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Original Article

Potential of *Phaleria macrocarpa* Leaves Ethanol Extract to Upregulate the Expression of Caspase-3 in Mouse Distal Colon after Dextran Sodium Sulphate Induction

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ABSTRACT

Ulcerative colitis (UC) is a part of incurable chronic inflammatory disease that has gained importance over the past few decades. A lot of research has been done to find effective treatments for UC, one of which is herbal medicine. *Phaleria macrocarpa (PM*), an Indonesian native plant, is thought to be an alternative therapy for UC because of its anti-inflammatory properties. Therefore, in this research, *Phaleria macrocarpa* Leaves Ethanol Extract (PMLEE) is used to assess its effect on UC by using Caspase-3 as apoptosis marker. PMLEE was made from dried material of *PM* that undergo maceration. Animals were separated into six groups: normal, negative control, positive control, and PMLEE groups (100, 200, 300 mg/kgBW). PMLEE was then injected to BALB/c mice that have been induced by dextran sodium sulphate (DSS) for 7 consecutive days. DSS is used to model UC in mice colon tissue. All animals were sacrificed and their colons were collected then stained with anti-Caspase-3. The stained sections were subsequently examined with ImageJ based on color intensity which generated H-Score as the results. Based on H-Score of each group, PMLEE 300mg has significantly upregulate the expression of Caspase-3 compare to the negative control (p=0.015). PMLEE also has a tendency to be dose dependent based on the significant difference between PMLEE doses. Therefore, it concludes that PMLEE is able to upregulate the expression of Caspase-3 in colon cells as in this study it was directly proportional.

Keywords: Mahkota Dewa, Inflammation, Apoptosis, Ulcerative colitis.

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INTRODUCTION

UC has gained importance over the past few decades which is a part of incurable chronic inflammatory disease.[1] The pathogenesis of UC is not yet clear, but it is thought to be caused by various factors, such as the condition of the colon environment, genetics, or problem in tissue structure that ultimately causes prolonged inflammation and cell proliferation.[1] The incidence and the prevalence of UC in Southeast Asia each even reached 0.68 cases per 100,000 individuals/year and 6.67/100,000 individuals.[2] The prevalence of UC in Indonesia has even tripled from 1990.[3] This chronic disease, aside from having an effect on Quality of Life (QoL)[4], also requires high medical cost. In fact, the mean annual cost per patient reaches € 15,775 for treatment with anti-tumor necrosis factor (anti-TNF) which is a common drug to treat UC.[5] If not treated, patients with UC can increase the risk of colectomy or can even cause colorectal cancer which increases the risk of death.[1,6]

Over the past few decades, a lot of research has been done to find effective treatments for UC. Currently, the common therapy for UC is aspirin administration.[7] However, this drug has several severe side effects, such as nausea, vomiting, headaches, hepatitis, and male infertility.[7] In addition, due to phenotypic variability, different varieties of therapeutic modalities can involve in UC treatment.[4] Other therapies that are currently being developed are herbal therapies derived from plants.[8] In addition for being natural and inexpensive, herbal medicine therapies have been extensively investigated for their efficacy and safety.[9] One of Indonesia's native plants that is widely associated as an anti-inflammation is Mahkota Dewa (*Phaleria macrocarpa*).[10] Its role as an anti-inflammatory is thought to be able to be an alternative therapy for UC.

Until now, research on the development of *PM* as an anti-inflammatory therapy is still very minimal. In fact, research on the pharmacological effects of the *PM* remains to be developed further. Therefore, the authors conducted a study of the *PM* that have been made in the form of an extract. The extract used is *Phaleria macrocarpa* Leaves Ethanol Extract (PMLEE). PMLEE is administered into mice's colon tissue that has been modelled UC using Dextran Sodium Sulphate (DSS). DSS is a proinflammatory agent that widely known to cause prolonged inflammation and cell proliferation. Furthermore, to assess its relationship with UC, the Caspase-3 protein was chosen as an apoptosis marker. Caspase-3 is a tissue biomarker associated with the apoptosis process, especially in UC.[11,12] Caspase-3 would induce apoptosis hence would decrease cell proliferation.[12,13] Inducing apoptosis and decreasing cell proliferation

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are the key factor to stabilize inflammation process in UC which will prevent progression to cancer. Therefore, the effect of PMLEE on UC can be assess by the Caspase-3 expression level as a histopathological marker. This research is expected to be able to provide insight into the mechanism of PMLEE in regulating inflammation, as well as the basis for developing *PM* as a potential alternative therapy for UC.

