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Polyphenol Compounds of *Mahkota Dewa* (*Phaleria macrocarpa* [Scheff.] Boerl) Up-regulated Caspase-3 and Apoptosis Index in Balb/c Strain Mice

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ABSTRACT

Background: Polyphenol compounds of *Mahkota Dewa* (*Phaleria macrocarpa* [Scheff.] Boerl (PMD) can potentially be used as anticancer treatment by scavenging radical molecules. The effect *in vivo* is still limited to Indonesia.

Purpose: This research was aimed to validate the activity of PMD in increasing caspase-3 expression and apoptosis in Balb/c mice, induced by Benzo(a)pyrene (BaP).

Methods: A post test control group was implemented and used by 40 Balb/c mice at the age of 1-2 weeks, with the body weight of 20-30 g. The tumor induction was administered to the mice using BaP. The animals were randomized into two groups called the control group and the PMD treatment group, the latter of which was given a dosage of 50mg. Lung tumor growth was assessed through surgery at week 8, 17, and 26. The results of caspase-3 expression and apoptotic index from IHC-TUNEL staining were analyzed using Kruskal-Wallis, Mann-Whitney, One-way ANOVA, and Post hoc test LSD with significant levels of $p < \alpha$ (0,05). This research was approved by Ethical Clearance.

Results: Oral administration of 50mg PMD significantly increased caspase-3 expression and apoptotic index in the treatment group animals at weeks 8, 17, and 26. Carcinogenesis incidence in the control group were respectively found at $2,32 \pm 0,26$ and $3,93 \pm 0,46$ at weeks 8 and 26, while those of the treatment group were $1,88 \pm 0,38$ and $0,88 \pm 0,22$ ($p=0,001$). The apoptotic index in the control group was $0,00 \pm 0,00$ at 8 weeks and $0,92 \pm 0,22$ at 26 weeks, whereas the indexes of the treatment group were $1,12 \pm 0,71$ and $2,02 \pm 1,05$ ($p=0,001$). In the control group, the caspase-3 expression at weeks 8 and 26 were $0,28 \pm 0,17$ and $0,56 \pm 0,16$, while those in the treatment group were $0,60 \pm 0,14$ at week 8 and $2,52 \pm 0,33$ at week 26 ($p=0,001$).

Conclusion: The treatment of PMD effectively induced cell apoptosis in the Balb/c mice via up-

regulation of the caspase-3 expression, thereby increasing the apoptotic index. This shows that PMD has anticancer abilities to inhibit lung carcinogenesis in Balb/c mice.

Keywords: polyphenol, mahkota dewa (*Phaleria macrocarpa* [Scheff.] Boerl), cell proliferation, apoptotic index, Balb/c strain mice, Benzo(a)pyrene (BaP).

INTRODUCTION

As seen within this epidemiology study, lung cancer is the most threatening cancer type in the world when compared to prostate, breast and colorectal cancer [1]. The overall survival of lung cancer patients is 15% within 5 years. However, these statistics can be even worse when diagnosed at an advanced stage of this cancer [2]. Surgery and chemotherapy, alone or in combination with radiotherapy, are the therapeutic choices for lung cancer patients [3]. Unfortunately, at the advanced stages of lung cancer, these types of therapy are less effective in prolonging the survival rate in patients [4]. A study by Syahrudin E, *et al* (2005) reported that overall survival and therapeutic response in various chemotherapy modalities demonstrated similar results [5]. Similarly, Masuda N, *et al* (2000) revealed that in non-small cell lung cancer (NSCLC), chemotherapy resulted in a good response, whereas in small cell lung cancer (SCLC), the therapeutic response was dependent on the individual patient [6]. Consequently, current novel strategies to suppress lung cancer focus on preventive work.

