The Effect of Pomegranate Peel Ethanol Extract to TNF-α Expression of Mice Colonic Epithelial Cells Induced Using Dextran Sodium Sulfate (DSS)

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ABSTRACT

Introduction: The conventional drugs for inflammatory bowel disease (IBD) have many side effects that impact patient’s quality of life, leading to the emergence of alternative therapies such as pomegranate peel ethanol extract (PPE). This study aims to investigate the anti-inflammatory effect of PPE by observing TNF-α expression in mice induced chronic inflammation of the colon using dextran sodium sulfate (DSS).

Methods: 28 Swiss Webster mice samples were taken and divided into five groups, the control group (6 mice), the negative control group (5 mice), the group that was given DSS and aspirin (6 mice), the group was given DSS and a high dose of PPE (5 mice), and the group was given DSS and a low dose of PPE (6 mice). In mice, distal colonic tissue was taken and then stained immunohistochemically against TNF-α and observed with light microscopy at 400x magnification, and TNF-α expression was assessed using the H-Score. Results: TNF-α expression was significantly lower in the group given a high dose of PPE than the negative control group (p < 0.05), with mean rank scores of 3.00 and 8.00. There was no significant difference between the group given PPE with a high dose and aspirin (p > 0.05). Conclusion: TNF-α expression in colonic epithelial cells of mice given DSS decreased upon treatment of a high dose of PPE, indicating a mechanism of decreasing inflammation. PPE also has the same effect as aspirin in reducing inflammation.

Keywords: Pomegranate peel ethanol extract, Colonic epithelial cells, Dextran sodium sulfate, TNF-α, Mice.

INTRODUCTION

Inflammatory bowel disease (IBD), which consists of Ulcerative colitis (UC) and Crohn’s disease (CD), is a condition of remitting immunologic disorders that generate chronic inflammation of the gastrointestinal (GI) tract. UC and CD are differentiated by which portion of the GI tract is affected and how much impact they have on the bowel wall. IBD used to be known as a Western disease because it is most common in Oceania, North America, and Europe. In the Western world, the prevalence of CD is around 50-200 per 100,000 persons, and for UC, it is around 120-200 per 100,000 persons. However, in the 21st century, IBD has indicated an increasing tendency of incidence and prevalence rates in Asia and Africa. Therefore, with a stable number of new cases worldwide, IBD is considered a global healthcare issue.

Epidemiological data on IBD in Indonesia are still challenging to find; however, data can be obtained through hospital reports of colonoscopies taken by patient visits in several National Hospitals. In general, the incidence of UC is higher than the CD. The case report In Cipto Mangunkusumo Hospital Jakarta (RSCM), from 1541 colonoscopies, IBD is found as much as 8.3%. In Dr. Sardjito Hospital Yogyakarta (RSUP Dr. Sardjito), from 269 colonoscopies, IBD is found as much as 44%. In Jambi General Hospital (RSUD Jambi), IBD is found as much as 17% from 364 colonoscopies.

Microscopically, the lamina propria of patients with active IBD, is revealed to have pronounced infiltration of natural killer T cells, neutrophils, dendritic cells and macrophages. Along with the activation and increased number of these cells, interleukins 23-TH17 pathway cytokines, interferon-γ, interleukin-1β, and tumor necrosis factor-α (TNF-α) also increased.

The inflammation process in IBD begins with recognizing the gut's microbial agents by tolls like receptor (TLR) and nucleotide-binding oligomerization domain-like receptor (NLR) located on the surface of the intestinal epithelium. The recognition is followed by TLR signals inducting myeloid differentiation primary response 88 (MyD88), transforming growth factor beta-activated kinase 1 (TAK1), and expression of nuclear factor-kappaB (NF-κB). Activation of NF-κB contributes to the emergence of inflammatory diseases and oncogenesis. NF-κB that is activated will produce inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor-α (TNF-α), which have been shown to play a role in the pathogenesis of inflammatory diseases and oncogenesis.

