Potential Inhibition by Phaleria macrocarpa Leaves Ethanol Extract on Ki-67 Expression in Distal Colon Mouse

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ABSTRACT
Ulcerative colitis (UC) has been an important aspect of an incurable chronic inflammatory disease over the last few decades. To find useful therapies for UC, one of which is herbal therapy, many researches have been conducted. Due to its anti-inflammatory effects, Phaleria macrocarpa (PM), an Indonesian indigenous herb, is considered to be the alternative therapy for UC. Phaleria macrocarpa Leaves Ethanol Extract (PMLEE) is then used in this research to determine its effect on UC by using Ki-67 as a marker of proliferation. PMLEE was created from dry PM content undergoing maceration. The animals were classified into six categories: normal, positive control, negative control and PMLEE group (100, 200, 300 mg/kgBW). PMLEE was then injected for 7 consecutive days into BALB/c mice that were caused by dextran sodium sulphate (DSS). DSS is used for modeling UC in the colon tissue of mice. All mice were terminated and then stained with anti-Ki-67 after their colons were extracted. Subsequently, the stained parts were analyzed with ImageJ based on the color intensity produced by the results of H-score. Based on H-score, PMLEE 300mg and 200mg has significantly decreased the expression of Ki-67 compare to the negative control (p=0.001 and p=0.01). PMLEE also has a tendency to be dose dependent based on the significant difference from PMLEE 300mg and 100mg (p=0.002). It then concludes that PMLEE is related to Ki-67 expression in cells, as it was inversely proportional in this analysis.

Key words: Mahkota Dewa (Phaleria macrocarpa), Inflamation, Dextran sodium sulphate, Ki-67.

INTRODUCTION
Over the last few years, UC has grown in significance as part of chronic incurable inflammatory diseases.1 UC pathogenesis has yet to be seen, but several causes, such as the colon environment, biology, and tissue structure problem which in the end leads to prolonged inflammation are known to be causing them.1 The prevalence of UC in South East Asia even reached 6.67/100,000 individuals.3 In Indonesia, the UC prevalence has also tripled since 1990.1 This chronic condition often demands high treatment costs, apart from having an effect on the quality of life (QoL).4 The annual average costs of care with an anti-tumor necrosis factor (TNF), typical medicine for UC, are EUR 15,775.5 If not treated, patients suffering from UC may be at risk of colectomy, or may also be at risk of death by causing colorectal cancer.6

Many researches have been carried out over the last few years in order to identify successful UC therapies. Aspirin is presently the common UC therapy.7 However, this drug has some significant side effects, starting from nausea, vomiting, hepatitis, to male infertility, and can require a variety of clinical approaches for the treatment of UC because of phenotypic variability.8 Other therapies currently under development include plant-based herbal therapeutics.8 Herbal therapy has also been researched extensively for the effectiveness, efficacy, and safety.9 Mahkota Dewa (Phaleria macrocarpa) is one of Indonesia’s most widely-associated tropical plants, of which are considered an alternative anti-inflammatory therapy.10 To date, PM literature as an anti-inflammatory treatment remains limited. In fact, there is a need for more studies on the pharmacological effects of PM. The authors then carried out an analysis of the PM extract. The extract used is known as the Phaleria macrocarpa Leaves Ethanol Extract (PMLEE). PMLEE is given to the colon tissue of mice, which has UC modeled with Dextran Sodium Sulphate (DSS). DSS is a pro-inflammatory agent generally believed to cause long-lasting inflammation. In addition, the Ki-67 protein was chosen as marker to determine PMLEE relationship with UC. This protein has a role in inflammatory process that can be assessed by immunohistochemistry.11,12 Accordingly, the effect of PMLEE on UC could be defined by the measurement of Ki-67 expression. This study can provide insights into PMLEE’s anti-inflammatory function and the basis for the development of PM as a possible alternative treatment for UC.

