

## THE POTENCY OF INDONESIA'S POMEGRANATE PEEL ETHANOL EXTRACT (*PUNICA GRANATUM* LINN.) AS ANTI-INFLAMMATORY AGENT IN MICE COLON INDUCED BY DEXTRAN SODIUM SULFATE: FOCUS ON CYCLOOXYGENASE-2 AND INOS EXPRESSIONS

KUSMARDI KUSMARDI<sup>1</sup>, DONY HERMANTO<sup>2\*</sup>, ARI ESTUNINGTAS<sup>2</sup>, ARYO TEDJO<sup>3</sup>,  
BAMBANG P PRIOSOERYANTO<sup>4</sup>

<sup>1</sup>Department Anatomical Pathology, Faculty of Medicine, Universitas Indonesia, 10430, Indonesia. <sup>2</sup>Department of Pharmacology & Therapeutics, Faculty of Medicine, Universitas Indonesia, 10430, Indonesia. <sup>3</sup>Department of Medical Chemistry, Faculty of Medicine, Universitas Indonesia, 10430, Indonesia. <sup>4</sup>Department of Veterinary Pathology, Faculty of Bogor Agricultural University, Bogor, Indonesia. Email: normality\_04@yahoo.com

Received: 16 July 2017, Revised and Accepted: 20 September 2017

### ABSTRACT

**Objective:** Inflammatory disease occurs in the mucosal of the colon, or ulcerative colitis (UC) is one of 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 effectiveness with aspirin and pure ellagic acid.

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

© 2017 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>) DOI: <http://dx.doi.org/10.22159/ajpcr.2017.v10i12.21390>

### 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 (CAC) 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 1930 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 according to the patient's visit 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 23% [6].

The etiology of UC is not well understood, but evidence suggests that 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- $\kappa$ B). NF- $\kappa$ B dimers are kept in an inactive cytoplasmic complex by inhibitory proteins, the inhibitor protein kappa B (I $\kappa$ B) family, in resting cells [10]. NF- $\kappa$ B 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 I $\kappa$ B. This occurrence leads to the rapid dissociation of the complex accompanied by proteolytic degradation of I $\kappa$ B and release of NF- $\kappa$ B that subsequently translocates from the cytoplasm into the nucleus. NF- $\kappa$ B 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 $\beta$ , TNF- $\alpha$ , IL-6, IL-8, IL-12, cyclooxygenase (COX-2), and iNOS [11].

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 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 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 flavonols [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- $\kappa$ B [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 condition at temperature 22 $\pm$ 2°C with relative humidity at 65 $\pm$ 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-diamine 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 2<sup>nd</sup> week. In Group 3 (ASP), mice were administered with aspirin 43 mg/kg/d orally, and DSS was administered in 3 cycles started in the 2<sup>nd</sup> 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 2<sup>nd</sup> week. In Group 5 (DOSES-1), mice were administered pomegranate peel ethanol extract 240 mg/kg/d orally, and DSS was administered in 3 cycles started in the 2<sup>nd</sup> 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. 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, slice sections were blocked endogenous peroxidase activity with 0.3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) 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 1:1000) 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 chromogenic 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  $\times$ 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

- 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 immunohistochemically assessment performed by H-score assessment, 10 fields were chosen at random at  $\times 400$  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  $\pm$  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