Apoptosis is an integral molecular process, involving death receptor and mitochondrial pathway [7,8]. It employs receptor-ligand proteins (*i.e.* FasL/CD95L with Fas/CD95) [9], regulator proteins (*i.e.* Bcl-2, Bcl-xL, Bax, Apaf-1, PUMA, Noxa, Smac-Diablo) [10, 11], and several proteases that play a crucial role in the apoptotic process, including caspase 9, 8, 7, 6 and 3 [12, 13]. The apoptotic mitochondrial pathway is initiated by the activation of the tumor suppressor protein p53, an essential regulator for cellular stress [2]. The p53 activation will be accompanied by up-regulation of pro-apoptosis proteins (Bax, Bad, Bid, PUMA, Apaf-1) and/or down-regulation of anti-apoptosis protein (Bcl-2, Bcl-xL) [14] as a response to DNA damage [15] which induces cytochrome c release from mitochondria [16]. The cytochrome c release will trigger caspase activation, thereby inducing the apoptotic process [13, 17]. In contrast, the death receptor pathway is induced by the death receptor's activation on the cell surface, such as *Tumor Necrosis Factor* (TNF) and Fas ligand (FasL) [14]. Active FasL recruits intracellular *Fas-associated death domain* (FADD) to form a complex with procaspase-8 [18, 19]. Procaspase-8 transactivation produces active caspase-8 that directly cleaves procaspase-3 into caspase-3. After subsequent activation, apoptosis will occur or induce more activation signal via Bid cleavage [20]. Moreover, according to Owen-Schaub LB, *et al* (2005), the importance of p53 is that it can induce activation in both the mitochondrial and death receptor pathways, thereby activating caspase-8 [21].

The caspase protein plays crucial roles in apoptosis signaling and execution [22]. Reduced caspase expression influences apoptosis process and disturbs cell proliferation [23]. Caspase-3 was found in low grades of malignancy, which can be an indicator for a good prognosis with lung carcinoma [24, 25]. However, high caspase-3 expression is related to a worse prognosis in resected NSCLC.

Mahkota dewa (MD) (*Phaleria macrocarpa* [Scheff.] Boerl is one of the Indonesian traditional herbal plants reported to have anti-cancer activity. Its activity is hypothesized to be related to polyphenol compounds. Based on phytochemistry analysis of MD extract, polyphenol was one of the abundant compounds found in MD. However, the activity of polyphenol compounds from MD (PMD) to modulate lung cancer cell apoptosis *in vivo* has not been explored. Thus, the current study aims to investigate the activity of PMD in the *in vivo* lung cancer model.

MATERIAL AND METHODS

Carcinogenic model [26,27]: Every Balb/c mouse at the age of 24-48 hours was subcutaneously injected at sub-scapula region with 0.02 ml suspension containing 0.2 mg in 1% Benzo (A) Pyrene (BaP) solution (single dose). A carcinogen was used during an hour period after emulsification. Then, mice were raised together with their mother, supplemented with water and food *ad libitum*. At the average time of 4 weeks, male and female mice were then separated and divided into two groups.

The first group was the carcinogenic control, which were not given the oral PMD, but injected only with BaP up to the 26th week. In contrast, the second group was the PMD treatment group was given PMD orally by dosage of 50mg/mouse/day starting from the 5th week of BaP injections until the 26th week. At weeks 8, 17, and 26, parameters for both groups were determined. Each group consisted of 30 animals, and 15 of them were sacrificed at each time point for histopathology and immunohistochemistry analysis.

Experimental design: Treatment of PMD began at week 5 after a BaP injection with the dosage of 50mg in each mouse, every day, and continuously up to the 26th week. This research was approved by Ethical Clearance. Animals were determined by the following the guidelines from the Ethic Commission, Komisi Etik Penelitian Kesehatan (KEPK) Faculty of Medicine, Diponegoro University dan Dr. Kariadi Hospital, Semarang (No. Reg: 38/EC/FK/RSDK/2010).

Histopathology evaluation of the lung [27]: Histopathology evaluation was conducted using five lobus of the lung. Each lobus was washed using phosphate buffered saline (PBS) and placed on in blotting paper to remove remaining blood. The lung tissues were fixed in 10% normal formalin buffer for 24 hours in dehydrated and graded ethanol concentration, washed using xylene, and finally put in paraffin. The five lung lobus were cut, put on a slide and stained. Four serial region was used for *hematoxylin-eosin* (HE) staining, microscopic evaluation under light microscope, histopathology evaluation, AgNORs expression analysis, and apoptotic index (AI).

