INTRODUCTION

Inflammatory disease occurs in the mucosal of the colon, or ulcerative colitis (UC) is one of the subtypes of inflammatory bowel disease [1]. The serious of UC is colorectal cancer, the appearance of which seems to be related with chronic inflammation of the colon mucosa [2]. The risk of colorectal cancer (CAG) for any patient with UC is known to be elevated and is estimated to be 2% after 10 years, 8% after 20 years, and 18% after 30 years of disease [3]. The incidence of UC in these 4 countries, i.e., Hong Kong, Japan, Korea, and India has demonstrated an increase from 0.02 cases per 100,000 people per year in 1961 to 6 cases per 100,000 people per year in 2008 [4]. In Europe, the incidence of UC has significantly increased from 0.6 to 24.3 cases per 100,000 people from 1990 to 2008 [5]. The incidence of UC in Indonesia is still unknown.

The data on UC are still based on reports of several hospitals that are doing the colonoscopy. According to data, the incidence of UC at Cipto Mangunkusumo hospital Jakarta (RSCM) showed 5.4% from 2001 to 2006, Hasan Sadikin hospital Bandung and Sardjito hospital Yogyakarta during 2007 also showed 8.33% and 5.4% from 2001 to 2006, Hasan Sadikin hospital Bandung according to the patient's visit doing the colonoscopy. According to data, the incidence of UC is influenced by a complex interaction of genetic, gut microbiota, environmental, and immunologic factors. The hypotheses have arisen that primary deregulation of the mucosal immune system leads to excessive immunologic responses to normal microbiota. In addition, that change in the composition of gut microbiota and deranged epithelial barrier function elicit pathologic responses from the normal mucosal immune system. The microbiota and dysfunction of regulatory T cells are implicated in the development of chronic intestinal inflammation [7-9].

One of the central transcription factors mediating inflammatory responses is nuclear factor kappa B (NF-κB). NF-κB dimers are kept in an inactive cytoplasmic complex by inhibitory proteins, the inhibitor protein kappa B (IκB) family, in resting cells [10]. NF-κB protein is activated by a wide range of stimuli, such as the proinflammatory cytokines, tumor necrosis factor (TNF), interleukin-1 (IL-1) and the bacterial endotoxin, lipopolysaccharide (LPS), or leads to phosphorylation cause phosphorylation of IκB. This occurrence leads to the rapid dissociation of the complex accompanied by proteolytic degradation of IκB and release of NF-κB that subsequently transmigrates from the cytoplasm into the nucleus. NF-κB regulates the transcription of a series of genes involved in acute responses to injury and in chronic intestinal inflammation including the genes for IL-1, TNF-α, IL-6, IL-8, IL-12, cyclooxygenase (COX-2) and iNOS [11].

ABSTRACT

Objective: Inflammatory disease occurs in the mucosal of the colon, or ulcerative colitis (UC) is one of the subtypes of inflammatory bowel disease. The numerous of drug side effects for treatment of colitis give rise to use medicinal herbs as alternative therapies. Pomegranate peel extract has been used for the treatment of pain and inflammatory conditions. This study aimed to investigate the anti-inflammatory effects of pomegranate peel ethanol extract on mice colon through inflammation pathway which reduce inflammation score in mice model of chronic inflammation induced by dextran sodium sulfate (DSS).

Methods: Thirty Swiss Webster mice divided randomly into 6 groups: Normal, aspirin 43 mg/kg/d (ASP), ellagic acid 26 mg/kg/d (ELG), DSS 2% b/v (DSS), pomegranate peel ethanol extract 240 mg/kg/d (DOSES-1), and 480 mg/kg/d (DOSES-2). All groups were given DSS 2% over 3 cycles except normal group (where each cycle in the DSS group consisted of 2% DSS in drinking water for 7 days, followed by a 7-day interval with normal drinking water). At the end of the experiment, colon samples were washed with water then buffered neutral formalin 10% fixed and paraffin embedded for histological analysis.

