Original article

Treatment of *Pimpinella Alpina* Molk Improve Oxidative stress and Inhibit Liver Cellular Apoptosis in Rats Following UVB Irradiation: Is there Any Correlation?

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Abstract

Objective**:* The effect of *Pimpinella alpina* Molk (PaM) in improvement of oxidative stress has been established, but did not for cellular apoptosis. This investigation was conducted to evaluate the effect of PaM on GPx and XO activities, hepatocytes apoptosis and their correlation following UVB radiation. ***Methods: Forty male rats were assigned into 8 groups: Nor-G, Neg-7, Neg-15, PaM50-7, PaM100-7, PaM150-7, PaM100-15, and PaM150-15. UVB irradiation was given 10 minutes to each rat daily, blood and liver organ samples were taken in day 8 and day 16 to evaluate all variables. ***Results*:** Post Hoc statistical analysis showed that GPx activity in PaM150-15 was not significant lower compared to that of Nor-G, $p > 0.05$. Conversely, the activity of XO in PaM150-7 was not significant higher compared to that of Nor-G, $p > 0.05$. Expression of Bax and Caspase3 mRNA in PaM100-15 and PaM150 were comparable to that of Nor-G, $p > 0.05$. The strong negative correlation (-795, $p < 0.01$) was occur between GPx and XO. Similarly, there were strong negative correlations (-697 and -715, $p < 0.001$) between GPx and the expression of Bax and Caspase3 mRNA. ***Conclusion:*** PaM treatment with 100 - 150 mg daily dose for 15 days capable of improving oxidative stress marked by increase in GPx activity and decrease in XO activity and inhibit apoptosis characterized by decrease in the expression of Bax and caspase3 mRNA. There were strong negative correlations between antioxidant activity and apoptosis on Sprague male rats after UVB irradiation.

Keyword: *Pimpinella Alpina* Molk; Apoptosis; Oxidative stress; UVB; Oxidative stress; apoptosis

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Introduction

Pimpinella alpina Molk (PaM), known as Purwoceng, is a medicinal plant growing in Dieng plateau Central Java Indonesia. Male population particularly who aged 40 years or above have traditionally consumed PaM to rejuvenate and increase their vitality[1]. According to the compression-of-morbidity hypothesis, reducing the cumulative lifetime morbidity is possible, as long as primary prevention measures is able to postpone the onset of chronic illness[2,3]. Most evidence indicates that daily intake of flavonoids from plants could prevent degenerative diseases and chronic illness.[4-6] Considering, degenerative diseases and chronic illness are tightly associated with oxidative stress, therefore, daily intakes of flavonoids in midlife and late adulthood are important predictors of subsequent degenerative diseases and disability.[3] The effect of PaM in improvement of oxidative stress following UVB and UVC irradiation has been established,[7] but did not for cellular apoptosis. Considering, severe oxidative stress is prerequisite for apoptosis triggered by UVB and posses an important role in aging process and frailty,[8,9] therefore, further research is needed to investigate the effect of PaM on antioxidant activity and hepatocyte cells apoptosis, and their relationship following UVB irradiation.

Forty five percent of the total sunlight spectrum is UV light, consisting of UVA ranging from 315 - 400 nm, UVB ranging from 280 - 315 nm), and UVC ranging from 100 - 280 nm. The earth's ozone

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layer efficiently absorbs UV radiation up to about 310 nm. Accordingly, all UVC and most of UVB light are consumed by ozone before they reach the surface of the earth.[10,11] However, current depletion of the ozone layer is significantly increasing the amount of UVB radiation that reaches the surface of earth. This condition potentially induces acute and chronic skin disorders such as inflammation, erythema, hyper pigmentation, premature aging, and cancer.[12-14]. These disorders were mostly attributable to photoproducts such as [cyclobutane pyrimidine dimers (CPD)] and (6-4) pyrimidine-pyrimidone [(6-4)PPs], increase in production of reactive oxygen species (ROS) and alteration of antioxidant defensive system leading to cellular oxidative stress. Oxidative stress is defined as the imbalances in the equilibrium between pro-oxidants and antioxidants status in cellular systems, in favor of oxidants which results in damaging the cells.[15,16]. The increase in ROS constitutes a major cause of DNA fragmentation and initiates cellular damages leading to apoptosis.[17] There are growing evidences that ROS and ROS-modulated molecules participate in both intrinsic and extrinsic apoptotic pathways.[18] Considering ROS, particularly OH• which is resulted from Fenton reaction are vastly distributed in systemic circulation following UVB radiation, thus the cellular damages, DNA fragmentation, and apoptosis occur not just in skin but also in other deeper cells[19]. There are various evidences that increase in ROS such as $OH^{\bullet}$, O_2^- and H_2O_2 is associated with the arrest of cell proliferation. Similarly, generation of oxidative stress in response to various external stimuli has also been implicated in the activation of transcription factors and to the triggering of apoptosis.[20,21] A study was reported by Higuchi and co workers indicated that increase in ROS inducing apoptosis mediated by xanthin oxidase (XO) and caspase$_3$ activity.[22] On the other hand, study was reported by Wedi et al. pointed out that cellular apoptosis (eosinophil) can be blocked by glutathione and *N*-acetylcysteine.[23] Moreover, the study was reported by Nasihun and Widayati indicated that treatment of PaM capable of improving oxidative stress following UVC radiation in rats. The improvement of oxidative stress was marked by decreased in concentration of MDA and 8-OHdG, otherwise increased in concentration of total antioxidant capacity (TAC) and super oxide dismutase (SOD) activity[7].