Briefly, HE staining was completed by putting the tissue section on a slide, soaked in xylol for 5 minutes; xylol II for 5 minutes; washed with graded alcohol for 3x2 minutes, aquadest for 2 minutes; soaked in HE Lillie-Mayer for 5 minutes; washed in flowing water for 2 minutes, 0.4% acidic alcohol for 3x, flowing water for 2 minutes, saturated Lithium carbonate for 3 x, flowing water for 2 minutes, 50% alcohol for 3x2 minutes, 70% alcohol, eosin, 70% alcohol 3x, carbon xylol, xylol 3x, xylol for 20 minutes, Canadian balsam; and at the end, cover with glass slide.

Immunohistochemistry: Immunohistochemistry staining was completed from lung tissue section according to protocol by Mohan KPVC, and et al. [28,29]. The tissue section of the slide

was fixed using formalin for 20 minutes. After this, it was washed with Phosphate-buffered saline (PBS), incubated at -20°C for 5 minutes and followed by acetone for 3-5 minutes, and then washed again using PBS. Prior to the blocking step, H_2O_2 was applied at 5°C for 15 minutes. Non-specific antibody binding was blocked using normal serum for 10 minutes. The Caspase-3 expression in the tissue was detected by monoclonal antibodies. Incubation using primary antibodies was conducted for an hour and secondary antibodies for 30 minutes. To visualize the protein expression in tissue, peroxidase was applied for 30 seconds and diaminobenzidine (DAB) was added for 3-5 minutes. Tissue section was counterstained using Mayer Hematoxylin Solution for 4 minutes, washed in alcohol and xylol, and then sealed using EZ mount.

The expression of caspase-3 in the lung tissue was observed using a light microscope connected to a direct screen (Mitsubishi), with 400x magnification from five different fields. The brown color of the cell's cytoplasm indicated the positive expression of the caspase-3 protein, which came from the specific binding of monoclonal antibodies labeled horseradish with Caspase-3 proteins expressed in the cytoplasm. Addition of DAB substrate will react with horseradish and give brown color. In contrast, cells did not express caspase-3 protein will colored purple. Scoring was given to cells with positive brown staining in 100 counted cells.

Apoptosis detection: The apoptotic index was calculated from the number of cells undergoing apoptosis. Apoptotic cells are characterized by condensed and fragmented nuclei which are labeled using TUNEL and colored brown. Positive brown staining from the tumor tissue section is counted in a percentage of the apoptotic cells. Observation and counting of apoptotic cells come from five different fields, using a light microscope with 400x magnification.

Statistical analysis: The results from the cell proliferation and the apoptotic index were presented on a ratio scale. Descriptive analysis was presented using mean, median, mode, and standard deviation. From the histogram and plot, the normal data and data that is normal not are determined. Data analysis includes the Kruskal-Wallis test followed by the Mann-Whitney test. For the apoptotic index assessment, one-way ANOVA was used and followed by *Post Hoc Test* LSD (*Least Square Difference*). All statistical analysis was completed using SPSS *vers.* 19 by significance $p < 0,05$.

RESULTS

Evaluation of Caspase-3 expression using IHC: The higher apoptosis incidence in cells, the higher the p53 expression, which influences the ratio between Bax and Bcl-2 that induces caspase-3 expression. The effect of PMD on the caspase-3 expression was evaluated by histopathology observation from immunohistochemistry staining. The up-regulation of caspase-3 expression was observed in the PMD treatment group. As shown in the figure below (Figure 1), the cells stained positive in the bronchiolar region and were less in the PMD treatment group compared to the carcinogen control group. Quantitative analysis of caspase-3 expression revealed that the expression in carcinogen control at weeks 8, 17, and 26 was $0,28 \pm 0,17$, $0,20 \pm 0,14$, and $0,56 \pm 0,16$, respectively. While in the PMD treatment group, caspase-3 expression was higher in each analysis by weeks 8, 17, and 26 and was $0,60 \pm 0,14$, $0,96 \pm 0,16$, and $2,52 \pm 0,33$, respectively. These results were summarized in a boxplot graph (Figure 2).