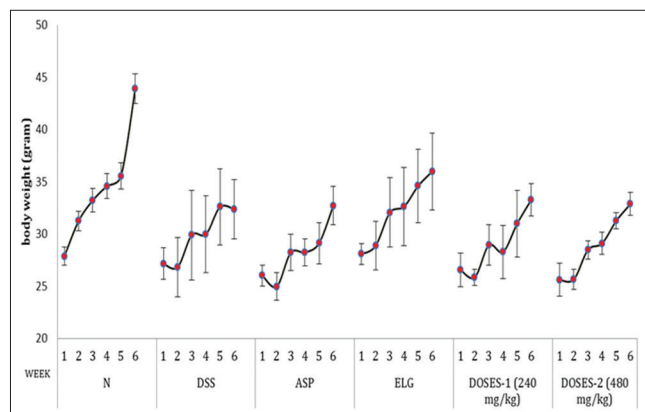
### Body weight changes

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 2<sup>nd</sup> and 6<sup>th</sup> week. Body weight loss is also occurred in the ASP group, at 2<sup>nd</sup> week. Body weight loss in the DOSES-1 group occurred at 2<sup>nd</sup> and 4<sup>th</sup> 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

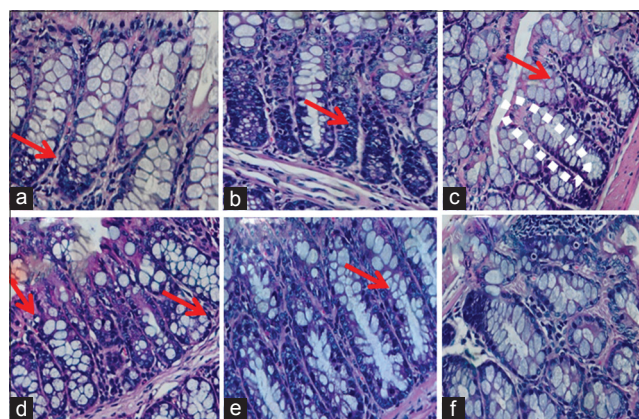


**Fig. 1: Effect of aspirin, pure ellagic acid, pomegranate peel ethanol extract (DOSES-1 and DOSES-2) on the body weight of mice during the study period.**

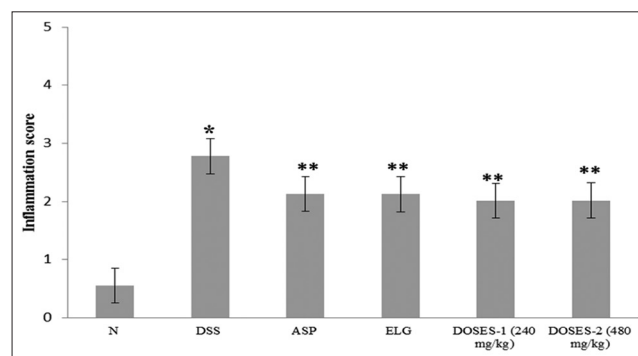
of colonic organs. Microscopic on colon cells and inflammatory scores are presented in Figs. 2 and 3. As shown in Fig. 2, DSS 2% through drinking water in each group caused inflammation. The DSS group showed inflammatory cells cover several crypts and goblet cells are not visible, whereas in the aspirin (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



**Fig. 2: Histological sections of mice colonic samples stained with hematoxylin and eosin. (a) Control group showing normal histology of mice colon. (b) Dextran sodium sulfate (DSS) 2% sample. Mucosal injury produced after DSS administration with loss of crypts and epithelial integrity as well as severe inflammatory cell infiltration. (c) ASP (aspirin 43 mg/kg/day and DSS 2%). (d) ELG (pure ellagic acid 26 mg/kg/day and DSS 2%). (e) DOSES-1 (pomegranate peel ethanol extract 240 mg/kg/day and DSS 2%). (f) DOSES-2 (pomegranate peel ethanol extract 480 mg/kg/day and DSS 2%). Morphological alteration associated with DSS treatment is reduced on aspirin, pure ellagic acid, DOSES-1, and DOSES-2 administration keeping colonic architecture and reducing epithelium loss. Original magnification:  $\times 400$ . Red arrow: Inflammatory cells. White circle: Crypts**



