Original article:

Comparison of Bax and Caspase-3 Protein Expression in Liver Cells following UVB Irradiation for 7 days and Treatment of *Pimpinella alpina* Molk during 7 and 15 days

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Abstract:

Objective: The effect of *Pimpinela alpina* Molk (PaM) on decrease in Bax and Caspase-3 protein expression in liver cells apoptosis have been proven. However, the difference result between 7 and 15 days treatment duration of PaM need to be confirmed. This study aimed to confirm that treatment of PaM during 15 days is more effective decreasing Bax and Caspase-3 protein expression in liver cells following UVB irradiation. ***Methods:*** In the post test only control group design, 35 Sprague Dawley male rats, 300 gram body weight were divided into two arms, consisting of three groups respectively. First arm comprise Neg-7, PaM7-100, and PaM7-150. Second arm comprise Neg-15, PaM15-100, and PaM15-150. Nor-G was added as normal control neither exposed to UVB nor PaM treatment. In negative group was only radiated to UVB and PaM groups were exposed to UVB and treatment with 100, and 150 mg PaM per oral for 7 and 15 days respectively. At day 8 (first arm) and 16 (second arm), liver organ was taken and Bax and Caspase-3 protein expression assessed by Immunohistochemical staining method. ***Result:*** Post Hoc LSD analysis indicated that Bax and Caspase-3 protein expression in PaM15-100 and PaM15-150 was significant lower compared to that of Nor-G, PaM7-100, and PaM7-150, $p < 0.05$. ***Conclusion:*** Ttreatment of PaM with doses 100 and 150 mg for 15 days was better in decreasing Bax and Caspase-3 protein expression of liver cells following UVB irradiation.

Keywords: *Pimpinella alpina* Molk; antiapoptosis; antioxidant; immunohistochemistry

Bangladesh Journal of Medical Science Vol. 19 No. 02 April'20. Page : 296-303
DOI: https://doi.org/10.3329/bjms.v19i2.45011

Introduction

Pimpinella alpina Molk (PaM) treatment with 100-150 mg daily dose during 7 days have been proven capable of inhibiting liver apoptosis characterized by decrease in mRNA protein expression of Bax and Caspase-3 following ultraviolet B (UVB) light irradiation in rats.[1] Considering cell is able to denovo synthesis protein and other building blocks in order to regenerate their own self structure following damage from variety insults,[2-5] therefore the emerging question is, whether treatment with PaM in the same doses (100-150 mg daily) during 15 days will reduce apoptosis marked by Bax and Caspase-3 protein expression significantly compared to those of 7 days treatment following UVB irradiation. In addition, in this study, identification of Bax and Caspase-3 protein expression in liver cells will be assessed by immunohistochemical (IHC) staining

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method to confirm the previous result were measured by Rt-PCR method.

Ultraviolet B light is continuously expose to human skin and may induce skin inflammation and photoaging.[6,7] The most powerful and damaging effect of UVB on skin is mostly attributed to reactive oxygen species (ROS) particularly radical hydroxyl ($OH^{\bullet}$).[1] Another ROS which is also produced in skin triggered by UVB are hydrogen peroxide (H_2O_2) and oxygen superoxide (O_2^-) resulted from mitochondrial oxidative phosphorilation.[1,8] Furthermore, H_2O_2 is rapidly circulated in systemic circulation and may be degraded to $OH^{\bullet}$ through Fenton or Heber Weis reaction following UVB radiation.[9] Consequently, the cellular damages, DNA fragmentation, and apoptosis triggered by UVB occur not just in skin but also in liver, kidney, and other deeper cells.[9]