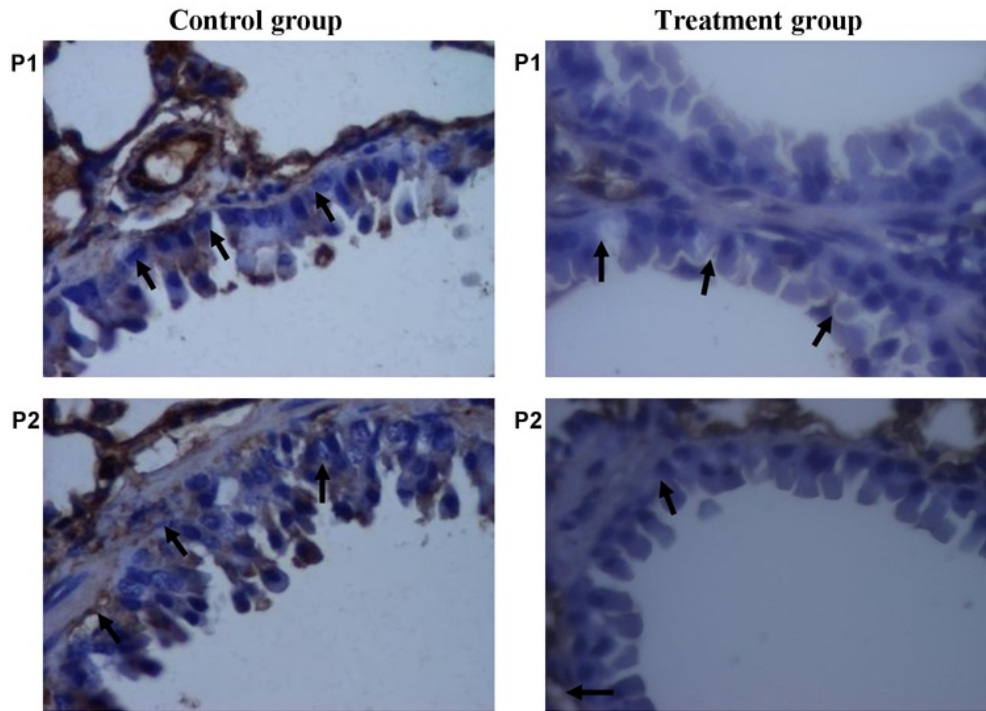


Figure 1. Localisation of caspase-3 expression in bronchioles cell in control and PMD treatment groups stained using immunohistochemistry. The PMD treatment induced the caspase-3 expression at the bronchial epithelium as shown by brown color in cytoplasm. Observation was done under 400x magnification. (P1= observation in pretest, and P2 = observation in post test)