Results: DOSES-1 and DOSES-2 were significantly reduced inflammation score in colon mice induced by DSS (p<0.05), mean score 2.01 and 2.02. Expression of cyclooxygenase (COX-2) was significantly decreased (p<0.05), mean score 27.48 and 17.77. Expression of iNOS was also significantly decreased (p<0.05), mean score 54.01 and 36.69. There were no significant differences between DOSES-1 and DOSES-2 groups with ASP and ELG group (p>0.05).

Conclusion: Pomegranate peel ethanol extract has an anti-inflammatory agent by reduces inflammation score, inhibiting COX-2 and iNOS expression on mice colon by DSS induced. Furthermore, pomegranate peel ethanol extract has equivalent effectivenes with aspirin and pure ellagic acid.

Keywords: Inflammatory, Pomegranate peel, DSS, Cyclooxygenase-2, iNOS.

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As demonstrated earlier, UC is a complex and chronic condition, which not only affects the quality of the life of patients but also increases the risk of developing CAC. The current treatment is still conventional depends on the extent of the disease. Corticosteroids and 5-ASA drugs such as mesalazine, sulfasalazine, olsalazine, and balsalazide as anti-inflammatory agents are first-line therapies [12,13]. The administration of 5-ASA drugs has uncomfortable side effects such as nausea, vomiting, diarrhea, and myalgia [13]. Furthermore, 5-ASA as a first-line in some patients may be poorly responsive to this therapy, so the use of this drug is still controversial. Patients with colitis-associated colorectal cancer are also given aspirin to reduce inflammation. However, aspirin also causes side effects such as ulceration and gastrointestinal bleeding [14].

The numerous of drug side effects for treatment of colitis give rise to use medicinal herbs as alternative therapies. Currently, some medicinal herbs are widely studied as UC therapies such as apples, blackberries, green tea, curcuma, and pomegranate [15]. Some of the medicinal herb mentioned, pomegranate is one of the plants that have been studied many effects such as anti-inflammatory, anticancer, antibacterial, antioxidants, and neuroprotective [16-19]. Pomegranate peel possesses higher content of polyphenols. This part contains ellagitannins and ellagic acid. Pomegranate also contains other polyphenols, such as anthocyanins (3-glucosides and 3, 5-glucosides of delphinidin, cyanidin, and pelargonidin) and flavones [16].

Ellagitannins are hydrolyzed to ellagic acid in the gut, thus resulting in a prolonged release of this acid into the blood. In humans and different animal models, it has been found that ellagic acid is metabolized by the colon microflora to form urolithins A and B. In vitro study showed that ellagic acid 13% w/w is contained in the pomegranate peel has many activities such as antibacterial, anti-inflammatory, and anti-allergic [17]. In vivo study reported that ellagic acid also improves UC in mice after induced by dextran sodium sulfate (DSS). Ellagic acid inhibits intestinal inflammation, lowers COX-2 and iNOS expressions, and blocks inflammatory signal pathway NF-κB [20]. Pomegranate peel extract inhibits COX-2 expression on the colon and visceral adipose tissue at 6 mg daily in mice for 4 weeks [21].

Considering this perspective, this study provides an in vivo study to assess the effects of pomegranate peel ethanol extract, especially anti-inflammatory effect like UC. This study used white pomegranate peel ethanol extract from Indonesia as anti-inflammatory agent through reduced inflammation in mice colon. Pomegranate peel ethanol extract has chosen because it has higher ellagic acid content than water extraction [22]. This study also used aspirin and pure ellagic acid powder as positive control which have anti-inflammatory effect. If this study of pomegranate peel ethanol extract proven to have any effect in mice colon induced by DSS, then it can be used for further research in human.

METHODS

Ethics statement and experimental animals

Male 12-week-old Swiss-Webster mice with an average weight of 20 g bred in the Research and Development Institute of Health (LITBANGKES) were used. The mice were acclimatized for a week before the start of the experiments. The animals were housed under standard laboratory conditions at temperature 22±2°C with relative humidity at 65±10%. Standard pellet rodent diet and water were provided to the animals ad libitum. The experimental protocols were approved by the Institutional Animal Ethics Committee of Universitas Indonesia.