There are growing evidences that necrosis and apoptosis is a classical pathological feature in a wide variety of acute and chronic liver diseases caused by various factors such as drugs, viruses, ROS, and alcohol.[10-12] In the acute liver injure necrosis constitute a prominent feature, whereas apoptosis is major feature in chronic infection.[12] Moreover, excessive apoptosis has also been proven occur in acute and chronic viral and alcoholic hepatitis. Persistent apoptosis has also been associated with the development of hepatic fibrosis. In contrast, deficient apoptosis has been proven linked with development and progression of liver cell tumors.[11] Liver cells apoptosis can be roughly classified into three groups according to their sources: extrinsic factors, intrinsic factors, and immune factors lie between extrinsic and intrinsic factors.[10] Overproduction of ROS which is induced by UVB may insult mitochondria and result in cytochrome *c* release and activate caspase-9.[13] The activated caspase-9 further stimulates Caspase-3 activation and apoptosis.[10,13] Execution of apoptosis is tightly regulated by Bcl2 protein family and p53.[14,15] An imbalance between proapoptotic (Bax, Bad, Bak, Bid, and Bcl-Xs) and antiapoptotic protein (Bcl-2, Bcl-XL, Bag-1, and Bcl-W) capability is a prominent characteristic of liver injury.[16]

Flavonoids, is a polyphenols secondary metabolite resulted from plants that have been proven capable of inhibiting and or inducing cellular apoptosis,[1,17,18] can be utilized to maintain protein pro and antiapotosis in balance condition. It is plausible, considering polyphenols are able to counteract oxidative stress by scavenging the excess of ROS, therefore suppress the oxidative stress responsive gene and cell proliferation can be inhibited. On the other hand, polyphenol is capable of inducing ROS production, accordingly as an intolerable level of oxidative stress is reached, cellular apoptosis can be induced.[15-17] PaM, in central Java Indonesia known as Purwoceng, is a medicinal plant that believed capable of improving vitality in male.[1] Therefore, majority of male population consume PaM as a panacea to increase their fitness, vitality, and sexual potencies. There are growing evidences that PaM treatment was proven able to increase testosterone level, antioxidant concentration, and reduced apoptosis in penile, prostate, and hepatocyte cells in rat's model.[1,18] Apoptosis which is triggered by UVB irradiation may activate and stabilizes p53 in nucleus and cytoplasm. Consequently, p53 drive intrinsic and extrinsic apoptotic pathway through amplifying the apoptotic signal consisting of apaf 1, cell death receptor Fas (CD95), PUMA, Bax, and caspases3 mediated by activation of stress activated protein kinases (SAPK).[19]

PaM is a botanical product contained flavonoids, constituting a potential antioxidant, radical scavenger, and metal ion chelator agent[1,18]. Accordingly, PaM is able to inhibit apoptosis mediated by reducing ROS Level and oxidative stress in any cells and tissues.[1,18] There are some evidences that the rate of aging process in various organ is prominently determined by the rate of apoptosis. Low rate of apoptosis result in dysfunctional cells, by contrast high rate of apoptosis may induce tissues degeneration.[20,21] Consequently, maintenance cellular apoptosis in appropriate condition is warranted for healthy cell and organs.

Based on these previous data, this study was proposed to elucidate that PaM treatment for 15 days is better in inhibiting liver cells apoptosis characterized by decrease in protein expression of Bax and Caspase-3 identified with IHC staining method after UVB irradiation during 7 days. IHC staining method was widely used in specific molecular events such as proliferation and apoptosis.

Matrial and Methods

Animal Models and Research Procedure

In the *post test only control group design*, 30 Sparague Dawley male rats, 6 months old, with ± 300 gram body weight were divided into two arms 20 and 15 male rats respectively. First arm consist of four groups: Nor-G, Neg-7, PaM100-7, and PaM150-7; Second arms consist of three groups:

Neg-15, PaM100-15, and PaM150-15. Nor-G was not exposed to UVB, Neg-7 and Neg-15 was only exposed to UVB for 7 days respectively. Whilst in PaM groups were exposed to UVB and treatment with 100, and 150 mg PaM per oral for 7 and 15 days respectively. At day 8 and 16 liver organ was taken and protein Bax and Caspase-3 assessed by IHC staining method.

Pimpinella Alpina Molk Exstract

Pimpinella alpina Molk was obtained from Dieng Plateu Central Java and extracted by Soxhlet method from the whole plant with ethanol as a solvent. Ethanol was evaporated by using a rotary evaporator, leaving a small yield of extracted plant material.