There are several options in treating IBD. Unfortunately, in the long run, a substantial number of patients will not be helped by the available drugs. It has been observed that primary nonresponse is found in 20-30% of patients, and 30% of patients get refractory because of secondary response loss. Moreover, the available conventional drugs have numerous side effects that impact life quality of the patients. This challenging situation of treating patients with IBD due to the disease complexity and severity gives rise to an urgent need to develop drugs that are more efficacious and safer.
Studies on alternative IBD treatments therapies, such as herbs medication been conducted.\textsuperscript{11,12} Herbs medication are considered more natural, less toxic and less likely to have undesirable side effects of conventional therapy.\textsuperscript{12} Recently, pomegranate is the most widely recognized for its anti-cancer, antioxidant, antimicrobial and anti-inflammatory benefits. The health benefits of pomegranate are believed due to the high content of polyphenol in pomegranate. Polyphenols are proven to decrease pro-inflammatory cytokines such as cytokine CXCL8 in TNF stimulated Caco-2 cells and inflammatory mediators like IL1α, IL6 and CXCL8.\textsuperscript{13} Up until today, researches have not focused on the expression of TNF-α cytokines after administration of pomegranate peel extract. Meanwhile, contents of polyphenols are found higher in its peel. Based on this background, the effect of pomegranate peel ethanol extract (PPE) on TNF-α cytokines expression in mice colon induced by dextran sodium sulfate (DSS) can be investigated. DSS is toxic to the colonic epithelial cells, thus creating chronic inflammation. This study of pomegranate peel ethanol extract can be further used for research in human if it is proven to generate any effect in mice colon induced by DSS.\textsuperscript{14}

**METHODS**

**Study design**

This study used an in vivo experimental research design conducted at the Anatomical Pathology Department Faculty of Medicine, University of Indonesia. This study used stored biological material from a previous study. The study was conducted to assess pomegranate peel extract’s effect on the colon of mice induced by DSS and compare it with positive control that was given aspirin.

Research was carried out in Laboratory of Anatomical Pathology Department, Faculty of Medicine, University of Indonesia, Jakarta.

**Source of research data**

The data source was the primary data obtained by observing the colon tissues histopathological image of Swiss Webster mice given several treatments.

**Research sample**

This study used stored biological material by Kusmardi et al. The tested animals were Swiss-Webster mice in the Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia with male mice, aged 12-16 weeks, and weighing 20-30 grams. The Inclusion criteria was histological preparations that were well colored (observable) and the exclusion criteria was histological preparations that were difficult to observe.

**Sample size**

The calculation of the number of samples was obtained with the Federrer formula \( (t-1) \times (n-1) \geq 15 \), if \( n \) is the sample size for each group and \( t \) is the number of treatment groups, the calculation is:

\[
\begin{align*}
(t-1) \times (n-1) & \geq 15 \\
(n-1) & \geq 5-1 \\
(n-1) & \geq 3.75 \\
n & \geq 4.75
\end{align*}
\]

Thus, the minimum sample size for each treatment group was five samples, so that the minimum sample size required was 25 Swiss-Webster mice. This study used 28 Swiss-Webster mice.

**Sampling technique**

Sampling was done using a simple random sampling technique where the sample was taken randomly to avoid bias. Mice that have been obtained following the required number will be assigned to the treatment groups randomly, where the first mice are included in the normal group without treatment, the second mice are in the negative control group, the third mice are in the aspirin group, the fourth and fifth mice are in the pomegranate peel extract group. This group’s division was repeated according to the sequence until all the mice were in their respective groups.

**Dose calculation**

The aspirin dose was determined based on a study of aspirin as a chemo-preventive for colon cancer, which was 75-325 mg/day.\textsuperscript{15} The dose was converted by body weight of 60 kg to mg/kg to 1.25-5.42 mg/kg and converted into a dose of mice with the formula:\textsuperscript{16}

\[
\text{Mice dosage (mg/kg)} = \frac{\text{human dosage (mg/kg) \times human Km (constanta)}}{\text{mice km (constanta)}}
\]

Based on the formula, a low dose of 15.4 mg/kgBW was obtained, and the highest dose was 66.8 mg/kgBW, and the researchers chose a dose of 43 mg/kgBW for the study.

The pomegranate peel ethanol extract (PPE) dosage of 240 mg/kgBW was calculated based on ellagic acid level in the pomegranate extract, which was 208.432 ppm atai 11.047%. Based on a previous study, the dose of ellagic acid as an anti-inflammatory in mice was 26 mg/kg. Thus, the PPE dose was calculated using the formula: 100/11.047x 26 mg/kg = 24 mg/kgBW. Meanwhile, the PPE dose of 480 mg/kgBW was obtained by calculating based on a counting series of doses of 240 mg/kgBW.

