

ACUTE TOXICITY OF CHITOSAN NANOPARTICLES CONTAINING MAHKOTA DEWA (*PHALERIA MACROCARPA*) LEAF EXTRACT AND ANTI-INFLAMMATORY EFFECTS IN A DEXTRAN SODIUM SULFATE-INDUCED MOUSE MODEL OF ULCERATIVE COLITIS

ARI ESTUNINGTYAS^{1*}, SANTI WIDIASARI², KUSMARDI KUSMARDI³

¹Department of Pharmacology, Faculty of Medicine, Universitas Indonesia, Jakarta 10430, Indonesia. ²Student of Biomedical Science Magister Program Universitas Indonesia, Jakarta 10430, Indonesia. ³Department of Anatomic Pathology, Universitas Indonesia, Jakarta 10430, Indonesia. Email: ari_handoyo@yahoo.com

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ABSTRACT

Objective: The plant mahkota dewa (*Phaleria macrocarpa*) is known to have anti-inflammatory effects. This study aimed to determine whether chitosan nanoparticles containing mahkota dewa leaf extract would yield superior anti-inflammatory effects in the colon of a mouse model of dextran sodium sulfate (DSS)-induced ulcerative colitis, compared with ethanol extract alone after testing the acute toxicities (lethal dose) of both preparations.

Methods: For acute toxicity testing, 10 Sprague-Dawley rats were administered 6000 mg/kg body weight (BW) of leaf extract alone or with nanoparticles. Subsequently, mice were divided into the following six groups to determine the anti-inflammatory effects: Untreated, negative control (DSS 2% w/v), leaf extract at 12.5 or 25 mg/kg BW, and leaf extract in chitosan nanoparticles at 6.25 or 12.5 mg/kg BW. To induce colitis, DSS (2% w/v) was administered through drinking water for 6 weeks. The anti-inflammatory effect was observed histopathologically by imaging the inflammatory cells of the mice colon with hematoxylin-eosin (HE) staining.

Results: For acute toxicity testing, 10 Sprague-Dawley rats were administered 6000 mg/kg BW of leaf extract alone or with nanoparticles. Subsequently, mice were divided into the following six groups to determine the anti-inflammatory effects: Untreated, negative control (DSS 2% w/v), leaf extract at 12.5 or 25 mg/kg BW, and leaf extract in chitosan nanoparticles at 6.25 or 12.5 mg/kg BW. To induce colitis, DSS (1% w/v) was administered through drinking water for 6 weeks. The anti-inflammatory effect was observed histopathologically by imaging the inflammatory cells of the mice colon with HE staining.

Conclusion: Chitosan nanoparticles containing mahkota dewa leaf extract can be included in the practically non-toxic class of materials. However, an ethanol extract of mahkota dewa leaf effectively inhibited DSS-induced inflammation in the mouse colon, regardless of delivery vehicle.

Keywords: Mahkota dewa leaf extract, Chitosan nanoparticles, Acute toxicity, Inflammation, Histopathology.

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INTRODUCTION

Ulcerative colitis (UC) is a subtype of inflammatory bowel disease (IBD) characterized by chronic inflammation of the large intestinal mucosal layer. Patients with chronic UC face a high risk of colorectal cancer development within 10–30 years [1-4]. To date, UC has been treated through surgical procedures and corticosteroid and 5-aminosalicylic acid therapy. Although drug therapy may reduce inflammation, the effect may be short-lived and the patient can experience a relapse. The currently available drugs can also cause serious side effects, such as gastric ulcers and gastrointestinal bleeding.

Several studies have investigated the use of natural and apparently safe ingredients for the prevention and treatment of UC. One such ingredient, the plant mahkota dewa (*Phaleria macrocarpa*) [5,6], contains flavonoid compounds such as kaempferol and quercetin, and an extract of the leaves has been shown to exert anti-inflammatory effects by suppressing the activity of nuclear factor kappa beta (NF- κ B), cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) [7-9]. In a study by Suprapti *et al.*, a 25 mg dose of mahkota dewa leaf extract was shown to reduce the levels of iNOS, β -catenin, and COX-2, although a high-dose extract was fatal to some animals; accordingly, the survival rate was <100%. This mortality was attributed to the lethal effects of extract components on other organs [10].