UVB Irradiation

UVB radiation was delivered using UV light sources fluorescent sun lamp FS72T12-UVB-H emitting a UVB wave length ranging from 280-320 nm, with a peak of 312.5 nm at the distance of 25 cm, was measured from cage floor. The average flux intensity at cage floor measured with digital UV light meter YK-35UV was 9.3 j/m2/sec. Hairless rats in 7 experimental groups were placed in plastic cage exposed to 1.6 kJ per M2 for 10 minutes per day for seven days.

Bax and Caspase-3 Protein Expression

Bax and Caspase-3 protein expression were identified by IHC stained method. Slides were prepared by deparaffining process for totally 110 minutes, and then the slides were immersed in Mayer Hematoxylin Eosin stain for 6 minutes. It was washed in running tap water, dehydrated, cleared, and mounted.

Statistical Analysis

The total number of Bax and Caspase-3 protein expression were calculated and presented as the mean ± SD. Differences between groups were analyzed for statistical significance, using a Anova and Post Hoc LSD. A level of $p < 0.05$ was considered as statistically significant.

Ethical clearance: The study was ethically approved by the ethical committee of Sultan Agung Islamic University Medical faculty, Semarang Central Java, Indonesia.

Results

The result of Bax and Caspase-3 protein expression following UVB irradiation for 10 minute per day and concomitantly with PaM treatment during 7 and 15 days can be seen in figure 1, whereas their mean can be seen in table 1.

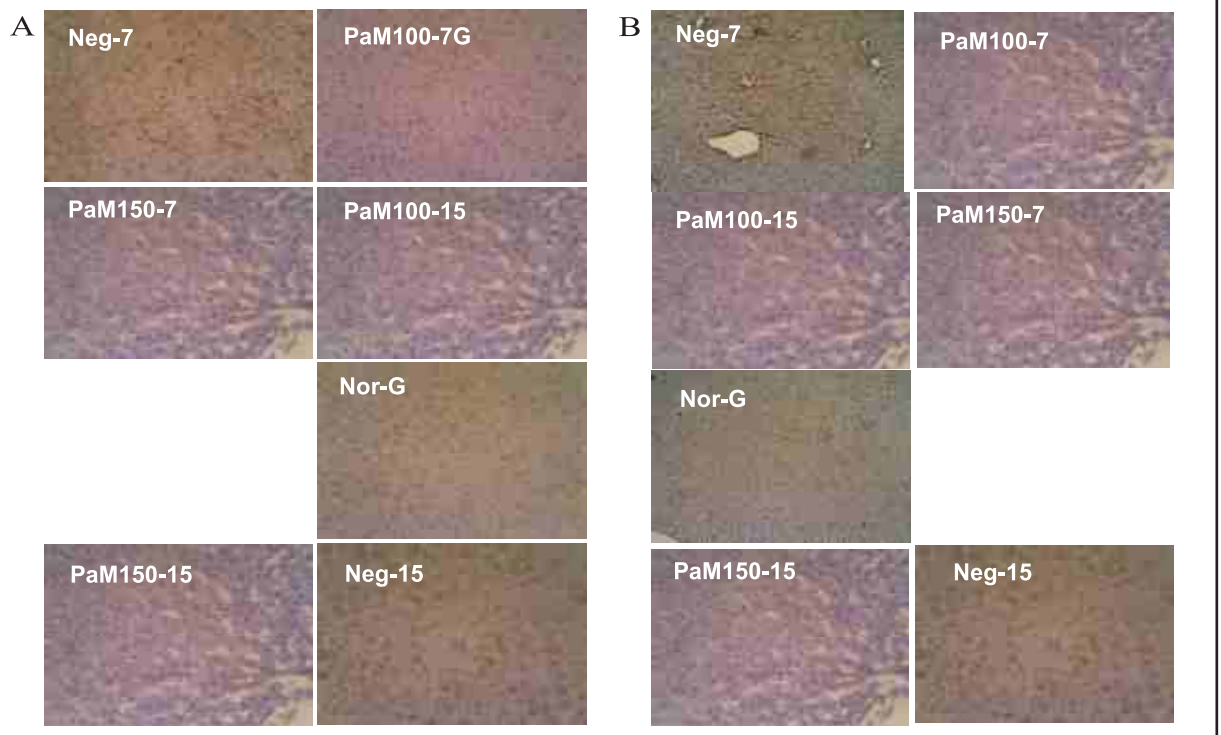

Figure 1. Bax and Caspase-3 Protein Expression of Liver Cells in each Group.
A: Protein expression of Liver Cells Bax; B: Protein expression of Liver Cells Caspase-3