MATERIALS AND METHODS
Experimental animal
BALB/c mice in the Animal Laboratories, National Institute of Medicine, Research and Development, Ministry of Health, Indonesia were adapted and
studied for one week (eating, drinking, movement and body weight) prior to induction of dextran sodium sulphate (DSS). Treatment and conservation of mice in compliance with the Reference to the Care and Usage of Laboratory Animals by the Animal Care and Use Committee, including by controlling temperatures of 25°C, 12 hours of light/dark period, 55% humidity, as well as normal food and water.

**Chemical material**

Reagents used in this study include dextran sodium sulphate BM 500,000 (Sigma Aldrich), Aspirin (Bratco Inc.), sodium carboxymethylcellulose / CMC Na (Bratco Inc.), anti-Ki-67 (Abcam), formaldehyde (Bratco Inc.), ether (Bratco Inc.), xylol (Merck), absolute alcohol (Merck), 70% alcohol (Merck), paraffin solidium (Bratco Inc.).

**Plant extract preparation**

PM leaves were collected from the Traditional Medicine Crops Research Institute, Ministry of Agriculture in Indonesia. Using purified water, it was washed and then allowed to shade dry. The dried material was homogenized into fine powder and kept at room temperature. The dried material is then macerated using a process adapted from the Wilson method.19

The dried powder was saturated with 3 Liters of 70% ethanol solvent. After 24 hours, the extract was filtered using the Whatman filter paper. The residue was further extracted for 24 hours. The extract is then purified and the filtrate is extracted. The filtrate was then thickened using a rotary evaporator until it became a thick extract (16 percent moisture content). The extract obtained by maceration provided phenol with a grade of 4.4103% or 44.103 GAE/g, flavonoids with a level of 0.3429% or 3.429 mgQE/g and an IC50 of 219.716 μg/mL (moderate antioxidant intensity).19

**Study design and DSS administration**

The experimental protocols have been accepted by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia. Animals were induced with 2% DSS for 7 consecutive days, except for the normal group. Animals were classified into six categories: normal control, negative control, positive control (aspirin) and PMLEE (100, 200, 300 mg/kg BW), with each group consisting of 5 mice. Normal group received PMLEE (100, 200, 300 mg/kg BW), with each group consisting of 5 mice.

**Tissue handling and immunohistochemistry**

In this process, the distal third of colon was washed and fixed using a 10% formalin solution, embedded in the paraffin medium, sliced transversely using a 4 mm microtome, and mounted on a slide for further immunohistochemical staining (IHC). The IHC was performed by deparaffinization, rehydration, antigen retrieval, and blocking. The slides were then incubated with anti-Ki-67 antibodies in phosphate buffer solution (PBS) for 2 hours at room temperature, then visualized with 3,3’-diaminobenzidine (DAB) for 10 minutes.20 The sample sections were dipped in Lilie Mayer haematoxylin solution as counterstain for 2 minutes and then rinsed again with water.13 Next, the sections were dipped in lithium carbonate for 60 seconds and then rinsed. Slides were dehydrated using ethanol and xylol clearing. Finally, the pieces were covered by a liquid mask, which is an aqueous mounting medium. Subsequently, the stained slides were analyzed for histopathological change.

Quantiﬁcation of KI-67 expression

Each slide was examined using a light microscope with high power field (400x magnification) and filmed using a Leica LAZ EZ software computer and a camera combined with the Leica DM750 microscope. Photographs were taken randomly for a limit of five visual fields per slide. The brown color intensity was then measured using the plugin software in Image J. IHC profiler, which quantifies the color intensity in the image. The quantification results are in the form of the H score.

**Statistical analysis**

All data are displayed in mean ± standard deviation. Data were evaluated using variance analysis (ANOVA) in SPSS 20.0 followed by Tamhane’s Post Hoc test to compare the variations between treatments. Differences of p<0.05 shall be deemed statistically significant.