MATERIALS AND METHODS

Experimental animal

BALB/c mice in the Animal Laboratories, National Institute of Health, Research and Development, Ministry of Health in Indonesia were adapted and observed for one week (eating, drinking, movement, and body weight) before induction of dextran sodium sulphate (DSS). The treatment and maintenance of mice in accordance with the Guide for the Care and Use of Laboratory Animals by the Animal Care and Use Committee, namely by monitoring the temperature of 25°C, 12 hours of light/dark cycle, 55% humidity, as well as standard food and drink.

Chemical material

Reagents used in this study include dextran sodium sulphate BM 500,000 (Sigma Aldrich), Aspirin (Brataco Inc.), sodium carboxymethylcellulose / CMC Na (Brataco Inc.), anti-Caspase-3 (Abcam), formaldehyde (Brataco Inc.), ether (Brataco Inc.), xylol (Merck), absolute alcohol (Merck), 70% alcohol (Merck), paraffin solidum (Brataco Inc.).

Plant extract preparation

Leaves of *PM* was obtained from Traditional Medicine Crops Research Institute, Ministry of Agriculture in Indonesia. Using distilled water, it was washed then allowed to shade dry. The dried material was homogenized into fine powder and then stored at room temperature. The dried material then undergo maceration using method adapted from the method of Wilson.[14]

The fine dried powder (1000g) was soaked with 3 L 70% ethanol solvent. After 24 hours, the extract was filtered through using Whatman filter paper no.1 to take the filtrate. The remaining residue was extracted further for 24 hours. The extract is then filtered and the filtrate is taken. The filtrate then underwent thickening process using rotary evaporator until it becomes a thick extract (16% moisture content). The extract obtained by maceration produced phenol with a grade of 4.4103% or 44.103 GAE/g and flavonoids with levels of 0.3429% or 3.429 mgQE/g, and have IC_{50} 219.716 µg/mL (moderate antioxidant intensity).[14]

Study design and DSS administration

The experimental protocols were approved by the Ethics Committee of Faculty of Medicine, Universitas Indonesia. The animals were induced (2% in drinking water) with DSS for 7 consecutive days, except the normal control group. Animals were separated into six groups (n = 5 per group): normal control, negative control, positive control (aspirin), and PMLEE groups (100, 200, 300 mg/kg body weight). Normal control group received 0.9% sterile saline and do not receive an oral exposure of DSS.[14] Animals were randomized and acclimated one week before inducing by DSS 2%. After induce by DSS for 7 consecutive days, positive control group received aspirin solution and PMLEE group received PMLEE (100, 200, 300 mg) by oral administration daily for 2 weeks. In the end of treatment, all animals were sacrificed and the colons were collected.

Tissue handling and immunohistochemistry

In this process, distal third of the colon was washed and fixed using 10% formalin solution, implanted into the paraffin medium, cut transversely using a microtome 4 mm thick, and put on slide for further immunohistochemical staining (IHC). IHC procedure was carried out by deparaffinization, rehydration, antigen retrieval, and blocking. Then, the sections were incubated with anti-Caspase-3 antibodies in phosphate buffer solution (PBS) for 2 hours at room temperature then visualized using 3,3'-diaminobenzidine (DAB) for 10 minutes.[14,15] The sample sections were dipped in a Lilie Mayer haematoxylin solution (counterstain) for 1-2 minutes then rinsed again with water.[16] Next, the sections were dipped in lithium carbonate for 60 seconds then rinsed. The sections were dehydrated using ethanol and clearing with xylol. Finally, the sections were covered using liquid cover, which is aqueous mounting media. The stained sections were subsequently examined for histopathological change.