The oral administration of mahkota dewa leaf extract is expected to suppress the activity of specific inflammatory proteins in the colon.

However, this method of administration is also expected to be affected by gastrointestinal absorption, particularly in the stomach and small intestine, before the compound reaches its target in the colon [7-9]. Therefore, a more colon-specific delivery has been investigated to ensure that the extract reaches its target and produces the desired effect.

One rational option for extract delivery involves packing the extract in the form of chitosan nanoparticles. Allegedly, this format will allow delivery of the compound to the colon followed by release from the nanoparticles. The nano-scale size is thought to facilitate the diffusion of the extract into cells in the colon, where it will target inflammatory proteins. In addition, a more targeted delivery method could reduce the dose required to yield anti-inflammatory effects [11-13]. In this study, we will investigate the effects of a mahkota dewa leaf extract delivered through chitosan nanoparticles on colonic inflammation in a mouse model of dextran sodium sulfate (DSS)-induced UC. Particularly, we will evaluate the histopathology of the gastrointestinal organs, especially the colon, using hematoxylin-eosin (HE) staining to visualize morphologic changes. This type of staining is very useful for identifying the morphologic and cellular components of organs and facilitating the diagnosis of histopathological abnormalities [14,15].

We note that herbal preparations are required to undergo safety testing. Therefore, this study also evaluated the acute toxicities (lethal dose [LD₅₀]) of the mahkota dewa leaf extract delivered alone or within chitosan nanoparticles before the mouse model experiment. Acute oral toxicity

testing aims to determine the intrinsic toxicity of a substance, target organ, and species sensitivity. It is also used to obtain hazard information after acute exposure, preliminary information that can be used to establish the dose rate and subsequent toxicity tests and identify the LD50 values of materials and preparations. Finally, such testing determines the classifications of materials and preparations, as well as the labeling information.

METHODS

This experimental study featured a completely randomized design. The research was conducted in 2017 at the Animal Laboratory of Center for Health Research and Development, Ministry of Health, Jakarta with the approval of the Health Research Ethics Committee of the Faculty of Medicine Universitas Indonesia (approval no. 17/2/UN2.F1/ETIK/2017).

Oral acute toxicity test

Experimental animals

Female Sprague-Dawley rats aged 8–12 weeks with body weights (BW) of ≥ 120 g were used for the acute oral acute toxicity test. The animals were housed in a facility in which the temperature, humidity, light, and noise levels were maintained to ensure a healthy environment. The rats were provided with food and water *ad libitum* according to laboratory standards.

Reagents

Two forms of an ethanol extract of *P. macrocarpa* leaf were tested: Alone and delivered in chitosan nanoparticles. The ethanol extract was obtained from the Laboratory of IPB Biopharmaceutical Study Center (Bogor), and the nanoparticles were produced at PT Nanotech Indonesia (Puspipetek-Tangerang).

Testing follows the principle that the administration of a single dose of an orally tested agent may exhibit toxic effects. Therefore, the test preparations were administered at a single dose of 6000 mg/kg BW because the traditional medicinal extract of *P. macrocarpa*, which is considered relatively safe (non-toxic), has a single dose limit of 5000 mg/kg BW.

For the experiment, 10 rats were divided into two groups of five rats (one for preliminary testing and four additional animals). Group I directly received the ethanol extract of *P. macrocarpa* leaf, while Group II received the same extract delivered in chitosan nanoparticles. All rats were acclimated for at least 5 days before treatment. Before receiving the test preparation, the rats were fasted for 14–16 h (water was provided), weighed and administered the test preparation dissolved in water using sonde.

Observational analysis

Observations of the skin, fur, eyes, mucous membranes, respiratory system, autonomic nervous system, central nervous system, somatomotor activity, and behavior were made immediately and within 30 min and 4 h after test preparation administration followed by every 24 h for 14 days. The BWs of the rats were recorded every 2 days. Dead mice were also subjected to observational analyzes, and all surviving mice were sacrificed and subjected to autopsy and macroscopic observations at the end of the study. Histopathology examinations were conducted if suspicious findings were observed.