**Fig. 3: Inflammation score in each treatment group. \* Significantly different from the DSS group ( $p < 0.05$ ). Each bar represents the mean  $\pm$  SD of mice colon. \*\* There was no significant difference between the aspirin (ASP), pure ellagic acid, and pomegranate peel ethanol extract (DOSES-1 and DOSES-2) ( $p > 0.05$ )**



Table 1: Body weight changes in mice groups

Group	Body weight (g)					
	1 <sup>st</sup> week	2 <sup>nd</sup> week	3 <sup>rd</sup> week	4 <sup>th</sup> week	5 <sup>th</sup> week	6 <sup>th</sup> 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	29.97±3.68	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	32.06±3.32	32.63±3.75	34.60±3.52	36±3.68
DOSES-1 (240 mg/kg)	26.57±1.62	25.86±0.77**	28.94±1.93	28.29±2.53**	31±3.2	33.29±1.55
DOSES-2 (480 mg/kg)	25.63±1.58	25.66±0.97	28.49±0.86	29.11±1.06	31.26±0.79	32.89±1.12

Data are expressed as mean±SD. \*\*There was no significant difference of body weight changes in previous week ( $p>0.05$ )

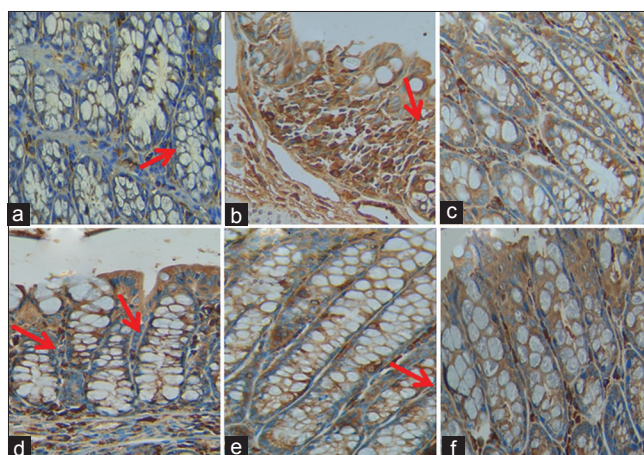


Fig. 4: COX-2 expression in colonic organ of mice with immunohistochemical staining in each treatment group.

Magnification:  $\times 400$ . (a) Normal group, (b) DSS Group, (c) ASP (aspirin 43 mg/kg/day and DSS 2%). (d) ELG (pure ellagic acid 26 mg/kg/day and DSS 2%), (e) DOSES-1 (pomegranate peel ethanol extract 240 mg/kg/day and DSS 2%). (f) DOSES-2 (pomegranate peel ethanol extract 480 mg/kg/day and DSS 2%). The red arrows indicate COX-2 expression in the form of brown-stained cells in the cytoplasm of crypts

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 in 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 Fig. 7.

## 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

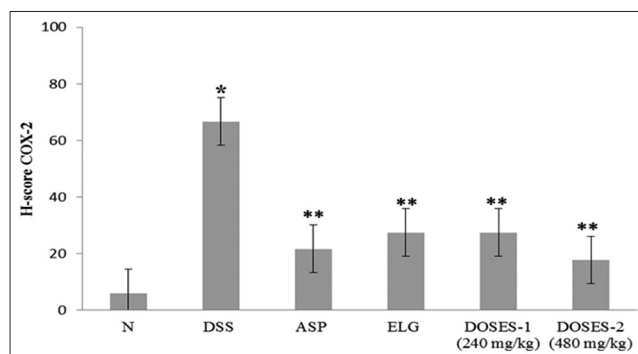


Fig. 5: Score of COX-2 expression in each treatment group. \*Significantly different from the DSS group ( $p<0.05$ ). Each bar represents the mean±SD of mice colon. \*\*There was no significant difference between the aspirin (ASP), pure ellagic acid (EG), and pomegranate peel ethanol extract (DOSES-1 and DOSES-2) ( $p>0.05$ )

