dipped into xylol I and xylol II solutions for about 5 min each. Finally, each section was covered with 1 drop of Ingelan and a cover glass.

Interpretation of hematoxylin and eosin staining

The histology of colon tissue samples was performed using a Leica light microscope and Sigma camera at 400× magnification. Examination was conducted in 10 fields of view for each sample. The amount of angiogenesis appearance was calculated manually by the Image-J program. After that, received data was interpreted to obtain a valid data conclusion.

Data analysis

The distributions of data were analyzed using a Shapiro-Wilk test, followed by a one-way ANOVA with a Tukey post hoc test to compare more than 2 groups. Nonhomogeneously distributed data were analyzed using a nonparametric Kruskal–Wallis test followed by a Mann-Whitney U test.

RESULTS

All of the doses of leaf extracts were found to reduce inflammation in mouse colon tissue induced by DSS (fig. 1). The amount of angiogenesis was significantly less in the EPM 12.5 ($p = 0.03$) and NPPM 6.25 ($p = 0.02$) groups compared with that in the NC group. By contrast, the EPM 25 ($p = 0.07$) and NPPM 12.5 ($p = 0.105$) groups showed no significant difference in angiogenesis compared with that in the NC group. No significant differences were found between the treatment groups. The histopathological features of angiogenesis appearance was presented in fig. 2.

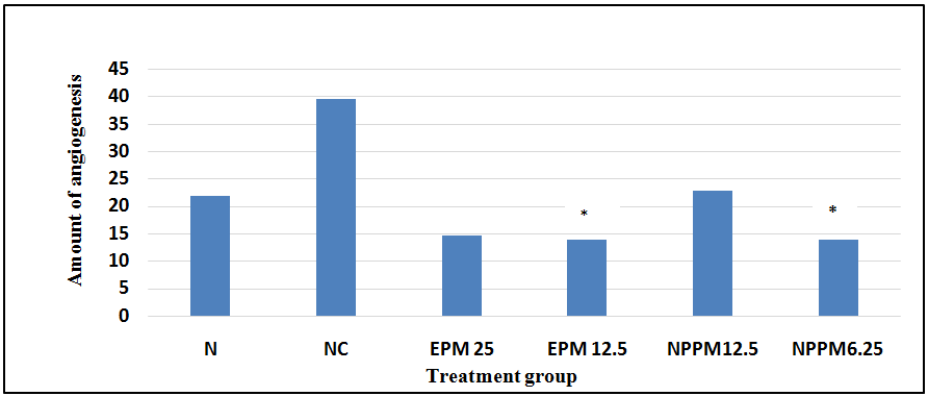

Fig. 1: Mean amount of angiogenesis on mice colon, Note: N = no treatment group; NC = negative control group; EPM 25 = *Phaleria macrocarpa* leaf extract dose 25 mg/mouse; EPM 12.5 = 12.5 mg extract/mouse; NPPM 12.5 = 12.5 mg extract in chitosan nanoparticles/mouse; NPPM 6.25 = 6.25 mg extract in chitosan nanoparticles; * = significant difference to NC group

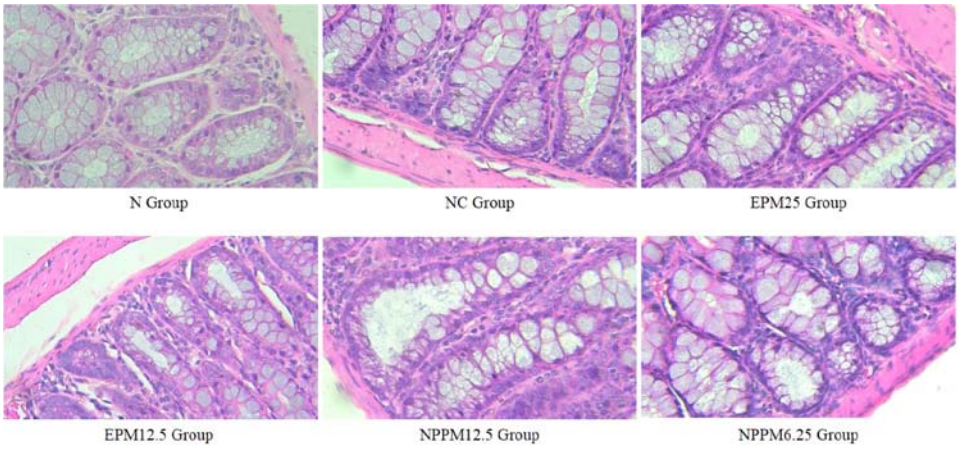

Fig. 2: Mice colon tissue after hematoxylin and eosin staining (400× magnification), Note: N = no treatment group; NC = negative control group; EPM 25 = *Phaleria macrocarpa* leaf extract dose 25 mg/mouse; EPM 12.5 = 12.5 mg extract/mouse; NPPM 12.5 = 12.5 mg extract in chitosan nanoparticles/mouse; NPPM 6.25 = 6.25 mg extract in chitosan nanoparticles; * = significant difference to NC group

DISCUSSION

P. macrocarpa leaf extract contains several flavonoid subtypes, such as quercetin, phalerin, and kaempferol which have anti-inflammatory, especially anti-angiogenesis properties. We used DSS, a polyamine dextran, to induce impairment of gastrointestinal permeability and colonic mucosal barrier function. This condition triggers cell dysfunction and immune system reactions. We used the extracts to reduce cell damage and immune responses. The extract is toxic at high dosage. Because of the toxicity, we used chitosan nanoparticles to carry the leaf extract to increase targeted drug delivery so that the efficacy of extract action could be enhanced.

Histopathology features showed the ability of the extract to suppress the amount of angiogenesis induced by DSS in mice colon *tissues*, possibly due to the anti-inflammatory effects of flavonoids, such as kaempferol, which can reduce inflammatory reaction by inhibiting of NF-κB activity [6]. The description of the inhibition of angiogenesis by *P. macrocarpa* leaf extract can be caused by the effect of *P. macrocarpa* on anti-inflammatory. The effect of *P. macrocarpa* on inflammation is shown by previous studies. This study showed an inhibition of the expression of iNOS protein in the cryptic colon epithelial cells induced by DSS [8,9]. The incidence of inhibitory effects is also in line with the increase in the extract dose of *P. macrocarpa*. This inhibition can prevent the aggregation of proinflammatory cells and cytokines. The amount of angiogenesis was found significantly reduced in all of the treatment groups, especially the EPM 12.5 and NPPM 6.25 groups.

CONCLUSION

Administration of *P. macrocarpa* leaf extract directly or carried by chitosan nanoparticles has the same ability to reduce the amount of angiogenesis in the inflammation reaction induced by DSS in mouse colon. EPM 12.5 and NPPM 6.25 groups showed significant reduction compared with that in the NC group.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

There are no conflicts of interest to declare

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