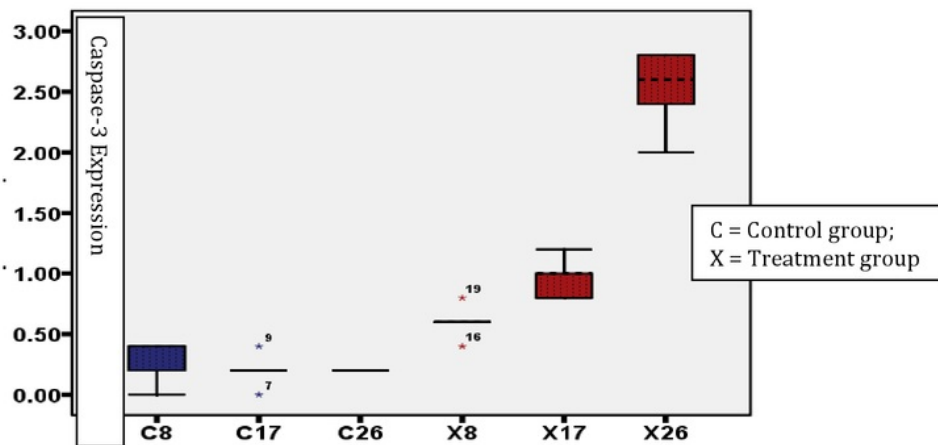


Figure 2. Boxplot graph of the PMD effect on caspase-3 expression in the control and PMD treatment groups from Balb/c mice induced by BP. (* represents outlayers among the animals being treated) Statistical analysis was done using the Kruskal-Wallis test, which resulted in significant increase ($p=0,001$) of caspase-3 expression after PMD treatment during lung carcinogenesis. Moreover, the Mann-Whitney test was used to test significant differences between groups. The

outcome exhibited that the increased caspase-3 expression at weeks 8, 17, and 26 in PMD treatment group was significantly different when compared to the control group, with significant value control-treatment of $p=0,016$, $p=0,008$, and $p=0,008$, respectively.

Apoptotic Index: The TUNEL assay was performed to determine the apoptotic cells with high specificity. The apoptotic cells could be counted out of every 100 cells. The apoptotic index was then calculated from an average of this count from five different fields.

In our study, apoptosis induction was observed in bronchial epithelium cells in PMD treated mice compared to the control group (Figure 3). In line, the apoptotic index would increase due to the higher apoptotic cell number in this group (Figure 4). The apoptotic index at 8 weeks was $0,00 \pm 0,00$ in the control group, while in PMD treatment group was $1,12 \pm 0,71$. During carcinogenesis, the apoptotic index in the PME treatment group was increasing, that was $2,28 \pm 0,83$ and $3,80 \pm 0,83$ at weeks 17 and 26, respectively.