Table 1. Mean Protein Expression of Bax and Caspase-3 of Liver Cells in each Groups

Variables	Groups							P (Anova)
	Nor-G n=5, χ± SD	PaM Treatment for 7 days			PaM Treatment for 15 days			
		Neg-7 n=5, χ± SD	100-7 n=5, χ± SD	150-7 n=5, χ± SD	Neg-15 n=5, χ± SD	100-15 n=5, χ± SD	150-15 n=5, χ± SD	
Liver Bax (%)	1.100 (0.223)	2.400 (0.285)	1.100 (0.418)	0.700 (0.209)	1.500 (0.306)	0.600 (0.136)	0.450 (0.209)	0.000
Liver Casp3 (%)	1.000 (0.176)	2.200 (0.325)	0.800 (0.418)	0.600 (0.136)	1.350 (0.335)	0.350 (0.136)	0.350 (0.136)	0.000

The highest protein expression of Bax and Caspase-3 were in Neg-7, followed by Neg-15, PaM100-7, Nor-G, and PaM100-15. The lowest protein expression of Bax was in PaM150-15, whilst the lowest protein expression of Caspase-3 was in PaM100-15 and PaM150-15. One way Anova statistical analysis both to Bax and Caspase-3 protein expression pointed out that there were significant differences among groups, $p < 0.05$. Moreover, Post Hoc statistical analysis should be done to identify which two groups that possess significant difference as depicted below.

Bax Protein Expressions

Post Hoc analysis indicated that Bax protein expression in Neg-7 and Neg-15 was higher compared to that of five other groups, < 0.05. On the other hand, Bax protein expression in Neg-15 was significant lower compared to that of Neg-7, $p < 0.05$. Bax protein expression in PaM100-15 and PaM150-15 was significant lower compared to that of PaM100-7, $p < 0.05$. Bax protein expression in PaM100-15 was lower compared to that of PaM100-7, however the difference was not significant, $p > 0.05$. Likewise, Bax protein expression in PaM100-15 was also lower compared to that of PaM150-7, nevertheless the difference was not significant, $p > 0.05$. Moreover, Bax protein expression in PaM100-7 and PaM7150 was not significant different compared to that of Nor-G, $p > 0.05$. Bax protein expression in PaM100-15 and PaM150-15 was significant lower compared to that of Nor-G, $p < 0.05$ (figure 2).

Caspase-3 Protein Expressions

Post Hoc analysis indicated that Caspase-3 protein expression in Neg-7 was higher compared to that of all other groups, $p < 0.05$. Caspase-3 protein expression in Neg-15 was lower compared to that of Neg-7, however the difference was not significant, $p > 0.05$. Caspase-3 protein expression in PaM100-15 and PaM150-15 was significant lower compared to that of PaM100-7, $p < 0.05$. Caspase-3 protein