**RESULTS AND DISCUSSIONS**

Qualitatively, the expression of Ki-67 can be shown by measuring the intensity of brown color in each field. The expression Ki-67 is represented by the brown color intensity of the cell cytoplasm. The variation in brown color intensity in the colon epithelial cytoplasm cells can be seen in Figure 1 (a-f). Figure 1(a) has a neutral color intensity. In comparison, Figures 1(c) and (d) have a high intensity. In the meanwhile, Figures 1(b), (e) and (f) have approximately the same intensity of brown colour. The results of this color intensity are grouped together and statistically analyzed by Imagej and SPSS.

Quantification of all Ki-67 expression in the images above are assessed by calculating the sum and average of Histo Score (H-score)21 for group. The results can be seen as in Table 1. All data from Table 1 have normal distribution based on the Shapiro-Wilk normality test (p = 0.337). However, based on the Levene’s test the data groups are not homogeneous (p < 0.001). Because the data groups were normally distributed but inhomogeneous, One Way ANOVA statistical analysis was carried out and continued with the post hoc Tamhane Test. ANOVA showed significant results (p<0.001). Meanwhile, Tamhane’s test results showed there were significant differences between positive control and negative control (p = 0.015), PMLEE 200mg and negative control (p = 0.01), PMLEE 300mg and negative control (p = 0.001), PMLEE 100mg and PMLEE 300 mg (p = 0.002) as shown in figure 2.

**Ulcerative colitis model with DSS and aspirin**

UC is a chronic inflammation that occurs in the gastrointestinal tract. In this study, UC was carried out by DSS administration. The effects of DSS administration can mainly be seen in the negative control group. The negative control group also showed a significant difference with the positive control group (p = 0.015). This shows that the administration of DSS and aspirin each succeeded in providing a good model.

The DSS administration can cause chronic inflammation, which is determined by several factors.22 Administratice dose is one of the reasons involved. The suggested dosage of DSS is 1.5%-3%.23 In this study, the authors used a 2% dose. In addition, length of treatment and frequency of DSS administration are also contributing factors.23

**Table 1: H-Scores in each group.**

| Group       | N  | H-Score (%) | CI 95% |
|-------------|----|-------------|-------|
| Normal      | 5  | 148.50      | 31.39 | 109.5 - 187.5 |
| Positive control | 5 | 179.49      | 10.34 | 166.7 - 192.3 |
| Negative control | 5 | 213.68      | 11.12 | 199.9 - 227.5 |
| PMLEE 100mg | 5  | 186.51      | 3.90  | 181.7 - 191.4 |
| PMLEE 200mg | 5  | 176.11      | 10.99 | 162.5 - 189.8 |
| PMLEE 300mg | 5  | 156.89      | 6.77  | 148.5 - 165.3 |
Figure 1: Expression of Ki-67 on Mice Colonic Epithelial Cell with 400X Magnification. (a) normal; (b) positive control; (c) negative control; (d) PMLEE 100 mg; (e) PMLEE 200 mg; (f) PMLEE 300 mg.

Figure 2: Mean Difference between Groups based on Tamhane's Test. A= normal; B= positive control; C= negative control; D= PMLEE 100 mg; E= PMLEE 200 mg; F= PMLEE 300 mg. *p<0.05; **p≤0.01; ***p≤0.001.
The prescribed duration and frequency is 5-10 days with 4-5 repeat cycles.17,18 The strain of Mice is also one of the factors that affect the efficacy of DSS. In fact, according to research by Mähler et al, C3H1/HeJ, C57BL/6, and BALB/c have a strong ulcerative effect.19 This is consistent with the research performed by Vetsushi et al, which found that the proliferation index of these strains increased by 40-60 folds relative to normal mice prior to DSS administration.20

Aspirin has been known to suppress the mechanism of inflammation. As with Amalia et al, administration of aspirin at a dosage of 150 mg/kg for 4 weeks decreased the occurrence of dysplasia in the colon tissue.21 The same results are also shown in this study. The results showed a significant difference between the negative and positive control groups (p = 0.015). This difference indicates that aspirin given to positive controls has significantly decreased Ki-67 expressions compared with negative controls.