Quantification of caspase-3 expression

Each preparation was observed using a light microscope at a total magnification of 400X and documented using a computer with Leica LAZ EZ software and a camera that had been integrated with Leica DM750 microscope. Photographs were taken randomly with a total of five visual fields per one preparation. Then, the brown color intensity was calculated using the plugin program in Image J, IHC profiler, which will quantify the color intensity af an image. The results of quantification were converted into H-Score based on the formula. H-Score = (%low positive x 1) + (%positive x 2) + (%high positive x 3).

Statistical analysis

All data are presented in mean ± standard deviation. Data were analysed using analysed of variance (ANOVA) in SPSS 20.0 then followed by Tukey's Post Hoc test to compare the differences between treatments. Differences of p<0,05 are considered statistically significant.

RESULTS AND DISCUSSIONS

Qualitatively, the expression of Caspase-3 can be seen by comparing the brown colour intensity in each field of view. The Caspase-3 expression is depicted by the brown colour intensity in the cytoplasm of cells. In Figure 1 (a-f) can be seen the difference of brown colour intensity in the colon epithelial cells cytoplasm. Figure 1(a) and 1(b) has somewhat very low brown colour intensity. In contrast, Fig. 1(c) and (f) have a high brown colour intensity. Meanwhile, Fig. 1(d) and (e) have somewhat moderate brown colour intensity. All results of this colour intensity are grouped and statistically tested using ImageJ and SPSS respectively.

Quantification of all Caspase-3 expression in the images above are assessed by calculating the sum and average of Histo Score (H-Score)[17] 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= 0.845). Moreover, based on the Levene's test the data groups are homogeneous (p = 0.494). Because the data groups were normally distributed and homogeneous, One Way ANOVA statistical analysis was carried out and continued with the post hoc Tukey Test. ANOVA showed significant results (p < 0.001). Meanwhile, Tukey's test results showed there were significant differences between PMLEE 300mg and PMLEE 200mg (p = 0.049), PMLEE 300mg and PMLEE 100mg (p = 0.009), positive control and PMLEE 200mg (p = 0.038), positive control and PMLEE 100mg (p = 0.007), negative control and PMLEE 300mg (p = 0.015), negative control and positive control (p = 0.011), normal and PMLEE 300mg (p <0.001), normal and positive control (p<0.001) as seen in Figure 2 (right).

Ulcerative colitis model with DSS and Aspirin

UC is a damage in colon tissue that occurs in the gastrointestinal tract and ultimately cause by apoptosis and inflammation.[18] In this study, DSS, a well-known proinflammatory agent is used to model UC.[19] The administration of DSS in mice can cause colonic tissue damage due to apoptosis reduction, cell proliferation enhancement, and pro-inflammatory mechanism. DSS-induced cell proliferation will cause

Table 1: H-Scores in Each group.

Group	N	H-Score (%)		CI 95%
		Mean	SD	
Normal	5	176.87	5.57	161.4 – 192.33
Negative control	5	190.94	5.47	175.29 – 205.66
Positive control	5	216.94	5.46	201.79 – 232.1
PMLEE 100mg	5	188.87	5.66	173.14 – 204.6
PMLEE 200mg	5	194.24	4.54	181.64 – 206.85
PMLEE 300mg	5	216.12	2.5	209.17 – 223.06