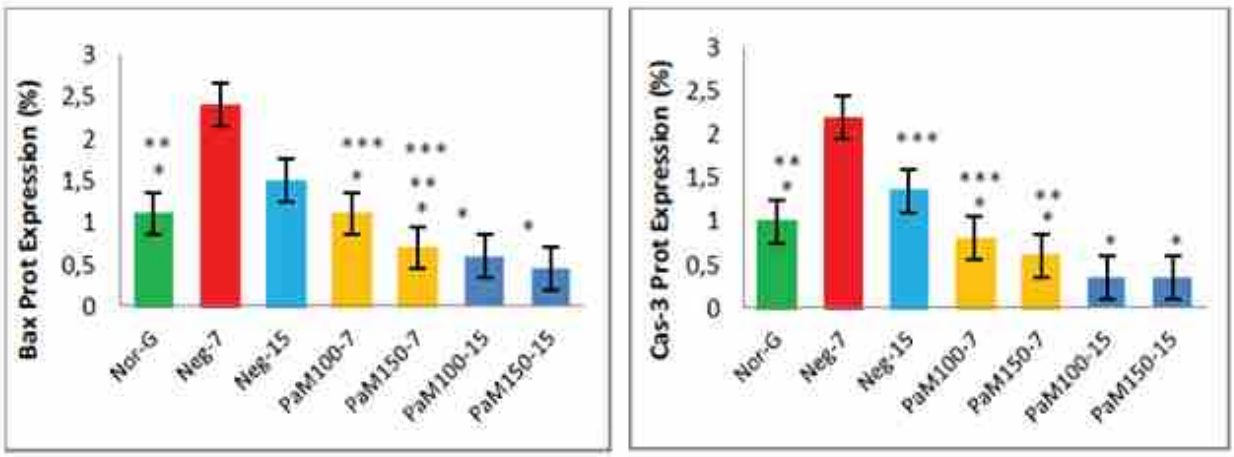

Figure 2. Protein Expression of Bax and Caspase-3 following UVB radiation and PaM Treatment during 7 and 14 days. * $P < 0.05$ Neg-7 and Neg-15 as Control ; ** $p > 0.05$ PaM100-7 as control; *** $P > 0.05$ Nor-G as control.

expression in PaM150-7 was lower compared to that of PaM100-7, but not significantly different, $p > 0.05$. Likewise, Caspase-3 protein expression in PaM100-7 compared to that of Nor-G was lower, but not significantly different, $p > 0.05$. On the other hand, Caspase-3 protein expression in Neg-15 was higher compared to that of Nor-G, but not significantly different, $p > 0.05$. Caspase-3 protein expression in PaM100-15 and PaM150-15 was significant lower compared to that of Nor-G, $p < 0.05$. However, there was no significant difference of Caspase-3 protein expression in PaM150-15 and PaM100-15, $p > 0.05$ (figure 3).

Discussion

In the present study, exposure to UVB radiation with the dose of 1.6 kJ per M2 for 10 minutes per day for seven days was capable of inducing apoptosis of liver cells. It was indicated by this result that Bax and Caspase-3 protein expression in Neg-7 increased significantly compared to those of other groups. Liver cell apoptosis was attributable to ROS production and stress oxidative triggered by UVB. A study was reported by Hattory et al. demonstrated that UVB radiation was able to produce $OH^{\bullet}$, O^{-}, and H_2O_2.[22] Another study was reported by Nasihun and Eni also demonstrated that UVB radiation induce oxidative stress marked by increase in MDA and 8-oxo-2'-deoxyguanosine (8OHdG), otherwise decrease in total antioxidant capacity (TAC) and GPx activity.[23] Moreover, there are growing evidences that UVB irradiation is able to induce DNA damage straightforwardly by absorption of energized UVB photons or circuitously through photosintizer molecule, thus creating an excited singlet state. DNA damages by an excited singlet may occur either through direct interaction with DNA molecule, thus induce free radical formation, or via energy transfer to molecular oxygen named as photodynamic action, leading to ROS production and subsequently DNA damage.[23,24] It is plausible since severe oxidative stress is prerequisite for apoptosis triggered by UVB.[25] On the other hand, Caspase-3 protein expression in Neg-15 was not significantly lower compared to that of Neg-7. It was indicated that seven days following UVB radiation halted a denovo synthesis for cells selves repairing occur; however its result was not optimal as for compared to that of PaM groups.