Drugs and chemicals

Pomegranate peel ethanol extract was purchased from PT Borobudur (Semarang, Indonesia). Ellagic Acid powder was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Aspirin was purchased from PT Bayer (Indonesia) DSS (DSS; molecular weight 60 kDa) was purchased from Sigma Aldrich (St. Louis, MO, USA). Primary antibody COX-2 (abcam, diluted 1:1000), primary antibody iNOS (abcam, diluted 1:100), horse serum (Gibco), universal link (Biocare), Streptavidin (Biocare), methanol (Merck), and 3,3-diamino benzidine (DAB) solution (Biocare).

Experimental design

The pure ellagic acid (ELG) dose used in this experiment was similar to previously described by Rosillo et al. [20], and conversion of the dose was according to US Food and Drug Administration [23]. The dose of aspirin (ASP) was based on previously published reports Al-Swayeh et al. [24]. The dose of pomegranate peel ethanol extract was calculated after first identification of ellagic acid levels in a preliminary study using high-performance liquid chromatography. The ellagic acid levels were obtained and then synchronized with a pure ellagic acid dose by Rosillo et al. [20]. A preliminary study of identification ellagic acid in pomegranate peel ethanol extract was based on previously published reports by Panichayupakaranant et al. [25].

Thirty animals were randomly divided into 6 groups containing 5 mice in each. All of drugs (ellagic acid, aspirin and pomegranate peel ethanol extract) were given early in the experiment except normal group. All experiment has done until 42 days. In Group 1 (normal), mice were administered only tap or normal water orally. In Group 2 (DSS), mice were administered with DSS over 3 cycles until 42 days (where each cycle consisted of 2% DSS in drinking water for 7 days, followed by a 7-day interval with normal drinking water) and started in the 2nd week. In Group 3 (ASP), mice were administered with aspirin 43 mg/kg/d orally, and DSS was administered in 5 cycles started in the 2nd week. In Group 4 (ELG), mice were administered with ellagic acid 26 mg/kg/d orally, and DSS was administered in 3 cycles started in the 2nd week. In Group 5 (DOSES-1), mice were administered pomegranate peel ethanol extract 60 mg/kg/d orally, and DSS was administered in 3 cycles started in the 2nd week. In Group 6 (DOSES-2), mice were administered pomegranate peel ethanol extract 480 mg/kg/d orally, and DSS was administered in 3 cycles started in the 2nd week. Animals were weighed regularly during the experiment. 3 days after the last of induction by DSS, animals were sacrificed with decapitation. The mice colons were taken, and the feces were removed from the colonic lumen and rinsed with water. The proximal-to-distal colon tissue fragments were fixed using 10% buffered neutral formaldehyde (BNF) for histopathology analysis purposes.

Histopathology and immunohistochemical analysis

Large intestine specimens were fixed in freshly prepared 10% BNF, embedded in paraffin, and sectioned at 3 mm of proximal to distal of colon. Colon sections were stained with hematoxylin and eosin for histopathological analysis; they were also stained immunohistochemically to determine the expression of COX-2 and iNOS. Briefly, paraffin-embedded slides were deparaffined and rehydrated, which was followed by antigen retrieval in Tris-EDTA buffer (pH 8.0) for 10 minutes in the decloaking chamber at 96°C. Before antigen retrieval, slide sections were blocked endogenous peroxidase activity with 0.3% hydrogen peroxide (H₂O₂) in methanol for 30 minutes. Sections were blocked with blocking background sniper for 15 minutes and incubated for 60 minutes with primary antibody COX-2 (diluted to 1:100) and iNOS (1:100) in a humidified chamber. After incubated, immunoreactivity was determined with the aid of HRP-conjugated for 15 minutes at 37°C. The chromogen reaction was developed with DAB solution, with a positive signal being defined as a brown color under a light microscope. Negative control sections were processed similarly with horse serum replaces the primary antibodies. All images were taken at ×400 magnification. The iNOS or COX-2 negatively expressed cells were manifested as blue-stained nuclei, and the positive cell was with brown-yellow cytoplasm or nuclear membrane.