For a tested substance, a smaller LD50 level indicates greater toxicity [16–19]. For medicines and other traditional ingredients (e.g., foodstuffs), acute toxicity categories in mice are stratified as follows: very toxic, oral LD50 ≤ 1 mg/kg BW; toxic, oral LD50 = 1–50 mg; medium toxicity, oral LD50 = 50–500 mg; light toxicity, LD50 = 500–5000 mg; practically non-toxic (PNT), oral LD50 = 5–15 g, and relatively no harm, and oral LD50 ≥ 15 g [19].

Histopathological examination

Experimental animals: Treatment

Swiss Webster mice aged approximately 12 weeks with an average BW of 25 g were acclimated for 1 week before treatment. The mice were obtained from the Animal Laboratory of the Center for Health

Research and Development, Ministry of Health, Jakarta, and maintained in accordance with the Guidelines for the Treatment and Use of Animal Laboratories of the Animal Committee. This research was approved by the Medical Research Ethics Committee of the Faculty of Medicine, University of Indonesia. The mice were housed in an environment with a controlled temperature of 25°C, 55% humidity, and a 12-light/dark cycle. All mice were provided standard feed and water *ad libitum*.

Thirty-six mice were divided into six groups to receive the following treatments (Fig. 1):

1. N=No treatment.
2. KN=Negative control: DSS 2% w/v (administered in drinking water for 1 week beginning on week 1, followed by no DSS for 1 week; this was repeated for up to 3 DSS cycles).
3. Ext MD 25 mg=Treatment 1: DSS 2% w/v as described above+*P. macrocarpa* leaf extracts 25 mg/kg BW given orally for 5 weeks, beginning on week.
4. Ext MD 12.5 mg=Treatment 2: DSS 2% w/v+*P. macrocarpa* extracts 12.5 mg/kg BW administered as described for Group III.
5. NPMD 12.5 mg=Treatment 3: DSS 2% w/v+*P. macrocarpa* leaf extracts 12.5 mg/kg BW in chitosan nanoparticles administered orally for 5 weeks, beginning on week 3.
6. NPMD 6.25 mg=Treatment 4: DSS 2% w/v+*P. macrocarpa* leaf extracts 6.25 mg/kg BW in chitosan nanoparticles administered as described for Group V.

Sample preparation

The mice were sacrificed in the 7th week through cervical dislocation. Colon tissues were harvested, cleaned, and rinsed with water. Tissue pieces were fixed in a 10% formalin buffer for 24–48 h, dehydrated, cleared in a stratified xylol solution and infiltrated with paraffin using an automatic tissue processor. Subsequently, the tissue was embedded into paraffin medium in a labeled block cassette. Prepared paraffin blocks were sliced using a microtome to yield slices with thicknesses of 3–5 μ m. The slices were placed in a water bath (40 °C–50 °C), affixed to glass slides and dried at 40 °C for 1 h.

HE staining

The processed tissue sections were immersed in xylol I and xylol II for 5 min each, and subsequently regraded in an ethanol gradient series (100%, 90%, and 75%) for 5 min per step. Subsequently, the preparations were stained by dipping into a hematoxylin solution for 5–10 min followed by a wash under running water for 5–10 min. The sections were then immersed in a solution of lithium carbonate followed by another wash under running water for 5–10 min. Finally, the samples were dipped into an eosin solution for 1–3 min. The next process of dehydration by dipping into the alcohol the concentration increases,

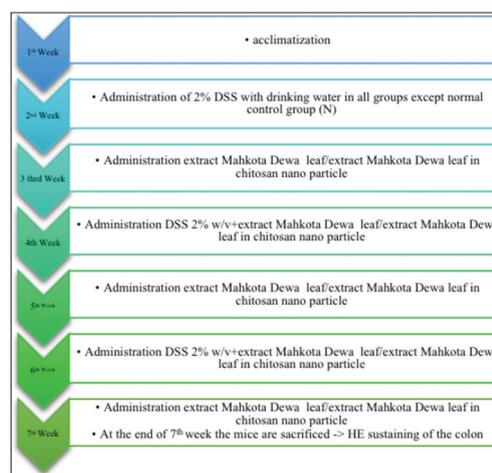


Fig. 1: Research flow chart

that is, 70%, 90%, and 100% each $\times 3-4$ dye. After re-dehydration, the sections were immersed into xylol I and xylol II solutions for 5 min each. Finally, the preparations were treated with 1 drop of Ingelan and covered with a glass coverslip.