PaM treatment with 100 and 150 mg daily dose orally during 7 days capable of decreasing Bax and Caspase-3 protein expression in liver cells. Likewise, administration of PaM with 100 and 150 mg daily dose during 15 days has also been able to decrease Bax and Caspase-3 protein expression in liver cells. It can be implied that treatment of PaM with 100 and 150 mg daily doses during both 7 and 15 days capable of decreasing apoptosis liver cells induced by reactive oxygen species (ROS) triggered by UVB. However, the decreases in apoptosis of liver cells were better in 15 days than that of 7 days of PaM treatment. It was plausible since the seven days longer of PaM treatment, beside provide cell an opportunity to undertake a denovo formation for self repairing after insulting of ROS was halted at day seven, also attributable to increase in antioxidant concentration due to duration of PaM treatment.[1] In this context, should be keep in mind that antioxidant activity of flavonoids can be altered to pro-oxidant activity in high concentration, therefore specific precaution should be paid when flavonoids is administered in the long term and high dose. In concern to circumvent the potential damage of cells due to pro-oxidant alteration, accordingly, PaM treatment should be administered in moderation. It was confirmed by the various studies that flavonoids is able to reducing cardiovascular events,[26] preventing and managing allergic diseases effectively when consumed in moderation.[27] Interestingly, in the present study treatment of PaM with the dose of 100 and 150 mg per day for seven days decreased Bax and Caspase-3 protein expression effectively, thus comparable to normal group.

Furthermore, in the present study also indicated that decreased in Bax in each group invariably followed by decreased in Caspase-3 protein expression. By Pearson statistical analysis demonstrated that the positive correlation was occur in all groups, however, in Neg-7 and PaM100-7 the positive correlation was strong, $r = 0.942$ and 0.859, $p < 0.05$ respectively (figure 3). It was suggested that liver cells apoptosis is runned by outweighed in the intrinsic or mitochondrial pathway than the extrinsic pathway. According to the available literature the mitochondrial pathway is induced as a response to cellular stress or DNA damage and brings about the activation of the pro-apoptotic BH3-only proteins. BH3-only proteins may in a straight line bind and