Scoring assessment

The severity and extent of inflammation were scored by a pathologist according to the following criteria [26]:

- Score 0: Healthy colon
- Score 1: Minimal inflammation with minimal to no separation of crypts

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• Score 2: Mild inflammation with mild separation of crypts
• Score 3: Moderate inflammation with separation of crypts, with or without focal effacement of crypts
• Score 4: Extensive inflammation with marked separation and effacement of crypts
• Score 5: Diffuse inflammation with marked separation and effacement of crypts.

For immunohistochemical assessment performed by H-score assessment, 10 fields were chosen at random at ×400 magnification, and the staining intensity in the cell cytoplasm was scored as 0, 1, 2, or 3 corresponding to the presence of negative, weak, intermediate, and strong brown staining, respectively. The total number of cells in each field and the number of cells stained at each intensity was counted. The average percentage positive was calculated and the following formula was applied [27]:

\[ \text{H-score} = (\% \text{ of cells stained at intensity category } 1 \times 1) + (\% \text{ of cells stained at intensity category } 2 \times 2) + (\% \text{ of cells stained at intensity category } 3 \times 3). \]

H-score between 0 and 300 was obtained where 300 was equal to 100% of cells stained strongly (3+).

**Statistical analysis**

Data were shown as mean ± SEM and were analyzed using Kruskal-Wallis test followed by Mann-Whitney test. Values of p<0.05 were considered as statistically significant. All the statistical analyses were performed using IBM SPSS software.

**RESULT**

**Body weight changes**

The body weight of mice measurements in each group was performed daily and gave different results. Calculation of mean body weight of the animals is done every week in each group so that it can be seen the body weight before and after given DSS (Fig. 1).

DSS 2% caused body weight loss in some groups. DSS group has body weight loss in 2nd and 6th week. Body weight loss is also occurred in the ASP group, at 2nd week. Body weight loss in the DOSES-1 group occurred at 2nd and 4th week. In contrast, in the ELG and DOSES-2 groups, there was no visible body weight loss of the animals (Table 1).

**Histological studies of the colon after pure ellagic acid powder, aspirin, and pomegranate peel ethanol extract treatment in experimental DSS model**

Some animals in each group give a different inflammatory feature. Inflammatory cells were measured using an average inflammatory score of colonic organs. Microscopic on colon cells and inflammatory scores are presented in Figs. 2 and 3. As shown in Fig. 2, DSS 2% through drinking water in each group caused inflammation. The DSS group showed inflammatory cells cover several crypts and goblet cells are not visible, whereas in the aspirin (ASP), pure ellagic acid (ELG), pomegranate peel ethanol extract (DOSES-1 and DOSES-2) groups, and some inflammatory cells are present only in crypts basal and goblet cells still visible.

**COX-2 and iNOS protein expressions are inhibited in colon tissue of DSS induced by pure ellagic acid, aspirin, and pomegranate peel ethanol extract treatment**

Fig. 4 shown that the aspirin (ASP), pure ellagic acid (ELG), and pomegranate peel ethanol extract (DOSES-1 and DOSES-2) groups were able to suppress COX-2 expression. The expression of COX-2 is characterized by the presence of brown-stained cells in the cytoplasm. In the DSS group, almost all cells are stained with a strong intensity that almost covers the crypts. This indicates that COX-2 is highly expressed in the DSS group. In contrast to the ASP, ELG, DOSES-1, and DOSES-2
groups, the presence of brown-stained cells was not as large in the DSS group with moderate-to-weak cell intensity. There were also some blue-stained epithelial cells with a nucleus still clearly visible in the ASP, ELG, DOSES-1, and DOSES-2 groups. Measurements of COX-2 and iNOS gene expression in each group were done through a scoring system of histology score (H-score). Scoring first tested the suitability of the results of cell calculations between observers. The statistical test used is the Wilcoxon test because the result data between observers are not normally distributed. From the statistical test, there were no significant differences between first and second observers with p>0.05. The results of the calculation of COX-2 expression score are shown in Fig. 5. Administration of DSS 2% also increased the expression of iNOS characterized by brown-stained in the cytoplasm (Fig. 6). Similar with COX-2, iNOS expression also occurs in DSS groups. Brown-stained cells strongly on sides of the crypts. This indicates that iNOS is very strongly expressed. In contrast to ASP, ELG, DOSES-1, and DOSES-2 groups, the presence of brown-stained cells was not as large in the DSS group with moderate-to-weak cell intensity. There were also some blue epithelial cells with a nucleus still clearly visible in the ASP, ELG, DOSES-1, and DOSES-2 groups.