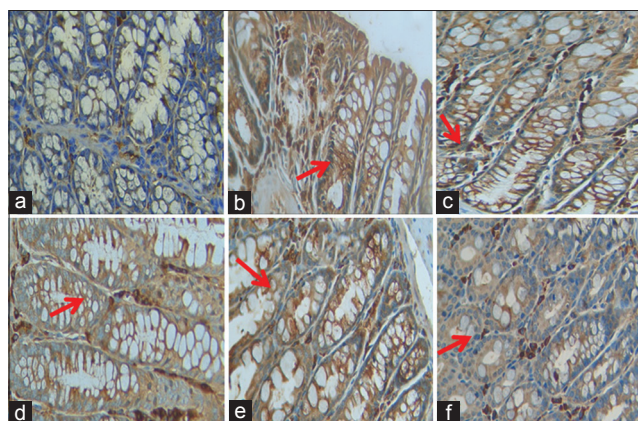
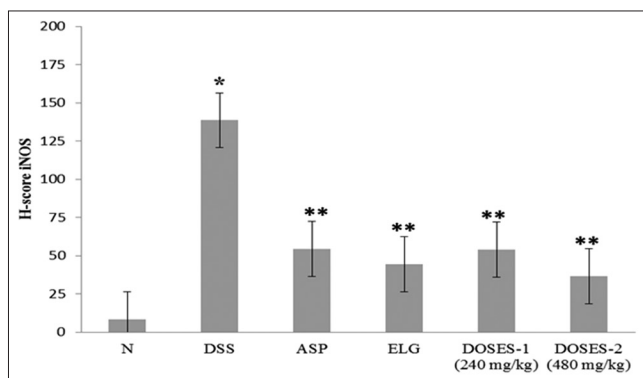


Fig. 6: iNOS expression in colonic organ of mice with immunohistochemical staining in each treatment group. Magnification:  $\times 400$ . (a) Normal group, (b) DSS group, (c) ASP (aspirin 43 mg/kg/d and DSS 2%), (d) ELG (pure ellagic acid 26 mg/kg/day and DSS 2%), (e) DOSES-1 (pomegranate peel ethanol extract 240 mg/kg/day and DSS 2%), (f) DOSES-2 (pomegranate peel ethanol extract 480 mg/kg/day and DSS 2%). The red arrows indicate iNOS expression in the form of brown-stained cells in the cytoplasm of crypts

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 causes cryptitis. Cryptitis reflects the migration of neutrophils into the mucosal epithelium [28,30].



**Fig. 7: Score of iNOS expression in each treatment group.**

**\*Significantly different from the DSS group ( $p < 0.05$ ). Each bar represents the mean  $\pm$  SD of mice colon. \*\*There was no significant difference between the aspirin (ASP), pure ellagic acid (EG), and pomegranate peel ethanol extract (DOSES-1 and DOSES-2) ( $p > 0.05$ )**

Aspirin given as a control drug has an anti-inflammatory effect by inhibiting the activity of NF- $\kappa$ B and downregulation expression of COX enzyme. The inhibitory activity of NF- $\kappa$ B occurs because of the interaction of aspirin with a complex IKK kinase [31]. IKK kinase complex (IKK) is an enzyme kinase consisting of IKK $\alpha$ , IKK $\beta$  (catalytic subunit), and IKK $\gamma$ /NF $\kappa$ B essential modulator (NEMO) (essential regulatory subunit) [32]. The binding of this enzyme by aspirin prevents phosphorylation of I $\kappa$ B and prevents the release of NF- $\kappa$ B that subsequently translocates from the cytoplasm into the nucleus. NF- $\kappa$ B regulates the transcription of a series of genes involved in acute responses to injury and chronic intestinal inflammation including the genes for IL-1 $\beta$ , TNF- $\alpha$ , 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 PGG<sub>2</sub> from *arachidonic acid* (AA). The acetylated COX-2 is not able to form PGG<sub>2</sub>, but it generates 15R-hydroxyicosapentaenoic acid (15R-HETE) from AA [37]. In endothelial cell, 15-epi-lipoxin A<sub>4</sub> (15-epi-LX<sub>4</sub>) has anti-inflammatory effect by inhibiting the proinflammatory genes such as IL8 through inhibition of NF- $\kappa$ B activity in the colon mucosal [38]. Kohnke *et al.* [39] reported that 15-epi-LX<sub>4</sub> 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 A<sub>2</sub>, COX, lipoxygenase, 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. Karlsson *et al.* [41] reported that ellagic acid is able to suppress COX-2 expression and the release of PGE<sub>2</sub> 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 PGE<sub>2</sub> 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 peroxide 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 ribosyl synthetase enzyme which results in increased permeability of epithelial cells. This affects the damage to the intestinal mucosa [43].