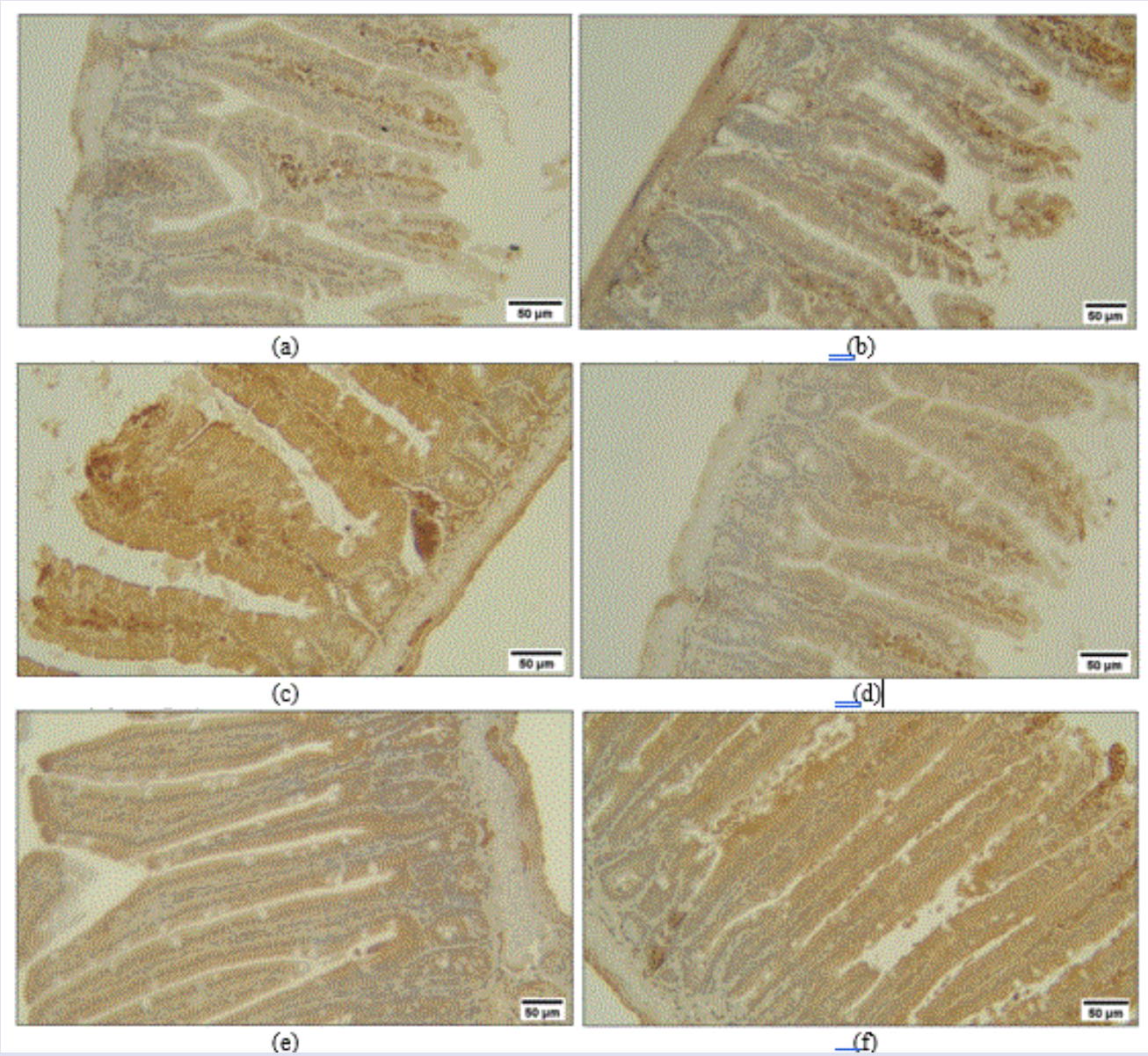

Figure 1: Expression of Caspase-3 on Mice Colonic Epithelial Cell with 400X Magnification. (a) normal; (b) negative control; (c) positive control; (d) PMLEE 100mg; (e) PMLEE 200mg; (f) PMLEE 300mg.