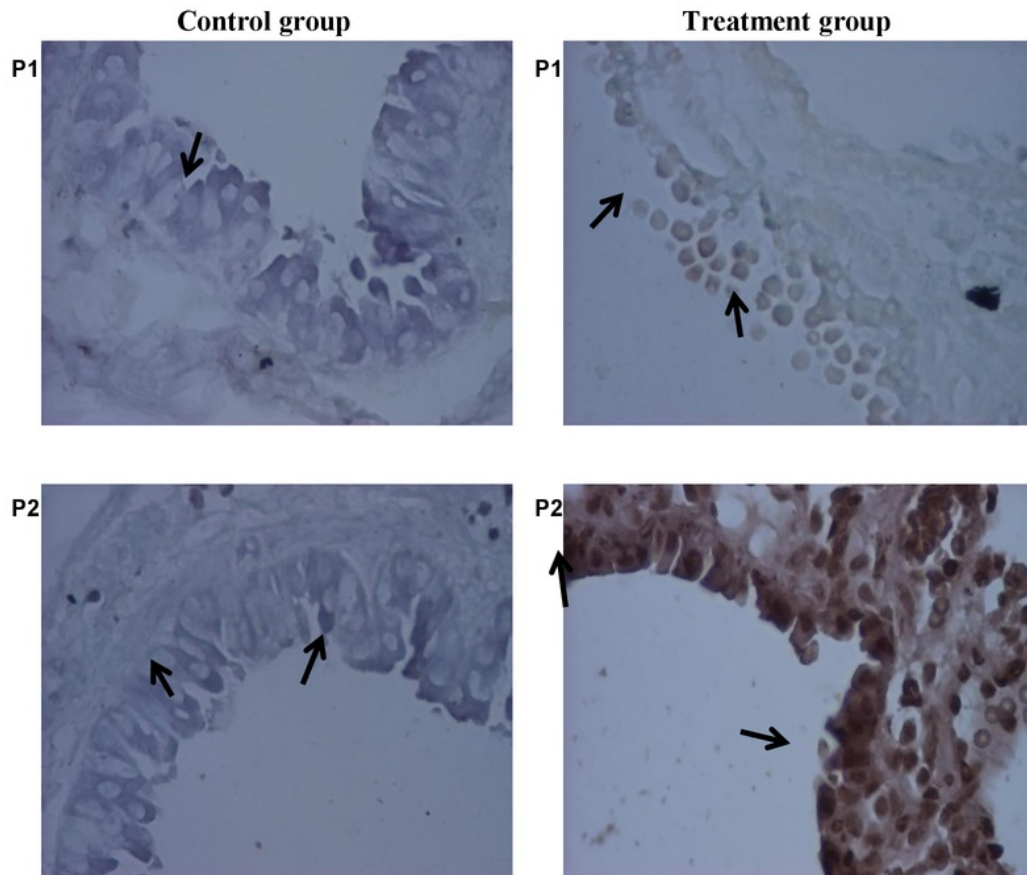


Figure 3. Apoptotic bodies were exhibited using TUNEL staining and brown coloring in the bronchial epithelium cells. Observation was done under 400x magnification.

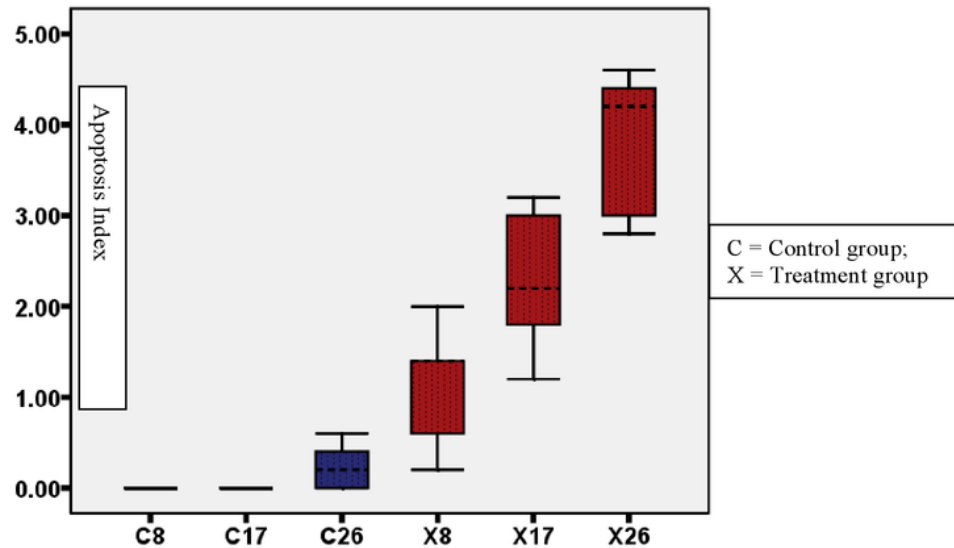


Figure 4. Boxplot graph of the PMD effect on the apoptotic index. The PMD treatment increased the apoptotic index in bronchioles region during lung carcinogenesis in Balb/c mice induced by BP.

Statistical analysis of apoptotic index resulted in normal distribution of the data and a similar variant. Thus, further analysis using one-way ANOVA followed by post hoc test LSD exhibited significant increase of the apoptotic index in the PMD treatment group compared to control carcinogenesis ($p=0,001$) at weeks 8, 17, and 26. As a result, it was revealed that the increase of the apoptotic index during PMD treatment was significantly different compared to the control group with significant levels of control-PMD treatment of ($p=0,005$), ($p=0,001$), ($p=0,001$) at weeks 8 17, and 26 respectively.

DISCUSSION

Our study used mice separated into two groups. The first group was the carcinogenic control group, which was not given the oral PMD but was only injected with BaP up to the 26th week. On the other hand, the second group was the PMD treatment group, which was given PMD orally by dosage of 50mg/mouse/day starting from the 5th week of BaP injection until the 26th week. At weeks 8, 17, and 26, parameters for both groups were determined. Tumorigenesis is a multistep progress of genetic mutations/alterations, which is divided in 3 stages. First, the cell's DNA regulates various enzymes. Secondly, there is an over-expression of enzymes involved in the synthesis and regulation of DNA nucleoties and differentiation processes. During the last stage, the cell proliferation is uncontrolled and undergoes metastasis.