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 Fischer male rats also suppresses 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 likelihood through inhibition of the NF- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B 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.

## ACKNOWLEDGMENTS

The authors would like to thank for Directorate of Research and Public Services Universitas Indonesia for PITTA 2017 Grand and Ministry of Research, Technology, and Higher Education for PUPT 2016 grand also thank all technicians of the clinical laboratory and provincial key laboratory of the pharmacology and Anatomical Pathology department in University of Indonesia for their technical supports.

## REFERENCES

- Mulder DJ, Noble A, Justinich C, Duffin JM. A tile of two disease: The history of inflammatory bowel disease. *J Crohn's Colitis* 2013;5:1-8.
- Itzkowitz SH, Yio X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: The role of inflammation. *Am J Physiol Gastrointest Liver Physiol* 2004;287(1):G7-17.
- Lakatos PL, Lakatos L. Risk for colorectal cancer in ulcerative colitis: Changes, causes and management strategies. *World J Gastroenterol* 2008;14(25):3937-47.
- Zhiqin W, Palaniappan S, Raja Ali RA. Inflammatory bowel disease-related colorectal cancer in the Asia-Pacific region: Past, present, and future. *Intest Res* 2014;12(3):194-204.
- Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, *et al.* Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012;142(1):46-54.e420.
- Perkumpulan Gastroenterologi Indonesia. Kelompok Studi Inflammatory Bowel Disease Indonesia, Konsensus Nasional Penatalaksanaan Inflammatory Bowel Disease (IBD) Di Indonesia. Jakarta: Perkumpulan Gastroenterologi Indonesia; 2011.