Surprisingly, the normal group did not show significant differences with either positive or negative controls. This can happen for several reasons, mainly because of variability. SD of the normal group reached 31.39 and significant Levene's test results (p < 0.001) showed possible variability due to outlier values.22 These outlier values might occur due to several factors, ranging from staining the preparation, image capture, to quantification with the results of Ki-67 expression.23 Based on the study of Kim et al, Ki-67 are more vulnerable to ischemia, so over fixation can cause irreversible damage to some epitopes.24 In addition, the scanned images of the samples must also be in a good quality. This is where the pathologist needs to do its role to ensure that each scanned photo meets a quality standard that will allow collection of meaningful and reproducible data before being processed by ImageJ.25,26 In addition to human error factors, this inhomogeneous data may also be due to system errors.27 Therefore, a result of the system sometimes requires validation from a pathologist.28

**Effect of PMLEE on Ki-67 expression**

The effect of PMLEE on the Ki-67 expression showed significant results. This can be seen in the result of Ki-67 expression between negative control and PMLEE 200mg (p = 0.01) and also between negative control and PMLEE 300 mg (p = 0.001). These results indicate that PMLEE is able to significantly reduce Ki-67 expression. The relationship between PMLEE and Ki-67 is inseparable from the compounds present in PM. The therapeutic effects of this plant have previously been discussed in several studies, especially in terms of anti-inflammatory effects. In fact, the study of Mariani et al states due to its affinity with the keto-protein receptor, the compound in PM, hydroxyl benzophenone glucoside, has minor anti-inflammatory activity.29 However, based on the study of Diamenti et al, benzophenone glucoside was not able to significantly reduce the proliferation of human cervical cancer cells.30 This suggests that the decrease in the expression of Ki-67, as a proliferation biomarker, may not be related to the activity of benzophenone glucoside.31 Besides benzophenone glucoside, it is often considered that other compounds such as flavonoids have anti-inflammatory effects.32 In fact, according to the study of Shaikh et al, flavonoids have a high suppression effect on the expression of Ki-67.33 One type of flavonoid, epigallocatechin gallate, is known to inhibit Ki-67 in (PRB)-E2F/DP pathway.34 The fact that PM in this study comprises flavonoids with levels of 0.3429% or 3.429 mgQE/g from the maceration phase further emphasizes this finding. The form of flavonoid found in PM, kaempferol, was thought to be one of the factors that influenced the decrease in Ki-67 expression, according to Kusmardi et al.35 This is supported by the study of Qin et al which showed a decrease in the expression of Ki-67 in cholangiocarcinoma cells treated with kaempferol.36

Besides showing a relationship with the expression of Ki-67, PMLEE appears to be dose-dependent as well. This can be seen in the significant difference in Ki-67 expression (p = 0.002) between the PMLEE 100 mg and the PMLEE 300mg. In fact, the expression of Ki-67, as seen in Figure 2, is inversely proportional to the dose of PMLEE. This can occur in one of them due to the activity of the compound phalerin and gallic acid in PM, according to the Altaf et al report.37 In a dose-dependent manner, the compound is thought to increase the development of BAX protein while down-regulating the Bcl-2 mRNA expression.38

**CONCLUSION**

PM is a medicinal plant which has been extensively studied as having anti-inflammatory effects. This effect was also related to the expression of Ki-67 in cells as in this study it was inversely proportional. The relationship between PM and Ki-67 still needs to be investigated. In addition, the dose of PM is also a determining factor for Ki-67 expression. The dose dependent characteristics of this plant on Ki-67 expression also need further research. Research related to the use of other inflammatory markers on UC, beside Ki-67, need to be done in order to understand anti-inflammatory properties of PM.

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**GRAPHICAL ABSTRACT**

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