Apoptosis is a word come from Greek and used to describe the "dropping off" or "falling off" of petals from flowers, or leaves from trees.[24] Moreover, in the biological system, apoptosis then used to describe the intrinsic cell suicide program, in contrast to necrosis. The morphological feature of apoptosis is characterized by global cell shrinkage, cell blabbing, and chromatin condensation accompanied by nuclear and DNA fragmentation into specific fragment sizes (180 – 200 base paired) and so called DNA ladder pattern.[24]. Apoptosis have a pivotal role in the aging process and age-associated carcinogenesis in mammals. The rate of apoptosis is prominent in most types of aging cell populations and organs, such as brain, cardio-vascular system, immune system, endocrine system, intestine, reproductive system and eye.[25,26] The increase in apoptosis in these various organs regarded as protective mechanism of organism against the accumulation and spread defective cell. In contrary, when the dismantling system is predominance the age-associated decline and deterioration in tissue and organ structure and function are induced. Accordingly, apoptosis has a pivotal role as sentinel homeostasis pathway in organ and tissues. However, there are growing evidence that high rate of apoptosis can result in tissue's degeneration,[26,27], while low rate of apoptosis allows either dysfunctional cells to accumulate or differentiated immune cells to persist.[28] Therefore, appropriate apoptosis is necessary in order to prevent age-related diseases and cancer.

Initiation of apoptosis by the cell is governed by gene activities, leading to cellular self-destruction through the intrinsic or the extrinsic pathways. Apoptosis process is initiated by various inducers from both internal and external millieu, followed by the cell death commitment and execution. DNA damage due to UVB irradiation activates and stabilizes p53 in nucleus and cytoplasm. Moreover, p53 may drive intrinsic and extrinsic apoptotic pathway through amplifying the apoptotic signal consisting of apaf 1, cell death receptor Fas (CD95), PUMA, Bax, Bak, and caspases. These protein pro-apoptosis are evoked by activation of stress activated protein kinases (SAPK) following UVB irradiation.[29,30] In intrinsic apoptosis pathway, the DNA damages induce mitochondrial permeability transition pore complex, which controls mitochondrial membrane potential to release cytochrome c. In concert with apaf-1, procaspase$_9$, and ATP, cytochrome c forms apoptosome death complex (ADD), that proteolitically convert procaspase9 to caspase9, and then activate caspase7,6 and caspase3 resulting in DNA fragmentation and cell death. On the other hand, in extrinsic apoptosis UVB and ROS induces

Fas and Fas ligand (FasL) or tumor necrosis factor (TNF) with TNF receptor 1 (TNFR1). Furtehrmore, Fas-FasL complex bound with Fas Associated Death Domain (FADD), subsequently activates caspase8 and other caspases executing apoptosis via Death Effectors Domain (DED).[31]

Studies about PaM have been documented particularly to increase expression of BcL_2 otherwise decreased expression of Caspase3 and inhibition of apoptosis in penile and prostate cells.[32,33] Indeed those PaM effect were unlikely through antioxidant capacity instead of hormone stimulation effect in castrated male rats. It was possible, because PaM contained stigmasterol, is a natural weak androgen that believed as androgen precursor. Stigmasterol may be converted to testosterone in peripheral tissues catalyzed by enzyme of 3-βOHsteroid and 17-βOH steroid dehydrogenase.[34] Testosterone is an androgen hormone capable of developing masculinity, increase sexual desire, vitality, and rate of fertility in male.[35,36] In addition to stigmasterol, another significant constituent of PaM is flavonoids[37], constituting a natural potent botanical antioxidant, radical scavenger, and metal ion chelator agent.[38] Flavonoids have been documented capable of reducing ROS level, oxidative stress, and biological aging.