**Experimental animals**

All laboratory animals (Swiss-Webster mice) were acclimatized at the Health Research and Development Institute (LITBANGKES) for one week. After that, the laboratory animals were divided into five treatment groups as follows:

- The normal group was a group of Swiss-Webster mice that were only given plain drinking water until the end of the treatment.
- The negative control group was a group of Swiss-Webster mice given 2% DSS induction treatment at the end of the 2\textsuperscript{nd}, 4\textsuperscript{th} and 6\textsuperscript{th} week.
- The aspirin group was the positive control group where Swiss-Webster mice were given 2% DSS induction treatment and given aspirin at a dose of 43 mg/kgBW/day at week at the end of the 2nd, 4th and sixth week.
- The low dose group was a group of Swiss-Webster mice given 2% DSS induction treatment and PPE at a dose of 240 mg/kg/day at the end of the 2\textsuperscript{nd}, 4\textsuperscript{th} and 6\textsuperscript{th} week.
- The high dose group was a group of Swiss-Webster mice given 2% DSS induction treatment and PPE at a dose of 480 mg/kg/day at the end of the 2\textsuperscript{nd}, 4\textsuperscript{th} and 6\textsuperscript{th} week.
- Three days after the last 2% DSS induction cycle was performed, Swiss-Webster mice were sacrificed with decapitation. Surgery was performed to remove the mice’s colon. The colonic tissues were fixed using a 10% formalin buffer solution for less than 24-48 hours.

**Tissue preparation procedure**

The colonic tissues Swiss-Webster mice that had been fixed for 24-28 hours were subjected to dehydration or excretion processes. After that, the tissues were put into a stratified alcohol solution, which was then put into a stratified xylool solution to draw the alcohol out of the tissue; this process is called the clearing process, where the tissue becomes...
clear. The next stage was the impregnation process, where paraffin infiltrates the colon organs' cavity so that the structure and texture of the cells were maintained during the cutting and staining process. The processes described were carried out with a machine, namely an automatic tissue processor machine.

The next process was planting the tissue into the paraffin medium. After that, the preparatory block was ready to be cut using microtome machines up to a thickness of 3-5 μm. The cutting results were then put into a water bath with a temperature of 40-50°C so that it expanded. After that, the cut results were attached to the object-glass and dried for one hour at 40°C.

Immunohistochemical staining

Immunohistochemical staining technique was performed to observe the expression of TNF-α protein. The colonic tissues attached to a particular glass object for the IHC technique were dried at 37°C and heated at 60°C on a slide warmer. The next process was deparaffinization and rehydration, in which the preparations are washed underwater heated at 60 oC on a slide warmer. The next process was deparaffinization for one hour at 40 oC.

After that, the cut results were attached to the object-glass and dried. Preparation was then put in a Tris-EDTA solution, which was then opened the epitope so that the primary antibody could recognize it. The solution mixed with methanol. Then the preparations were rewashed with water within 5 minutes, and antigen retrieval was carried out to open the epitope so that the primary antibody could recognize it. The preparation was then put in a Tris-EDTA solution, which was then heated at 96°C for 10 minutes in a decloaking chamber. The preparations were then cooled and washed with a phosphate buffer saline (PBS) with a pH of 7.4 within 5 minutes. The next process was background sniper blocking, and preparations were dropped by a primary antibody, which was diluted in horse serum within 1 hour. The preparations were washed with PBS solution, applied universal link secondary antibody for 15 minutes, and rewashed with PBS solution within 5 minutes. The horseradish peroxidase (HRP) reagent was dropped for 15 minutes and rewashed with PBS solution. Diaminobenzidine (DAB) solution was then dropped on the preparations for 2 minutes and rewashed for 10 minutes. Then, staining was done by immersing the preparations in a solution of hematoxylin for 2 minutes and rinsing with water.

After staining with hematoxylin, the preparations were immersed in a lithium carbonate solution within 1 minute and rinsed under running water for 3 minutes. After that, the dehydration and clearing processes were carried out for 5 minutes each. Finally, bonding was done by giving one drop of entellan*, then covered with a glass cover.