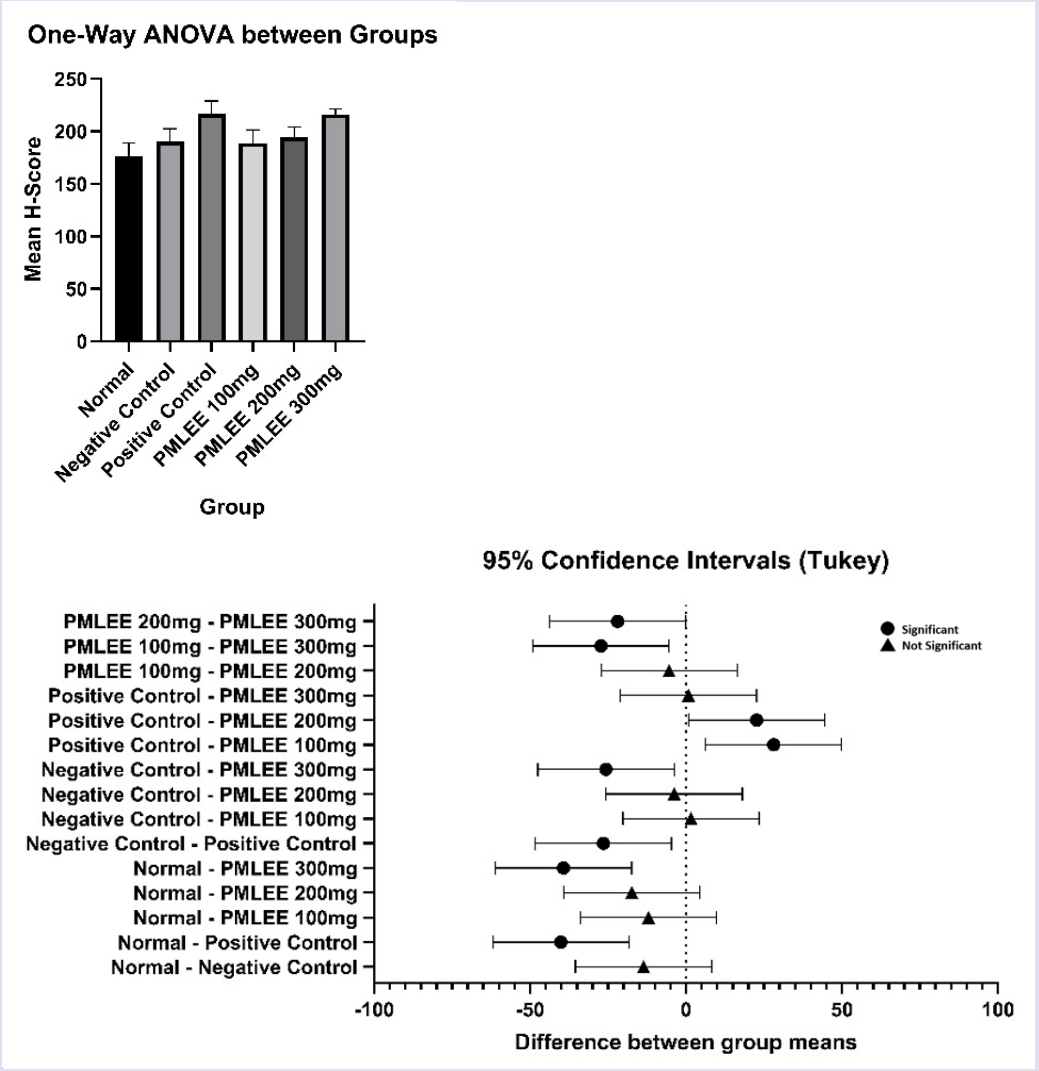

Figure 2: One-way ANOVA between groups (left) and the multiple comparison Tukey test (right). If an interval does not contain zero, the corresponding mean are significantly different.

instability in colonic tissue structure.[20] Normally, this is followed by an increase in apoptosis as an attempt to balance the number of colonic epithelial cells. Unfortunately, DSS also decrease cell apoptosis thus enhancing cell proliferation.[21] In UC, this event would cause cancer progression.[22] In this study, the effects of DSS administration, as already been explained above, can be seen in the negative control group. Based on Figure 2, even though not significant, expression of Caspase-3 in negative control is higher than normal group. This indicate that group given DSS was able to show an increase in Caspase-3 expression as an attempt to balance the number of colonic epithelial cells. The role of DSS to reduce Caspase 3 caused the increase in caspase 3 expression in the negative control group was not optimal. This may be the reason for the insignificance difference between normal and negative control. Besides the internal factor, DSS effects on the expression of Caspase-3 determined by some factors, including dose, duration, and mice strain. The recommended DSS dose is 1.5% -3%.[23] In this study, the authors used a 2% dose. In addition, duration of therapy and frequency of DSS administration are also contributing factors.[24] The recommended duration and frequency is 5-10 days with 4-5 repeating cycles.[24] Mice's strain is also one of the factors that influence the effectiveness of DSS. In fact, according to the research of Mähler et al, mice with strains of C3H/HeJ, C57BL/6, and BALB/c (used in this study) have a high ulceration effect.[25] This is consistent with the study of Vetuschi et al which showed that the proliferation index of mice with DSS increased 40-60 folds compared to normal mice.[26]