Based on these previous data, this study was proposed to elucidate that PaM treatment capable of decreasing XO activity, followed by increase in activity of GPx, leading to pro-oxidant antioxidant balance after UVB irradiation. This pro-oxidant and antioxidant balance providing cellular damages due to oxidative stress an opportunity to undertake denovo formation for self repair, indeed prevent cellular apoptosis.

Matrial and Methods

Animal Models and Research Procedure

In this experimental study, the post test only control group was adopted as design research. Forty Sprague Dawley (SD) male rats, 6 months old, and ± 300 gram body weight (BW), were assigned into 8 groups randomly as follow: 1). Normal group (Nor-G), rats got no UVB irradiation and no PaM treatment. 2). Negative control group for seven days (Neg-7) and 15 days (Neg-15), rats got UVB irradiation only. 3). PaM treatment group, was divided into two arms: first arm comprise three groups: PaM50-7, PaM100-7, and PaM150-7, in these groups all rats were radiated with UVB and treated with PaM 50 mg, 100 mg, and 150 mg doses per day respectively during 7 days; second arm comprise 2 groups: PaM100-15, and PaM150-15, all rats in these groups were expose to UVB for 7 days and administered PaM with dose of 100 mg and 150 mg respectively during 15 days. The seven days longer was proposed to provide cell an opportunity to undertake denovo formation for repairing. All rats were kept in acclimatization for one week with environmental controlled temperature (20^0C–24^0C), constant humidity (55 – 60%), and controlled photoperiod (12 h light and 12 h dark) properties before beginning of experiment. During the study all rats in all groups were given standard dietary intake (Ain 93) and tap water adlibitumly. At day 8 of study, blood samples and liver organs from rats in Nor-G, Neg-7, PaM50-7, PaM100-7, and PaM150-7 were taken and measured the activity of GPx and XO, and the expression of Bax and $capase_3$ mRNA, meanwhile, at day 16 with the similar method, blood samples from rats in Neg-15, PaM100-15, and PaM150-15 were taken for asses the same variables. All measurement of variables was undertaken at Inter University Program (PAU) of Gajah Mada University (UGM) Yogyakarta Indonesia. The study was ethically approved.

PaM Extract

Pimpinella alpina Molk was obtained from market and extracted by soxhlet methods from the whole plant with ethanol as a solvent.

UVB Irradiation

UVB radiation was delivered using UV light sources fluorescent sun lamp FS72T12-UVB-H emitting a UVB weave 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/m^2/sec. Hairless rats in all groups except in Nor-G were placed in plastic cage exposed to 1.6 kJ per M^2 or equivalent to minimal erythematic dose (MED) for 30 minutes per day during seven days.

GPx Measurement

A Glutathione peroxidase level was determined by the method of Paglia and Valentine, using a commercially available kit (Ransel; Randox Laboratories, UK). GPx catalyze the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to its reduced form with a concomitant oxidation of NADPH to NADP. The NADPH-consumption rate was monitored at 340 nm.

XO Measurement

Xanthine oxidase was determined by instruction method manufactured by BioVisison. The principal measurement of XO in this assay is XO oxidizes

xanthine to to hydrogen peroxide (H_2O_2) which react stoichiometrically with OxiRed™ probe to generate color (at ƛ = 570 nm) and fluorecence (at Ex/Em = 535/587 nm). Considering the color or fluorecence intensity is proportional to XO content, the XO activity can be accurately measured. This kit detects 1 – 100 mU XO in 100 µl reaction volume.

Detection Bax and Caspase3 mRNA Expression Using Real-time PCR

Total RNA was extracted from tissue samples using RNA extraction kit according to the manufacturer instruction (Favorgen). Real-time PCR was performed by use of the SYBR Green dye on the Exicycler™96 Bioneer machine. The real-time PCR reactions were carried out with a following 1 cycle of initial denaturation at 94°C for 10 min through 40 cycles and then held for 30 seconds at each temperature level. The forward and reverse primers used for PCR amplification of Bax and Caspase-3 were shown in table 1. Finally, melting curve analysis was performed over a gradient extending from an annealing to a denaturation temperature. The expression was calculated by using the relative standard curve method of quantification and reported as a fold change of gene expression.