The level of TNF-α expression was measured using a histological score (H-score). The first thing was to observe the tissue preparations given IHC staining under a light microscope with a magnification of 400x. Each tissue preparation was photographed using a camera from the InDexMicroView and ImageJ (IHC Profiler) application. Photos were taken in five different viewpoints at random. The researchers then assessed the number of epithelial cells that were blue and brown, blue as negative, and brown as positive. The intensity of brown in cells was assessed by a numerical score, including negative with a score of 1, low positive with a score of 2, positive with a score of 3, and high positive with a score of 4.45. The number of colored cells and color intensity was calculated using the ImageJ application with the IHC Profiler plugin, where the results were converted into percentage units.

After that, a semiquantitative assessment of the histology score (H-Score) was carried out using the following formula:

\[(\% \text{high positive x 4}) + (\% \text{positive x 3}) + (\% \text{low positive x 2}) + (\% \text{negative x 1})\]

The H-Score was assessed in each field with the formula above with a minimum score of 0 and a maximum of 400. After the H-Score for each field of view was obtained, the score was added and calculated on average to obtain each treatment group's score.

Data analysis

Choosing a statistical test relies on identifying how many variables are involved in the research question and what level of measurement the variables are. There are dependent and independent variables in this study, and therefore, the statistical analysis approach was bivariate. The measurement of variables in this research is categorical (qualitative) and metric (quantitative). The metric variables are the H-Score represented by the expression of TNF-α, and categorical variables are represented by five groups of treatments that were given to the laboratory animals. The Shapiro-Wilk (normality test) suggested that the distribution of the dependent variable in this study was not normally distributed for each group of the independent variable and this data has shown extreme outliers indicating that the data failed to meet the assumptions of the parametric test. Thus, non-parametric tests were taken.

Because there are more than two groups used and each group is independent to one and another, to compare H-Score between each group, the Kruskal-Wallis test was chosen because it is a rank-based nonparametric test that can be used to determine if there are statistically significant differences between two or more independent variables on a continuous or ordinal dependent variable when the data fails to meet the assumptions of the parametric test. After computed the Kruskal-Wallis test, Mann-Whitney U-test was used to determine if there are differences between the two groups. Mann-Whitney U-test is often presented as an alternative to the independent samples t-test, which can be used when the data fail the assumptions of the independent samples t-test. In this study, the normally distributed data assumption was violated, and hence, Mann-Whitney U-test can be used. The risk of bias should not be a concern because it had an adequate sample size based on Federrer Formula.

RESULTS

The results are obtained from data of five groups of mice that have been treated with different intervention. The groups consist of negative control group, normal group, aspirin group, low dose of pomegranate peel ethanol extract group, and high dose of pomegranate peel ethanol extract group. Each group has their own microscopic slides taken from mice's colon. Both negative control group and high dose of pomegranate peel ethanol extract group have 5 microscopic slides each. Normal group, aspirin group, and high dose of pomegranate peel ethanol extract group have 5 microscopic slides each. Each microscopic slide had been given Immunohistochemistry (IHC) staining. Each microscopic slides was analyzed with ImageJ application to calculate the H-score for identifying the expression of TNF-α.

Figure 1 shown that aspirin and low dose and high dose of pomegranate peel ethanol extract groups were able to decrease TNF-α expression. TNF-α expression is marked with the finding of cytoplasm that has brown-stained cells. Almost all cells in the negative control group showed a strong intensity staining that covers almost of the entire crypts. The strong intensity suggests a very high TNF-α expression in the negative control group compared to the aspirin and low and high dose of pomegranate peel ethanol extract.

The expression of TNF-α in the normal group were the lowest (Mean rank=6.00), followed by high dose of pomegranate peel ethanol extract group (Mean rank=8.80) and aspirin group (Mean rank=13.00). The expression of TNF-α was highest for negative control (Mean rank=23.80) and low dose of pomegranate peel ethanol extract group (Mean rank=21.50). Distributions of expression of TNF-α across the treatment groups were not similar as assessed by visual inspection. A Kruskal Wallis test found that the expression of TNF-α were significantly affected by their treatment (H(4) = 19.742, p = 0.001). A Mann-Whitney U-test was run to determine the differences in each treatment group.
The expression of TNF-α between normal and high dose of peel ethanol extract group

The expression of TNF-α between normal and high dose of peel ethanol extract group revealed that in a sample of 6 slides in the normal group and 5 slides in the high dose of peel ethanol extract group, the expression of TNF-α was lower for normal group ($Mdn = 123.98$) than for high dose of peel ethanol extract group ($Mdn = 132.35$). The expression of TNF-α is more variable for normal group (IQR=29.13) than for high dose of peel ethanol extract group (IQR=6.63). Distributions of the expression of TNF-α between normal and high dose of peel ethanol extract group were not similar, as assessed by visual inspection. The expression of TNF-α for normal group ($mean rank = 5.00$) was not statistically significantly lower than for high dose of peel ethanol extract group ($mean rank = 7.20$) $U = 21$, $z = 1.095$, $p = 0.329$, $r = 0.33$, using an exact sampling distribution for $U$.