The study of polyphenol compounds as anti-oxidants demonstrated that these compounds were able to induce cell survival, in contrast to pro-oxidant polyphenols, which induced apoptosis, cell death, and inhibited cell proliferation [30, 31]. Moreover, polyphenols are able to disrupt carcinogenesis through several mechanisms. In particular, during early carcinogenesis, polyphenols have the ability to counteract the oxidative pressure caused by a tumor [32, 33]. Consequently, by modulating the oxidative pressure in cancer cells, signal transduction,

transcription factors that are redox-sensitive, and gene expression related to apoptosis and proliferation would be disturbed, leading to carcinogenesis inhibition [34, 35].

Apoptosis is the key to cell death via DNA damage that will eliminate unhealthy cells [36, 37]. The decline of apoptotic cells in cancer play an essential role during each stage of carcinogenesis, due to the accumulation of defective DNA [38]. The disruption of apoptosis may indicate the early carcinogenesis occurrence and failure of tumor cell to undergo apoptosis, which will lead to its malignant form and chemotherapeutic resistant [39, 40]. In this phase, an apoptotic inducer can be used as an anti-carcinogenic agent to reverse the apoptotic incidence. In our study, PMD treatment was shown to enhance apoptotic incidence during each stage of lung carcinogenesis in Balb/c mice induced by BP at weeks 8, 17, and 26.

The apoptotic incidence is characterized by morphological change, including chromatin condensation, nucleus fragmentation, membrane blebbing, and cell shrinkage [19]. At the molecular level, apoptosis is described as a complex network that employ >100 proteins that actively contribute to signal transduction, zymogen cascade, and the execution process. One of the proteins of our interest is the caspase-3 protein, an active form of procaspase-3, which is important at the execution phase of apoptosis. Caspase-3 is activated after the assembly of aptosomal [41]. Caspase-3 and caspase-7 are substrate for CASP9 that is activated by proteolytic due to cleavage and binding of tetrameric enzymes.

In our study, oral PMD treatment in mice clearly increased caspase-3 expression compared to the control group. This result was confirmed by the statistical analysis $p=0,001$ that was below 0,05. As a result, our hypothesis which stated that PMD compounds induce apoptosis via up-regulation of caspase-3 expression, was demonstrated. Furthermore, PMD compounds were able to induce Bax and Bcl-2, so cytochrome-c would be release from mitochondria and activate caspase-3, as shown in Figure 5. The figure below indicates that PMD compounds induce apoptosis through two mechanisms which are via death receptor by activating caspase-8 and via mitochondrial pathway by inducing caspase-9. Both pathways will lead to activation of caspase-3 and apoptosis induction.

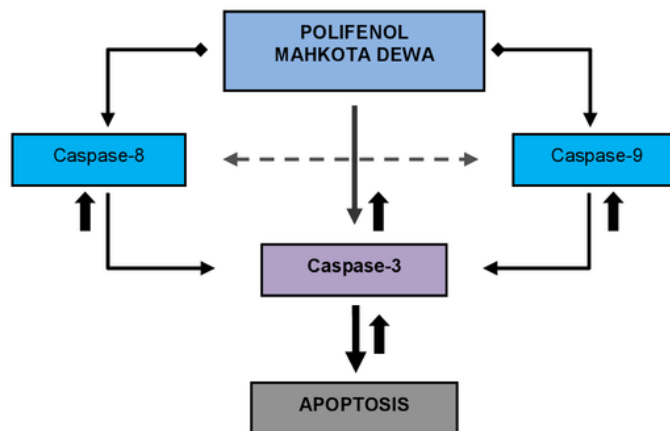


Figure 5.The mechanisms of PMDin inducing apoptosis were via death receptor pathway and mitochondrial pathway.