After being modeled by DSS, the main problem of UC was clearly seen in cell proliferation which was not accompanied by sufficient amount of cell apoptosis. Therefore, therapy with the aim of reducing proliferation and enhancing apoptosis is important. This therapy is expected to be able to control inflammation in UC and prevent cancer progression. In this study, aspirin used as positive control. Aspirin has been known to inhibit the inflammatory process as well as increase apoptotic effect.[27] As in Amalia et al, administration of aspirin at a dose of 150 mg/kg for 4 weeks reduced the incidence of dysplasia in the colon tissue.[28] Aspirin reduce the risk for cancer, due to their antiproliferative and apoptosis-inducing effects that involves the release of cytochrome c from mitochondria, which then interacts with Apaf-1 to activate caspase proteases that orchestrate cell death.[29] In this study, the effect of aspirin administration can be seen in the positive control group. Based on Figure 2, positive control shows significant difference with both normal and negative control ($p < 0.001$ and $p = 0.011$). This indicate

that aspirin could significantly increase Caspase-3 expression compare with normal and negative control. This finding is consistent with the study of Ding et al which show that myeloma cells exposed to aspirin treatment displayed concentration-dependent apoptosis, which was closely associated with activation of caspases, upregulation of Bax, and downregulation of Bcl-2 and VEGF.[30]

Effect of PMLEE on caspase-3 expression

The effect of PMLEE on the Caspase-3 expression showed significant results. This can be seen in the result of Caspase-3 expression between all three PMLEE groups. Different dose of PMLEE show different relations with other groups. The PMLEE group that has the most relationships with other groups in this study is PMLEE 300mg. Based on Figure 2, there is a significant difference between the Caspase-3 expression on PMLEE 300mg with both normal and negative control ($p = < 0.001$ and $p = 0.015$). This indicate that PMLEE given at 300 mg could increase the expression on Caspase-3 significantly compare to normal and negative control. This finding is strengthened by the fact that there is no significant difference between PMLEE 300mg with positive control. This indicate that the capability of PMLEE 300mg to increase expression of Caspase-3 is as strong as the aspirin. This effect may occur due to some of several compound in *PM*.

Previously, several studies have discussed the therapeutic effects of this plant related to its compound. In fact, the study of Mariani et al. states that the compound in *PM*, hydroxyl benzophenone glucoside, has slight anti-inflammatory activity due to its affinity with the ketoprofen receptor.[31] This study is also strengthened by Zhu et al. that show benzophenone has strong activity in inducing cellular apoptosis in colon cancer cells based on flow cytometry analysis.[32] Besides benzophenone glucoside, other compounds such as flavonoids are also considered to have effects with expression of Caspase 3.[33] In fact, according to Das et al, flavonoids are potential therapeutic agents for induction of apoptosis in human glioblastoma cells.[34] This statement is further strengthened by the fact that *PM* in this study contains flavonoids with levels of 0.3429% or 3.429 mgQE/g from the maceration process. According to Kusmardi et al, the type of flavonoid contained in *PM*, kaempferol, was thought to be one of the factors that upregulate Caspase-3 expression.[35] Henry et al also state that kaempferol-induced apoptosis is related to its ability to change the expression of apoptotic markers, such as caspase-3 (caspase-dependent) and AIF (caspase-independent).[36] In conjunction with apoptosis, Kaempherol increases the levels of pro-apoptotic enzymes and proteins, such as cleaved caspase-9, -7, -3, p21, p53, Bax, PARP, and p-ATM and decreased the levels of anti-apoptotic proteins Bcl2, polo-like kinase 1 (PLK-1), and pAKT.[37]

In contrast to PMLEE 300mg, other PMLEE dose, PMLEE 200mg and 100mg, show no significant difference with neither normal or negative control. In fact, PMLEE 200mg and 100mg instead show significant difference with both positive control and PMLEE 300mg. This indicate that PMLEE given at 200mg and 100mg could not decrease expression of Caspase-3 as low as aspirin and PMLEE 300mg. These interesting findings indicate that the effect of PMLEE on Caspase-3 expression has a tendency to be dose dependent. In fact, as seen in Figure 2, the expression of Caspase-3 is directly proportional to the dose of PMLEE. According to the study of Altaf et al, this can occur one of them because of the activity of the compound phalerin and gallic acid in *PM*. The compound is thought to increase BAX protein production in a dose dependent manner while down-regulating the expression of Bcl-2 mRNA.[38]