The expression of TNF-α between normal and negative control group

The expression of TNF-α between normal and negative control group revealed that in a sample of 6 slides in the normal group and 5 slides in negative control group, the expression of TNF-α was lower for normal group ($Mdn = 123.98$) than negative control group ($Mdn = 156.23$). The expression of TNF-α is more variable for normal group (IQR=29.13) than negative control group (IQR=10.49). Distributions of the expression of TNF-α between normal and negative control group were not similar, as assessed by visual inspection. The expression of TNF-α for normal group ($mean rank = 3.50$) was statistically significantly lower than negative control group ($mean rank = 9.00$) $U = 0.000$, $z = -2.739$, $p = .004$, $r = 0.83$, using an exact sampling distribution for $U$.

The expression of TNF-α between normal and low dose of pomegranate peel ethanol extract group

The expression of TNF-α between normal and low dose of pomegranate peel ethanol extract group revealed that in a sample of 6 slides in the normal group and 5 slides in low dose of pomegranate peel ethanol extract group, the expression of TNF-α was lower for normal group ($Mdn = 123.98$) than low dose of pomegranate peel ethanol extract group ($Mdn = 148.68$). The expression of TNF-α is more variable for normal group (IQR=29.13) than low dose of pomegranate peel ethanol extract group (IQR=10.82). Distributions of the expression of TNF-α between normal and low dose of pomegranate peel ethanol extract group were not similar, as assessed by visual inspection. The expression of TNF-α for normal group ($mean rank = 3.50$) was statistically significantly lower than low dose of pomegranate peel ethanol extract group ($mean rank = 9.50$) $U = 36.00$, $z = 2.882$, $p = 0.002$, $r = 0.83$, using an exact sampling distribution for $U$.

The expression of TNF-α between normal and aspirin group

The expression of TNF-α between normal and aspirin group revealed that in a sample of 6 slides in the normal group and 5 slides aspirin group, the expression of TNF-α was lower for normal group ($Mdn = 123.98$) aspirin group ($Mdn = 138.78$). The expression of TNF-α is more variable for normal group (IQR=13.20) than aspirin group (IQR=8.50). Distributions of the expression of TNF-α between normal and aspirin group were not similar, as assessed by visual inspection. The expression of TNF-α for normal group ($mean rank = 4.50$) was not statistically significantly lower than aspirin group ($mean rank = 8.50$) $U = 30.00$, $z = 1.922$, $p = 0.065$, $r = 0.55$, using an exact sampling distribution for $U$.

The expression of TNF-α between low dose of pomegranate peel ethanol extract group and aspirin group

The expression of TNF-α between low dose of pomegranate peel ethanol extract group and aspirin group revealed that in a sample of 6 slides in the aspirin group and 6 slides in the low dose of pomegranate peel ethanol extract group, the expression of TNF-α was lower for aspirin group ($Mdn = 138.78$) than low dose of pomegranate peel ethanol extract group ($Mdn = 148.68$). The expression of TNF-α is more variable for aspirin group (IQR=3.50) than low dose of pomegranate peel ethanol extract group (IQR=1.095). Distributions of the expression of TNF-α between aspirin and low dose of pomegranate peel ethanol extract group were not similar, as assessed by visual inspection. The expression of TNF-α for aspirin group ($mean rank = 4.50$) was statistically significantly lower than low dose of pomegranate peel ethanol extract group ($mean rank = 9.50$) $U = 36.00$, $z = 2.882$, $p = 0.002$, $r = 0.83$, using an exact sampling distribution for $U$.
variable for aspirin group (IQR=13.2) than low dose of pomegranate peel ethanol extract group (IQR=10.82). Distributions of the expression of TNF-α between blow dose of pomegranate peel ethanol extract and aspirin group were not similar, as assessed by visual inspection. The expression of TNF-α for aspirin (mean rank= 3.67) was statistically significantly lower than low dose of pomegranate peel ethanol extract group (mean rank =9.33) $U = 55.00$, $z = 2.722$, $p = 0.004$, $r = 0.79$, using an exact sampling distribution for $U$.