The results of our study were in line with several previous reports. In a study by Mohan KVPC *et al.*, apoptosis incidence was induced via the increased expression of caspase-3 that came from combination bLF and P-B in HBP carcinogenesis [28]. According to Benerjee S *et al.*, polyphenol compounds from black tea increased caspase-3 expression in the treatment group compared to the control group [42]. Moreover, in evaluation of anti-proliferative, anti-angiogenic, and apoptotic induction from black tea polyphenols, they were also able to enhance caspase-3 expression [29]. Other polyphenols from black tea, such as EGCG and theaflavin, were shown to induce apoptosis during skin carcinogenesis induced by BenzO(A) Pyrene (BAP) [43]. The results above exhibited that PMD might be responsible in apoptosis induction through caspase-3 modulation.

Up to the present, studies report that the use of caspase as a marker for lung cancer was limited. Theoretically, in SLCC *in vivo* study, caspase-3 expression was directly correlated with apoptosis that influenced overall survival [44]. Furthermore, the level of caspase-3 expression corresponded to lymph node metastasis, in which low caspase-3 expression led to poor prognosis [24]. As a result, it was suggested that the downstream pathway of caspase-3 may contribute to apoptosis resistance [45].

The cells undergoing apoptosis will have condensed chromatin, shrinkage of cells, and smaller nuclei. Membrane blebbing together with nucleus fragmentation will produce a clear apoptotic body that can be observed microscopically using histology staining, H&E staining, or TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick and labeling) immunohistochemistry assay. TUNEL assay that has a high specificity, combines molecular and immunohistochemistry detection to visualize apoptotic cells. The binding of fragmented DNA with degoxigenin aided by TdT enzyme (terminal deoxynucleotide transferase) marks molecular reaction, while immunohistochemistry reaction is marked by immunological reaction between antigen-antibody and chemical reaction between enzyme-substrate. The TUNEL assay uses TdT-FragE1 for detection in which TdT binds to 3-OH of fragmented DNA, then labeled biotin and unlabeled deoxy-nucleotide is added. Biotynilated nucleotide will be detected from its binding to the streptavidine-horseradish peroxidase. The addition of diaminobezidine substrate will give colour to the labeled sample which indicates the fragmented DNA [46]. By using TUNEL assay, we observed morphological changes in cells that underwent apoptosis, including membrane blebbing, cell shrinkage, an increase in permeability and decline in mitochondrial membrane potential. Therefore, it was proved that oral PMD treatment does induce apoptosis in mice of PMD treatment group.

The semi quantitative analysis of our study also showed the higher apoptotic index in the PMD treatment group compared to the control group in mice. This result was confirmed by statistical analysis using significant levels of $p=0,001$. Because the significant value was below 0,05, our hypothesis stated that PMD compounds increase apoptotic index was accepted. Our result was in accordance with a previous study that reported the ability of EGCG in raising the apoptotic index in human epidermis carcinoma cell, A431 [47]. In the human colon adenocarcinoma cell, HT29, apoptotic induction was observed after EGCG treatment [48]. In line, in *in vivo* study EGCG and theaflavin treatment exhibited similar results, suggesting that both polyphenols could inhibit cell proliferation and induce apoptosis leading to carcinogenesis disturbance [27].

CONCLUSION

The polyphenol compounds from mahkota dewa (PMD) were able to induce cell apoptosis through up-regulation of the caspase-3 expression, thereby increasing the apoptotic index in Balb/c mice induced by benzo(a)pyrene (BaP).

Competing interests: The authors declare that they have no competing interests with anyone or with any organizations.

Authors' Contributions: All authors contributed to this study. Indranila KS designed and conducted the study, performed the data analysis including the statistical analysis, and drafted the manuscript. Theopilus Watuguly initiated the study design and conducted the study. Lyle Craker supervised and co-designed the entire study, and also co-revised and edited the manuscript in its entirety. Judiono conducted the data analysis, including the statistical analysis, and drafted the manuscript.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7

PAGE 8

PAGE 9

PAGE 10

PAGE 11

PAGE 12

PAGE 13