Interpretation of HE staining

Mouse colon samples were subjected to a histopathologic examination using a Leica light microscope and Sigma microscope camera at $\times 400$ magnification. Five fields of view were obtained for each preparation, after which the assessment was done in accordance with a 2015 report by Rogers *et al.* in which each preparation was scored according to severity, extent, and inflammation as shown in Table 1.

Data analysis

The data distributions were analyzed using the Shapiro-Wilk test, followed by a one-way analysis of variance and Tukey's test. Non-homogeneously distributed data were analyzed non-parametrically using the Kruskal-Wallis test.

RESULTS AND DISCUSSION

Oral acute toxicity test

No adverse effects such as seizures, tremors, salivation, diarrhea, lethargy, weakness, sleep disorders, or coma were observed in the rats within 4 h of treatment with *P. macrocarpa* leaf extract delivered directly or through chitosan nanoparticles. Nor are there any animals did not hide or crawl on their bellies. No deaths or symptoms of toxicity were observed in either group after 14 days. The weights of rats in both groups during the 14-day observation period are presented in Figs. 2 and 3. After 14 days, all animals were sacrificed. Necropsy revealed no gross pathology in any test animals; therefore, no microscopic examination was performed.

Histopathological results

Both modes of *P. macrocarpa* leaf extract were found to reduce inflammation induced by DSS. Notably, a significant difference in inflammation was detected between normal group (1.33), Dewa leaf extract 25 mg/kg BB (Ext MD 25 mg) (2.1), leaf extract *P. macrocarpa* 12.5 mg/kg BB (Ext MD 12.5 mg) (1.633), leaf extract of *P. macrocarpa* in nano chitosan particle 12.5 mg/kg BB (NPMD 12.5 mg) (1.633), and *P. macrocarpa* leaf extract in nano chitosan particle 6.25 mg/kg BB (NPMD 6.25 mg) (1.697) compared with the negative control group (3.33) ($p < 0.05$) (Fig. 4). No significant differences were observed between the two forms of extract.

DISCUSSION

Our oral acute toxicity testing results indicated that the mixture of chitosan nanoparticles and *P. macrocarpa* leaf extract could be classified as a PNT material, as the test preparation did not cause adverse effects

Table 1: Inflammatory scoring according to Rogers *et al.*

| Score | Measurement criteria |
|-------|--|
| 0 | Healthy colon |
| 1 | Minimal inflammation with minimal to no separation of crypts (generally focal affecting <10% of mucosa) |
| 2 | Mild inflammation with mild separation of crypts (generally affecting 11–25% of mucosa or mild, diffuse inflammatory infiltrates with minimal separation of crypts) |
| 3 | Moderate inflammation with separation of crypts, with or without focal effacement of crypts (generally affecting 26–50% of mucosa or moderate, diffuse separation of crypts) |
| 4 | Extensive inflammation with marked separation and effacement of crypts (generally affecting 51–75% of mucosa) |
| 5 | Diffuse inflammation with marked separation and effacement of crypts (generally affecting >75% of mucosa) |

or death at a dose > 5000 mg/kg BW. Therefore, a human could safely consume a dose of either preparation up to 5000 mg/kg BW [19].