The expression of TNF-α between high dose of pomegranate peel ethanol extract group and negative control group

The expression of TNF-α between high dose of pomegranate peel ethanol extract group and negative control group revealed that in a sample of 5 slides in the negative control group and 5 slides high dose of pomegranate peel ethanol extract group, the expression of TNF-α was lower for high dose of pomegranate peel ethanol extract group ($Md n = 132.35$) than negative control group ($Md n = 156.23$). The expression of TNF-α is more variable negative control group (IQR=10.49) than high dose of pomegranate peel ethanol extract group (IQR=6.63). Distributions of the expression of TNF-α between high dose of pomegranate peel ethanol extract and negative control group were not similar, as assessed by visual inspection. The expression of TNF-α for high dose of pomegranate peel ethanol extract group (mean rank= 3.00) was statistically significantly lower than negative control group (mean rank =8.00) $U = 0.000$, $z = -2.611$, $p = 0.008$, $r = 0.83$, using an exact sampling distribution for $U$.

The expression of TNF-α between high dose of pomegranate peel ethanol extract group and low dose of pomegranate peel ethanol extract group

The expression of TNF-α between high dose of pomegranate peel ethanol extract group and low dose of pomegranate peel ethanol extract group revealed that in a sample of 5 slides in the high dose of pomegranate peel ethanol extract group and 6 slides in the low dose of pomegranate peel ethanol extract group, the expression of TNF-α was lower for high dose of pomegranate peel ethanol extract group ($Md n = 132.35$) than low dose of pomegranate peel ethanol extract group ($Md n = 148.68$). The expression of TNF-α is more variable in low dose of pomegranate peel ethanol extract group (IQR=10.82) than high dose of pomegranate peel ethanol extract group (IQR=6.63). Distributions of the expression of TNF-α between high dose of pomegranate peel ethanol extract and low dose of pomegranate peel ethanol extract group were not similar, as assessed by visual inspection. The expression of TNF-α for high dose of pomegranate peel ethanol extract group (mean rank= 3.00) was statistically significantly lower than low dose of pomegranate peel ethanol extract group (mean rank =8.50) $U = 0.000$, $z = -2.611$, $p = 0.008$, $r = 0.83$, using an exact sampling distribution for $U$.

The expression of TNF-α between low dose of pomegranate peel ethanol extract group and negative control group

The expression of TNF-α between low dose of pomegranate peel ethanol extract group and negative control group revealed that in a sample of 5 slides in the negative control group and 6 slides aspirin group, the expression of TNF-α was lower for high dose of pomegranate peel ethanol extract group ($Md n = 132.35$) than the aspirin group ($Md n = 138.78$). The expression of TNF-α is more variable in aspirin group (IQR=13.20) than high dose of pomegranate peel ethanol extract group (IQR=6.63). Distributions of the expression of TNF-α between high dose of pomegranate peel ethanol extract aspirin group were not similar, as assessed by visual inspection. The expression of TNF-α for high dose of pomegranate peel ethanol extract group (mean rank= 4.60) was not statistically significantly lower than aspirin group (mean rank = 7.17) $U = 8.000$, $z = -1.278$, $p = 0.247$, $r = 0.39$, using an exact sampling distribution for $U$.

The expression of TNF-α between aspirin group and negative control group

The expression of TNF-α between aspirin group and negative control group revealed that in a sample of 5 slides in the negative control group and 5 slides aspirin group, the expression of TNF-α was lower aspirin group ($Md n = 138.78$) than negative control group ($Md n = 156.23$). The expression of TNF-α is more variable for aspirin group (IQR=13.20) than negative control group (IQR=10.49). Distributions of the expression of TNF-α between aspirin and negative control group were not similar, as assessed by visual inspection. The expression of TNF-α for aspirin (mean rank= 4.17) was not statistically significantly lower than negative control group (mean rank =8.20) $U = 4.000$, $z = -2.008$, $p = 0.052$, $r = 0.61$, using an exact sampling distribution for $U$.