**DISCUSSION**

Administration of DSS 2% caused body weight loss, diarrhea, and bloody stool [28]. DSS molecules are polyanionic compounds of dextran derivatives containing negatively charged sulfate groups [29]. This sulfate group is highly toxic to the colonic epithelium and causes damage or erosion. The damage of epithelium increases the permeability of mucosa that permits the permeation of the substances in the lumen to colonic mucosa. Permeation of these substances causes damage to basal epithelial cells of crypts and induces an inflammatory response in the colonic mucosa. Histological changes due to DSS administration are depletion of goblet cells and cryptitis. Cryptitis reflects the migration of neutrophils into the mucosal epithelium [28,30].

### Table 1: Body weight changes in mice groups

| Group      | Body weight (g) | 1st week | 2nd week | 3rd week | 4th week | 5th week | 6th week |
|------------|-----------------|----------|----------|----------|----------|----------|----------|
| N          | 27.89±0.85      | 31.25±0.96| 33.22±1.13| 34.59±1.18| 35.55±1.25| 43.91±1.42|
| DSS        | 27.17±1.50      | 26.83±2.86**| 29.91±4.29| 28.26±1.28| 32.60±3.64| 32.37±2.87**|
| ASP        | 26.03±0.99      | 24.97±1.32**| 28.23±1.74| 28.26±1.28| 29.14±1.97| 32.71±1.84|
| ELG        | 28.09±0.98      | 28.89±2.32 | 32.6±3.32 | 32.63±3.75| 34.6±3.52 | 36±3.68  |
| DOSES-1    | 25.6±1.58       | 25.6±1.97 | 28.4±0.86 | 29.1±1.06 | 31.2±0.79 | 32.8±1.12 |
| DOSES-2    | 240 mg/kg       |          |          |          |          |          |
| DOSES-2    | 480 mg/kg       |          |          |          |          |          |

Data are expressed as mean±SD. **There was no significant difference of body weight changes in previous week (p>0.05)
Aspirin given as a control drug has an anti-inflammatory effect by inhibiting the activity of NF-kB and downregulation expression of COX enzyme. The inhibitory activity of NF-kB occurs because of the interaction of aspirin with a complex IKB kinase (IKK) [31]. IKK kinase complex (IKK) is an enzyme kinase consisting of IKKα, IKKβ (catalytic subunit), and IKKγ/ NFkappaB essential modulator (NEMO) (essential regulatory subunit) [32]. The binding of this enzyme by aspirin prevents phosphorylation of IKB and prevents the release of NF-kB that subsequently transmigrates from the cytoplasm into the nucleus. NF-kB regulates the transcription of a series of genes involved in acute responses to injury and chronic intestinal inflammation including the genes for IL-1β, TNF-α, IL-6, IL-8, IL-12, COX-2, and iNOS [33]. In addition, aspirin, but not other nonsteroidal anti-inflammatory drugs, is able to cause an irreversible inactivation of COX isozymes through the acetylation of a specific serine moiety (Ser529 of COX-1 and Ser516 of COX-2) [34]. In detail, aspirin binds to one monomer of COX-1 and COX-2 by the interaction with Arg120 residue and modifies covalently COX isozymes acetylating Ser529 (in COX-1) and Ser516 (in COX-2); the acetylated monomer becomes the allosteric subunit, and the partner monomer becomes the catalytic monomer [35,36]. Acetylation of the allosteric subunit of COX-1 by aspirin causes an irreversible inhibition of the COX activity and in turn of the generation of PGE2 from arachidonic acid (AA). The acetylated COX-2 is not able to form PGG2, but it generates 15R-hydroxyeicosapentaenoic acid (15R-HETE) from AA [37]. In endothelial cell, 15-epi- lipoxin A4 (15-epi-LXA4) has anti-inflammatory effect by inhibiting the proinflammatory genes such as IL8 through inhibition of NF-kB activity in the colon mucosal [38]. Kohnke et al. [39] reported that 15-epi-LXA4 in mice induced by DSS after administration of aspirin has an anti-inflammatory effect.