Previous research has determined that the *P. macrocarpa* leaf extract contains flavonoids [7], which are thought to exert several mechanisms such as antioxidative effects, direct free radical capture, leukocyte immobilization, and interactions with enzyme systems [20]. In this study, we induced UC using DSS, a polyamine dextran known to impair gastrointestinal permeability and interfere with the colonic mucosal barrier function, thus causing cellular damage and further triggering an immune response. DSS is a common means of inducing acute and chronic UC in animals [21]. In this study, 3 cycles of 2% DSS administration for 7 days, followed by 7 days with no administration, were applied according to reports by Zhang [21]. In this study, we employed targeted drug delivery through using nanoparticles, which were expected to increase drug concentration, and therefore efficacy, in the desired tissue through passive targeting while minimizing drug delivery to non-target tissues. Nanoparticles, which comprise colloidal polymer particles or solids, have diameters of 10–1000 nm and can be used in targeted delivery systems to improve bioavailability and control the drug release. Natural materials such as chitosan and Na TPP are commonly used as conductors; the positive charge of the chitosan amine group interacts with the negative charge of TPP to form complexes with sizes in the nanoparticle range [22]. Medical applications usually involve nanoparticle sizes < 200 nm (microcapillary width), and drugs are applied to or dissolved, captured, embedded, and/or encapsulated into the nano matrix. The properties and characteristics of the release

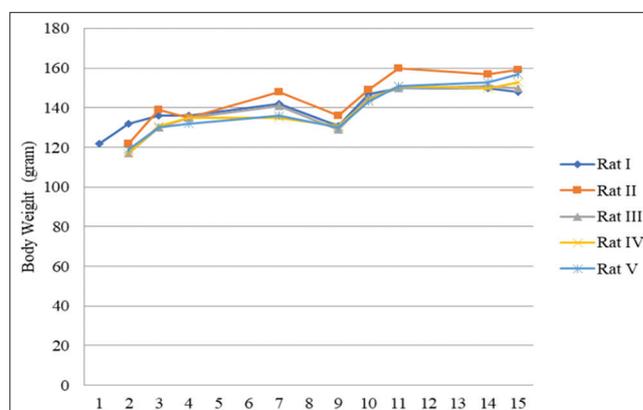


Fig. 2: Changes in body weight of rats who received the *Phaleria macrocarpa* leaf extract on an acute oral toxicity test (in 14 days)

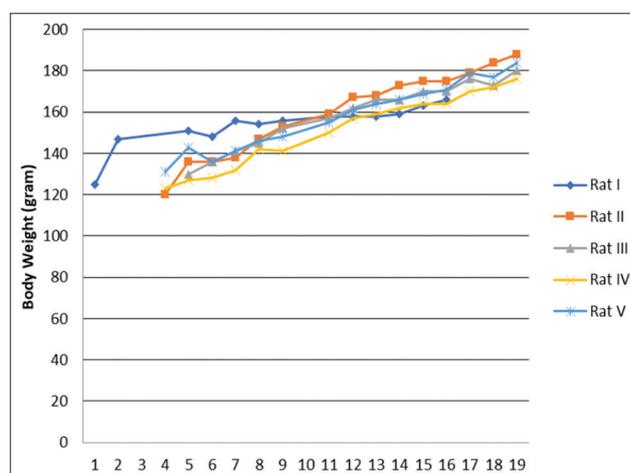


Fig. 3: Changes in body weight of rats who received the *Phaleria macrocarpa* leaf extract in chitosan nanoparticle on an acute oral toxicity test (in 14 days)

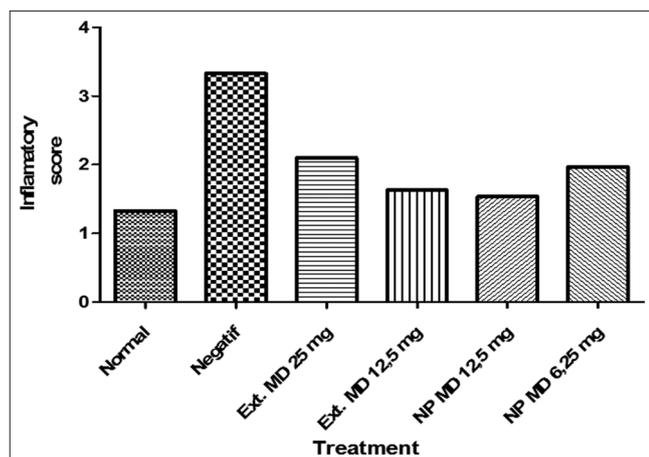


Fig. 4: Average inflammatory score

of the active substance can be adjusted depending on the manufacturing method [13].