**DISCUSSION**

Dextran Sodium Sulfate (DSS) administration has been proven to cause body weight loss, bloody stool and diarrhea.17 DSS has a very toxic sulfate group that is negatively charged as a constituent. The sulfate group is from the dextran derivatives in DSS polyanionic compounds molecules.18 The toxic sulfate group is able to create a significant damage to the epithelial cells of the colon. If the damage keeps on occurring, mucosa permeability will increase and allow substances in the lumen to permeate the colonic mucosa. Permeation that occurs induces disruption in the crypts basal epithelial cells and stimulates colonic mucosa inflammation. DSS administration will cause histological changes such as goblet cells depletion. The goblet cells depletion will cause cryptitis and will be reflected by neutrophils migration in the epithelium of the mucosa.19 Cryptitis is one of the histopathologic findings that can be seen in inflammatory bowel disease (IBD).20 It has been proven by Yan et al that in their study, it was demonstrated that DSS administration demonstrated increased inflammatory mediators including TNF-α, IL-1β, INF-γ, IL-10 and IL-12.21 Induction of inflammatory conditions by DSS carried out on the distal colon to obtain a negative comparison group was achieved in this study. The expression of TNF-α can prove this by showing a lower H-Score for the normal group ($Md n = 123.98$) than the negative control group ($Md n = 156.23$). The expression of TNF-α for the normal...
When aspirin binds to this enzyme, phosphorylation of IκB will be prevented and therefore preventing the release of NF-κB. Whereas, we know that NF-κB is very important for regulating the transcription of chronic inflammation genes in the intestine including TNF-α. These mechanisms underly how aspirin is an anti-inflammatory compound, which supposed to reduce the expression of TNF-α. In this study, a dose of 43 mg/kgBW of aspirin was given to the laboratory DSS-induced-mice orally to reduce inflammation in the mice's colon. This was performed in order to obtain a positive comparison group in this study.

The expression of TNF-α between aspirin group and negative control group revealed that in a sample of 5 slides in the negative control group and 6 slides aspirin group, the expression of TNF-α was lower in aspirin group ($Mdn = 138.78$) than negative control group ($Mdn = 156.23$). This suggests that the aim of giving aspirin to reduce inflammation in the colon induced by DSS as a positive comparison in this study has been achieved. However, it is somewhat surprising that Mann-Whitney U-test revealed that the reduction is not statistically significant ($U = 4000, z = -2.008, p = 0.052, r = 0.61$). The reason for this might be due to the usage of non-parametric test in this study. These results may also explain the possibility that the use of aspirin doses may not be sufficient to inhibit inflammation, which was previously reported by Hermanto et al. However, if the aspirin dose in this study was increased, it would certainly not inhibit not only COX-2 but also COX-1 will cause severe side effects on the gastrointestinal tract, kidneys, and platelet aggregation in the colon of mice in this study, which results in too severe complications and its effect to reduce inflammation as a positive comparator will not be achieved. Therefore, since the reduction was still observed there, as it can be observed that the reduce mean rank of TNF-α by half of mean rank of negative control group (8.20 to 4.11), we can still have said that aim of giving aspirin to reduce inflammation in the colon induced by DSS as a positive comparison in this study has been achieved. Therefore, since the reduction was still observed there, as it can be observed that the reduced mean rank of TNF-α in the aspirin group by half of the mean rank of the negative control group (8.20 to 4.11), we can still say that aim of giving aspirin to reduce inflammation in the colon induced by DSS as a positive comparison in this study has been obtained.

In comparison with pomegranate peel extract, this research revealed that, the expression of TNF-α was higher for low of pomegranate peel ethanol extract group ($Mdn = 148.68$) than aspirin group ($Mdn = 156.23$) but the difference was not statistically significant ($U = 7000, z = -1.461, p = 0.177$). Therefore, it can be said that pomegranate peel ethanol extract is correlated with a reduction in the expression of TNF-α but should be in the right amount of dosage.

Decreased expression of TNF-α expression with the treatment of pomegranate peel ethanol extract supports evidence from earlier studies such as by Saed et al. and Park et al. It was found that pomegranate can decrease the production of ROS and TNF-α in THP-1 cells influenced by PM10. Pomegranate peel ethanol extract has high polyphenols compounds mainly ellagitannins and ellagic acid which proven to have therapeutic effects. Polyphenol is proven to be able to inhibit the expression of pro-inflammatory cytokines by modulating the production of inflammation mediators and cytokines such as transcription factor NF-κB. Inhibition of NF-κB inhibits further production of proinflammatory cytokines including TNF-α, and hence supports the result of decreased level of TNF-α.