CONCLUSSION

PM is an herbal plant that has been widely studied as having anti-inflammatory effects. Based on H-Score of each group, PMLEE 300mg significantly upregulate the expression of Caspase-3 compare to the negative control. PMLEE also has a tendency to be dose dependent based on the significant differences between PMLEE doses. Therefore, it concludes that PMLEE is able to upregulate the expression of Caspase-3 in colon cells as in this study it is directly proportional.

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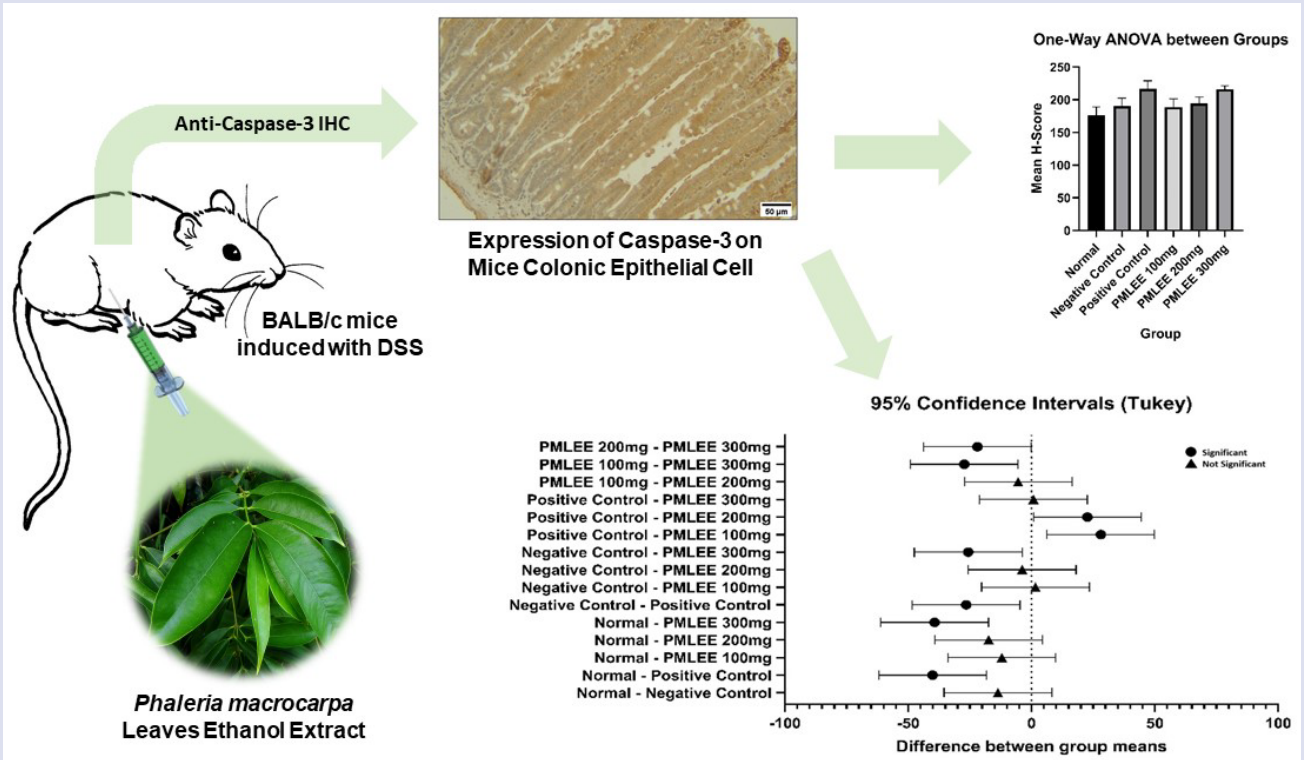
GRAPHICAL ABSTRACT



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