Our study results indicate no significant differences between the ethanol extract of *P. macrocarpa* leaf administered alone and delivered in chitosan nanoparticles in terms of the ability to inhibit inflammation in the mouse model colon. We attribute this to the size of the nanoparticles, which exceeded 200 nm. Our histopathologic observations of HE-stained colon tissues were assessed using the scoring criteria developed by Rogers *et al.* [23]. The administration of 2% DSS with molecular weight 60,000 (60 kDa) yielded significant inflammatory characteristics. These results are consistent with previous findings by Perse and Cerar, who reported that the molecular weight of DSS is a very important factor in colitis induction [24]. Notably, both forms of the *P. macrocarpa* leaf extract significantly decreased the inflammatory scores, possibly due to the effects of flavonoids such as kaempferol, which has anti-inflammatory effects *in vitro* and *in silico* by inhibiting of NF- κ B activity [25]. Specifically, kaempferol has the same binding energy and docking position as the NF- κ B inhibitor. The transcription factor NF- κ B is activated by various stimuli, including lipopolysaccharides from bacterial cell walls. In addition, kaempferol may inhibit the endothelial adhesion molecule intercellular adhesion molecule-1 (ICAM-1), which is upregulated during inflammation and facilitates the recruitment, migration, and activation of T lymphocytes, by inhibiting the expression of both ICAM-1 mRNA and proteins. Accordingly, the migration and activation of T cells would be inhibited [25].

Histopathologic observations revealed that the untreated group of mice also exhibited inflammation, with an average score of 1.33. This was attributed to environmental factors, such as food and drinking water, which are suspected to cause inflammation in the colon [26,27]. A previous study showed that foods with a high-fat content may increase the risk of IBD by modulating Toll-like receptors on macrophages [26]. Furthermore, high iron levels in drinking water can trigger IBD by stimulating the growth of inflammation-causing bacteria in the colon [27].

Black and red arrows indicate goblet cells and inflammation, respectively (Fig. 5).

CONCLUSION

P. macrocarpa leaf extract, administered directly or in chitosan nanoparticles, can be classified as a PNT material according to the results of the safety analysis. Furthermore, both means of leaf extract delivery equally and significantly reduced the inflammatory scores in a DSS-treated mouse model of UC, compared to the negative control. We, therefore, conclude that the ethanol extract and chitosan nanoparticle forms of *P. macrocarpa* leaf administration are equally effective inhibitors of DSS-induced inflammation in the mouse colon.

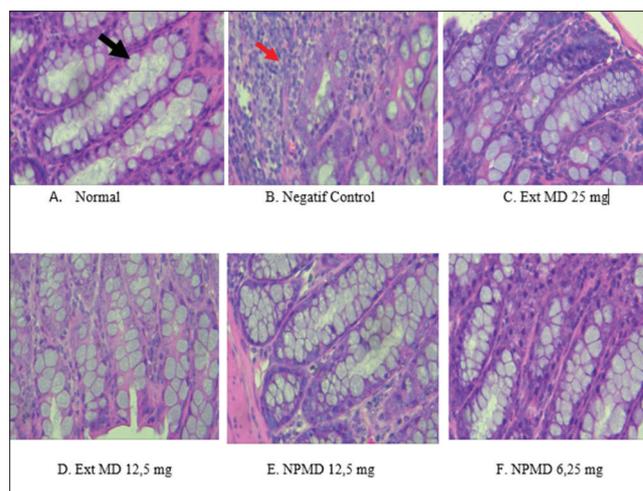


Fig. 5: Histopathologic analysis of colon sections from mice (HE staining, $\times 400$ magnification). (a) No treatment, (b) Negative control (DSS), (c) *Phaleria macrocarpa* leaf extract, 25 mg/kg BW, (d) *P. macrocarpa* leaf extract, 12.5 mg/kg BW, (e) *P. macrocarpa* leaf extract in chitosan nanoparticle, 12.5 mg/kg BW, (f) *P. macrocarpa* leaf extract in chitosan nanoparticle, 6.25 mg/kg BW

CONFLICTS OF INTEREST

All authors have none to declare.

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