The mechanism of NF-κB modulation by polyphenols is probably the most accepted mechanism. Ellagic acid in the pomegranate peel extract binds to p50 protein precursor, p105 protein to stimulate gene transcription. There is a hydrophobic interaction that occur when the binding of ellagic acid and p105 occurs. When the interaction is established, protein p50 will experience a formation failure and hence, NF-κB will not start the gene transcription process.

Another objective of this research is to compare of the expression of TNF-α on mice’s colonic epithelial cells induced using with Dextran Sodium Sulfate (DSS) with aspirin as a well-known anti-inflammatory agent and with pomegranate peel extract. The basis for selecting aspirin as a test animal treatment to produce a positive comparison group was because aspirin has been shown to reduce inflammation. Aspirin is a drug compound for the Non-Steroidal Anti-Inflammatory Drug (NSAID) class. Aspirin can cause irreversible inactivation of COX isoenzymes through specific serine acetylation, namely Ser516 for COX-2, COX-2, acetylated, is unable to form PGG-2 from arachidonic acid but produces 15R-HETE arachidonic acid. Aspirin also works by NF-kB inhibition. Aspirin can inhibit the activity of NF-kB through its ability to produce an interaction with a complex IκB kinase (IκK). IκB kinase (IκK) is a kinase enzyme that has more complex component.
CONCLUSION
This research showed that administration of pomegranate peel ethanol extract tends to decrease the expression level of TNF-α on mice’s colonic epithelial cells induced using Dextran Sodium Sulfate (DSS) and therefore, inhibiting inflammation and epithelial damage in IBD. A significant decrease found when pomegranate peel ethanol extract is given in a higher dose. Moreover, pomegranate peel ethanol extract with higher dose has equal effectiveness with aspirin in reducing inflammation, specifically on reducing the expression of TNF-α since the difference in both groups is insignificant. More studies should be conducted to determine the precise effects of pomegranate peel ethanol extract on other proinflammatory cytokines, especially in humans.

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REFERENCES
1. Kusmardi K, Hermanto D, Estuningytas A, Tedjo A, Priosoeryanto M. 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. Asian Journal of Pharmaceutical and Clinical Research. 2019;2019:370-371.
2. Jeong D, Kim S, Son M, Son C, Kim J, Kronbichler A, et al. Induction and maintenance treatment of inflammatory bowel disease: A comprehensive review. Autoimmunity Reviews. 2019;18(5):439-454.
3. Sýkora J, Pomahačová R, Kreslová M, Cvalínová D, Štych P, Schwarz J. Current global trends in the incidence of pediatric-onset inflammatory bowel disease. World Journal of Gastroenterology. 2018;24(25):2741-2763.
4. Konsensus Nasional Penatalaksanaan Inflammatory Bowel Disease (IBD) di Indonesia. Jakarta: Perkumpulan Gastroenterologi Indonesia 2011.p.1-2.
5. Chou J, Lai H, Chang C, Cheng K, Feng C, Chen T. Epidemiology and Clinical Outcomes of Inflammatory Bowel Disease: A Hospital-Based Study in Central Taiwan. Gastroenterology Research and Practice. 2019;10:1-8.
6. Abraham C, Medzhitov R. Interactions between the host innate immune system and microbes in inflammatory bowel disease. Gastroenterology. 2011;140:1729-1737.
7. Wang S, Liu Z, Zhang X. NF-κB signaling pathway, inflammation and colorectal cancer. Cell Mol Immunol. 2009;6:327-334.
8. Karin M. The κB kinase a bridge between inflammation and cancer. Cell Res. 2008;18:334-342.
9. Hernández-Ledesma B, Hsieh C, Lumen BO. Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. Biochem Biophys Res Comm. 2009;390(3):803-808.
10. SabinoJ, VerstocktB, VermeireS, FerranteM. Newbiologicsandsmall molecules in inflammatory bowel disease: an update. Therapeutic Advances in Gastroenterology. 2019;12:175628481985320.
11. 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;2016:39346470.
12. Lin CS, Cheifetz AS. The Use of Complementary and Alternative Medicine in Patients with Inflammatory Bowel Disease. Gastroenterology and Hepatology. 2018;14(7):415-425.
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**GRAPHICAL ABSTRACT**

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