7. Sartor RB. Mechanisms of disease: Pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol* 2006;3(7):390-407.
8. Coskun M. Intestinal epithelium in inflammatory bowel disease. *Front Med (Lausanne)* 2014;1:1-5.
9. Siegmund B, Zeitl M. Innate and adaptive immunity in inflammatory bowel disease. *World J Gastroenterol* 2011;17(27):3178-83.
10. Abraham C, Medzhitov R. Interactions between the host innate immune system and microbes in inflammatory bowel disease. *Gastroenterology* 2011;140(6):1729-37.
11. Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 1999;18(49):6853-66.
12. Carter MJ, Lobo AJ, Travis SP; IBD Section, British Society of Gastroenterology. Guidelines for the management of inflammatory bowel disease in adults. *Gut* 2004;53 Suppl 5:V1-16.
13. Sales-Campos H, Basso PJ, Alves VB, Fonseca MT, Bonfá G, Nardini V, et al. Classical and recent advances in the treatment of inflammatory bowel diseases. *Braz J Med Biol Res* 2015;48(2):96-107.
14. Friis S, Riis AH, Erichsen R, Baron JA, Sørensen HT. Low-dose aspirin or nonsteroidal anti-inflammatory drug use and colorectal cancer risk: A population-based, case-control study. *Ann Intern Med* 2015;163(5):347-55.
15. Kaulmann A, Bohn T. Bioactivity of polyphenols: Preventive and adjuvant strategies toward reducing inflammatory bowel diseases-promises, perspectives, and pitfalls. *Oxid Med Cell Longev* 2016;2016:9346470.
16. Turrini E, Ferruzzi L, Fimognari C. Potential effects of pomegranate polyphenols in cancer prevention and therapy. *Oxid Med Cell Longev* 2015;2015:938475.
17. Panichayupakaranant P, Tewtrakul S, Yuenyongsawad S. Antibacterial, anti-inflammatory and antiallergic activities of standardized pomegranate rind extract. *Food Chem* 2010;123:400-3.
18. Hamza RZ, Abd El-Azez AM, Hussien NA. Evaluation of the antioxidant potential for different extracts of al-taif pomegranate (*Punica granatum* L) induced by atrazine and Malathion pesticides in liver of male albino mice. *Int J Pharm Pharm Sci* 2015;7 Suppl 1:89-94.
19. Gadouche L, Djebli N, Zerrouki K. Neuroprotective effects of pomegranate juice on lead induced neurotoxicity in mice. *Int J Pharm Pharm Sci* 2016;9 Suppl 2:207-12.
20. Rosillo MA, Sánchez-Hidalgo M, Cárdeno A, Aparicio-Soto M, Sánchez-Fidalgo S, Villegas I, et al. Dietary supplementation of an ellagic acid-enriched pomegranate extract attenuates chronic colonic inflammation in rats. *Pharmacol Res* 2012;66(3):235-42.
21. Neyrinck AM, Van Hée VF, Bindels LB, De Backer F, Cani PD, Delzenne NM. Polyphenol-rich extract of pomegranate peel alleviates tissue inflammation and hypercholesterolaemia in high-fat diet-induced obese mice: Potential implication of the gut microbiota. *Br J Nutr* 2013;109(5):802-9.
22. Jimenez Del Rio M, Ramazanov A, Sikorski S, Ramazanov Z, Chkhikvishvili I. A new method of standardization of health-promoting pomegranate fruit (*Punica granatum*) extract. *Georgian Med News* 2006;140:70-7.
23. US FDA. Guidance for Industry: Estimating the Maximum Safe Starting Dose in Adult Healthy Volunteer. Rockville, MD: US Food and Drug Administration; 2005.
24. al-Swayeh OA, Clifford RH, del Soldato P, Moore PK. A comparison of the anti-inflammatory and anti-nociceptive activity of nitroaspirin and aspirin. *Br J Pharmacol* 2000;129(2):343-50.
25. Panichayupakarananta P, Issuriya A, Sirikatitham A, Wang W. Antioxidant assay-guided purification and LC determination of ellagic acid in pomegranate peel. *J Chromatogr Sci* 2010;48(6):456-9.
26. Rogers R, Anderson JE, De Voss J, Lesch J, Yan D, Xu M, et al. Image analysis-based approaches for scoring mouse models of colitis. *Vet Pathol* 2015;1:1-11.
27. Detre S, Saclani Jotti G, Dowsett M. A "quickscore" method for immunohistochemical semiquantitation: Validation for oestrogen receptor in breast carcinomas. *J Clin Pathol* 1995;48(9):876-8.
28. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. *Curr Protoc Immunol* 2014;104:1-16.
29. Solomon L, Mansor S, Mallon P, Donnelly E, Hoper M, Loughrey M, et al. The dextran sulphate sodium: An overview. *Comp Clin Pathol* 2010;19:235-9.
30. Perše M, Cerar A. Dextran sodium sulphate colitis mouse model: Traps and tricks. *J Biomed Biotechnol* 2012;2012:718617.
31. Paul-Clark MJ, Van Cao T, Moradi-Bidhendi N, Cooper D, Gilroy DW. 15-epi-lipoxin A4-mediated induction of nitric oxide explains how aspirin inhibits acute inflammation. *J Exp Med* 2004;200(1):69-78.
32. Pasparakis M. IKK, NF-Kb signaling in intestinal epithelial cells controls immune homeostasis in the gut. *Mucosal Immunol Nat* 2008;1:S54-7.
33. Dovizio M, Tacconelli S, Sostres C, Ricciotti E, Patrignani P. Mechanistic and pharmacological issues of aspirin as an anticancer agent. *Pharmaceuticals (Basel)* 2012;5(12):1346-71.
34. Patrono C, Baigent C, Hirsh J, Roth G. Antiplatelet drugs: American college of chest physicians evidence-based clinical practice guidelines (8th edition). *Chest* 2008;133 6 Suppl:199S-233.
35. Sidhu RS, Lee JY, Yuan C, Smith WL. Comparison of cyclooxygenase-1 crystal structures: Cross-talk between monomers comprising cyclooxygenase-1 homodimers. *Biochemistry* 2010;49(33):7069-79.
36. Rimon G, Sidhu RS, Lauver DA, Lee JY, Sharma NP, Yuan C, et al. Coxibs interfere with the action of aspirin by binding tightly to one monomer of cyclooxygenase-1. *Proc Natl Acad Sci U S A* 2010;107(1):28-33.
37. Sharma NP, Dong L, Yuan C, Noon KR, Smith WL. Asymmetric acetylation of the cyclooxygenase-2 homodimer by aspirin and its effects on the oxygenation of arachidonic, eicosapentaenoic, and docosahexaenoic acids. *Mol Pharmacol* 2010;77(6):979-86.
38. Gilroy DW. New insights into the anti-inflammatory actions of aspirin-induction of nitric oxide through the generation of epi-lipoxins. *Mem Inst Oswaldo Cruz* 2005;100 Suppl 1:49-54.
39. Köhnke T, Gomolka B, Bilal S, Zhou X, Sun Y, Rothe M, et al. Acetylsalicylic acid reduces the severity of dextran sodium sulfate-induced colitis and increases the formation of anti-inflammatory lipid mediators. *Biomed Res Int* 2013;2013:748160.
40. Bellik Y, Boukraâ L, Alzahrani HA, Bakhotmah BA, Abdellah F, Hammoudi SM, et al. Molecular mechanism underlying anti-inflammatory and anti-allergic activities of phytochemicals: An update. *Molecules* 2012;18(1):322-53.
41. Karlsson S, Nånberg E, Fjæraa C, Wijkander J. Ellagic acid inhibits lipopolysaccharide-induced expression of enzymes involved in the synthesis of prostaglandin E2 in human monocytes. *Br J Nutr* 2010;103(8):1102-9.
42. Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol* 1998;38:97-120.
43. Kolios G, Valatas V, Ward SG. Nitric oxide in inflammatory bowel disease: A universal messenger in an unsolved puzzle. *Immunology* 2004;113(4):427-37.
44. Miller MJ, Sandoval M. Nitric oxide. III. A molecular prelude to intestinal inflammation. *Am J Physiol* 1999;276:G795-9.
45. Larrosa M, González-Sarriás A, Yáñez-Gascón MJ, Selma MV, Azorín-Ortuño M, Toti S, et al. Anti-inflammatory properties of a pomegranate extract and its metabolite urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism. *J Nutr Biochem* 2010;21(8):717-25.
46. Marín M, María Giner R, Ríos JL, Recio MC. Intestinal anti-inflammatory activity of ellagic acid in the acute and chronic dextran sulfate sodium models of mice colitis. *J Ethnopharmacol* 2013;150(3):925-34.
47. Khan MK, Ansari IA, Khan MS, Arif JM. Dietary phytochemicals as potent chemotherapeutic agents against breast cancer: Inhibition of NF-κB pathway via molecular interactions in rel homology domain of its precursor protein p105. *Pharmacogn Mag* 2013;9:51-7.