Ellagic acid is a polyphenol compound that has some pharmacological activity such as anti-inflammatory. The activity of ellagic acid in suppressing inflammatory cells is likelihood through one or a combination of the following mechanisms of action: (1) Binding to free radicals, (2) modulation of inflammatory cell activity such as macrophages, lymphocytes, and neutrophils, (3) modulation of pro-inflammatory enzyme activity such as phospholipase A2, COX, lipoxigenase, and enzymes producing nitric oxide (NO) such as NOS, and (4) modulation of proinflammatory gene production [40].

Ellagic acid is able to suppress the expression of COX-2 and iNOS in crypts basal. Karlson et al. [41] reported that ellagic acid is able to suppress COX-2 expression and the release of PGE2 in human monocytes can be inhibited. COX-2 and iNOS are important enzymes during the inflammatory process. Activation of COX-2 will result in excessive PGE2 resulting in hyperemia, edema, and intestinal dysfunction [42]. Meanwhile, iNOS activation results in increased NO production, trigger reactive nitrogen species. High levels of NO in the gut cause interactions with superoxide anions to form peroxynitrite [43,44]. Nitric oxide is a powerful oxidant that interacts with proteins and lipids, and DNA can cause DNA strand damage. The occurrence of DNA strand damage stimulates the activation of the poly-ADP ribose synthetase enzyme which results in increased permeability of epithelial cells. This affects the damage to the intestinal mucosa [45].

Pomegranate peel ethanol extract has a beneficial effect on various diseases such as antioxidants, anti-inflammatory, and anticancer. Pomegranate peel contains the main polyphenol that is ellagitannins and ellagic acid. Ellagitannins are hydrolyzed to ellagic acid in the gut, thus resulting in a prolonged release of this acid into the blood. In humans and different animal models, it has been found that ellagic acid is metabolized by the colon microflora to form urolithins A and B [16]. Pomegranate peel ethanol extract able to suppress the expression of COX-2 and iNOS. Larrosa et al. [45] reported that pomegranate peel extract dose 250 mg/kg/day in Fisher male rats also suppressed the expression of COX-2 and iNOS. Another study by Marin et al. [46] also reported that ellagic acid given since the beginning of the study in C57BL/6 mice with DSS induction of chronic colitis model was able to reduce the loss of epithelial cells, decrease the expression of COX-2 and iNOS. The mechanism is likely through inhibition of the NF-kB pathway. Ellagic acid contained in the pomegranate peel extract binds to the p105 protein which is the precursor of the p50 protein in the cytoplasm [47]. p50 and p65 (RelA) are the NF-kB subunits that can trigger the transcription of genes. The binding of p105 occurs through hydrophobic interactions by H atoms of the OH group of ellagic acid with valine amino acids at position 61 and glycine 68. This interaction leads to the failure of protein p50 formation, and gene transcription process by NF-kB is not occurred [47]. Although pomegranate peel ethanol extract shown to inhibit COX-2 and iNOS, it remains to be seen whether pomegranate peel ethanol extract given to animals has only activity on COX-2 expression or COX-1. Pomegranate peel extract is expected to have effect as selective COX-2 inhibitor on colitis treatment.

CONCLUSION
Pomegranate peel ethanol extract from Indonesia has anti-inflammatory agent by reducing inflammation score and inhibiting COX-2 and iNOS. In addition, pomegranate peel ethanol extract has equivalent effectiveness with aspirin and pure ellagic acid.

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