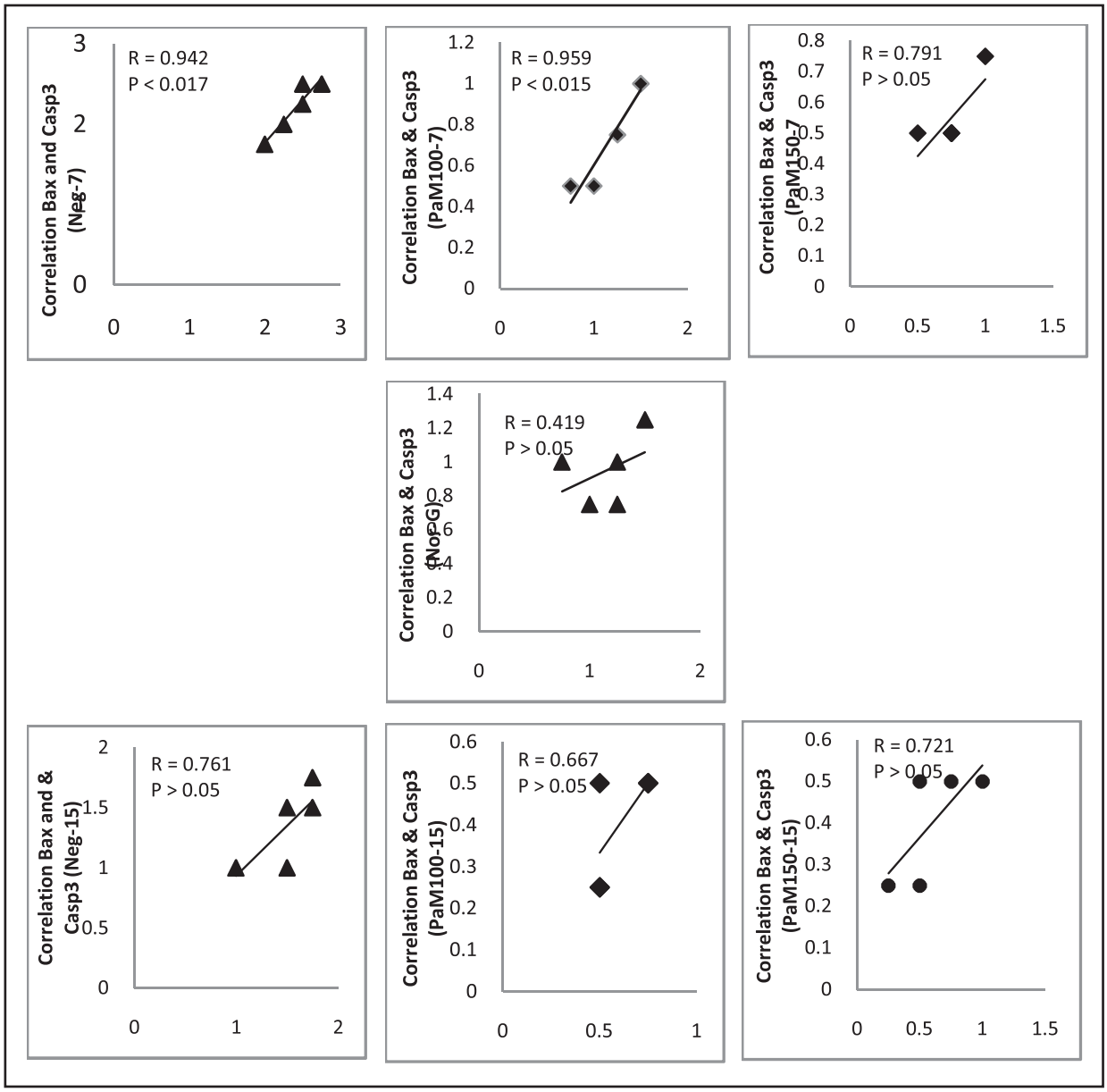

Figure 4. Peaarson Correlation of Bax and Caspase-3 Protein Expression in Each Groups

activate Bax and Bak and may also bind to the pro-survival Bcl-2-like proteins to indirectly activate Bax and Bak. Once activated, Bax and Bak oligomerize to form pores in the mitochondrial outer membrane that release cytochrome *c*. Cytosolic cytochrome *c* leads to Caspase-3 activation and subsequent cell death.[28]

The result of the present study is also corroborated by my previous study indicated that PaM treatment with 100-150 mg daily dose for 15 days capable of improving oxidative stress marked by increase in Glutathione peroxidase (GPx) activity and decrease in xanthin oxidase (XO) activity and inhibit liver cells apoptosis characterized by decrease in the protein expression of Bax and Caspase-3 marked by reduced mRNA.[1] In the previous study Bax and Caspase-3 protein expression were identified with Real-time Polymerase Chain Reaction (Rt-PCR) and in the present study both Bax and Caspase-3 protein expression were identified with IHC stained method. The resultant of IHC-staining method and Rt-PCR method in Bax and Caspase-3 protein expression are similar following PaM treatment. Concerning to those results, further statement can be made that identification of both Bax and Caspase-3 protein expression in liver and other cells apoptosis may be identified by Rt-PCR and IHC staining method

with the equivalent results. Accordingly, using IHC-staining method to confirm the previous study that used Rt-PCR to identified Bax and Caspase-3 protein expression may be accepted.

Conclusion

In conclusion, treatment of Pimpinela alpina Molk with the dose of 150 mg perday for 15 days was better compared to that of 7 days. However, PaM treatments with the dose of 100 and 150 mg for 7 days were comparable to normal. In addition, identification Bax and Caspase-3 protein expression using IHC staining and and Rt-PCR method were comparable.

Acknowledgements

The research was supported and funding by the institution of sultan agung Islamic University.

Source of fund: (if any)

Ministry of Research and Higher Education

Authors's contribution:

Data gathering and idea owner of this study: Taufiqurrachmand and Eny Widayati

Study design: Taufiqurrachman

Data gathering: Taufiqurrachman, Eny Widayati, Azizah Hikma Savitri, Nurina Tyagita

Writing and submitting manuscript: Taufiqurrachman

Editing and approval of final draft: Taufiqurrachman

Conflicts of Interests

No conflicts of interest were declared with relation to this work.

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