Table 1. **Primer sequences for RT-PCR**

Primer	Sequences (5' to 3')	PCR Fragments (bp)
Bax forward primer Bax reverse primer	5'-ATGTTTTCTGACGGCAACTTC-3' 5'-AGTCCAATGTCCAGCCCAT-3'	133
Caspase-3 forward primer Caspase-3 reverse primer	5'-TGTTTGTGTGCTTCTGAGCC-3' 5'-CACGCCATGTCATCATCAAC-3'	210

Statistical Analysis

Anova and Post Hoc test were adopted as statistical analysis in 95% of confidence interval or $P < 0.05$ was considered statistically significant. All statistical analysis were done use computer program. This study has been approved by the ethic committee, medical faculty of Sultan Agung Islamic University Semarang Central Java Indonesia.

Results

Effect of PaM on GPx, XO, Bax, and Caspase3 mRNA Expression

After treatment of PaM for 7 and 15 days, all variables including activities of GPx, XO in serum, and the expression number of Bax and $Caspase_3$ of liver organs were measured at day 8 and 16 and the results shown in table 2.

This result indicated that the highest level of GPx was occur in Nor-G, followed by PaM150-15, PaM100-15, PaM150-7, PaM100-7, PaM50-7, NeG-7, and the lowest was in Neg-15. In contrary the lowest level of XO, was found in Nor-G, followed by PaM150-7, PaM150-15 and PaM100-7, PaM100-15, PaM50-7, Neg-7, and the highest was in Neg-15 (table 2). The lowest expression of Bax mRNA was in PaM150-15, followed by Nor-G, PaM100-15, PaM150-7, PaM50-7, PaM100-7, Neg-15, and the highest was in Neg-7. Moreover, for the expression of $caspase_3$ mRNA, the lowest was occur in PaM150-15, followed by Nor-G, PaM100-15, PaM100-15, PaM150-7, Neg-15, PaM100-7, PaM50-7, and the highest was in

Table 2. Activity of GPx, XO, Expression of Bax and Caspase3 mRNA in Sprague Dawley Rats

Variables	Groups								P (anova)
	Nor-G (n=5)	Neg-7 (n=5)	Neg-15 (n=5)	PaM 50-7 (n=5)	PaM 100-7 (n=5)	PaM 150-7 (n=5)	PaM 100-15 (n=5)	PaM 150-15 (n=5)	
GPx U/ml (±SD)	87.48 (±2.54)	37.12 (±4.75)	28.60 (±4.60)	52.15 (±3.76)	57.19 (±3.76)	65.61 (±3.76)	72.34 (4.60)	82.43 (±3.76)	0.000
XO mU/ml (±SD)	0.08 (±0.04)	0.13 (±0.04)	0.20 (±0.05)	0.12 (±0.05)	0.10 (±0.02)	0.09 (±0.02)	0,11 (±0.01)	0,10 (±0.01)	0.000
Bax Σ (±SD)	8.96 (±3.82)	26.30 (±8.47)	16.45 (±2.99)	15.18 (±6.13)	16.18 (±3.99)	11.30 (±2.07)	10.29 (±2.09)	5.08 (±1.11)	0.000
$Caspase_3$ Σ (±SD)	6.53 (±1.93)	17.40 (±4.27)	14.49 (±2.93)	16.76 (±4.74)	14.86 (±2.39)	12.99 (±2.94)	9.85 (±3.43)	4.75 (±1.78)	0.000

Neg-7. One way anova statistical analysis pointed out that there were significant differences among groups, $p < 0.001$.

Activity of GPx and XO

Post Hoc statistical analysis showed that GPx activity in Neg-7 and Neg-15, were lower significantly compared with that of Nor-G, $p < 0.001$, whereas the GPx activity in Neg-7 were significant higher compared with that of Neg-15, $p < 0.001$. The GPx activity in PaM50-7, PaM100-7, and PaM150-7 was higher significantly compared to that of Neg-7 and Neg-15, $p < 0.001$. The GPx activity in PaM50-7, PaM100-7, and PaM150-7 was lowering significantly compared to that of Nor-G, $p < 0.001$. Interestingly, GPx activity in PaM150-15 was not significant lower compared to that of Nor-G, $p > 0.05$. (Figure 1)

The activity of XO in Neg-7 and Neg-15 were higher significantly compared to that of Nor-G, $p < 0.001$, whereas the activity of XO in Neg-7 was lower significantly compared to that of Neg-15, $p < 0.001$. In contrary, the activity of XO in PaM150-15, PaM100-15, PaM150-7, PaM100-7, and PaM50-7 was lowering significantly compared to that of Neg-7 and Neg-15, $p < 0.001$ respectively. The XO activity in PaM50-7 was higher compared to that of Neg-15, $p < 0.001$. Special for the level of XO in PaM150-7 was higher compared to that of Nor-G; however that difference was not significantly, $p > 0.05$. (Figure 1)

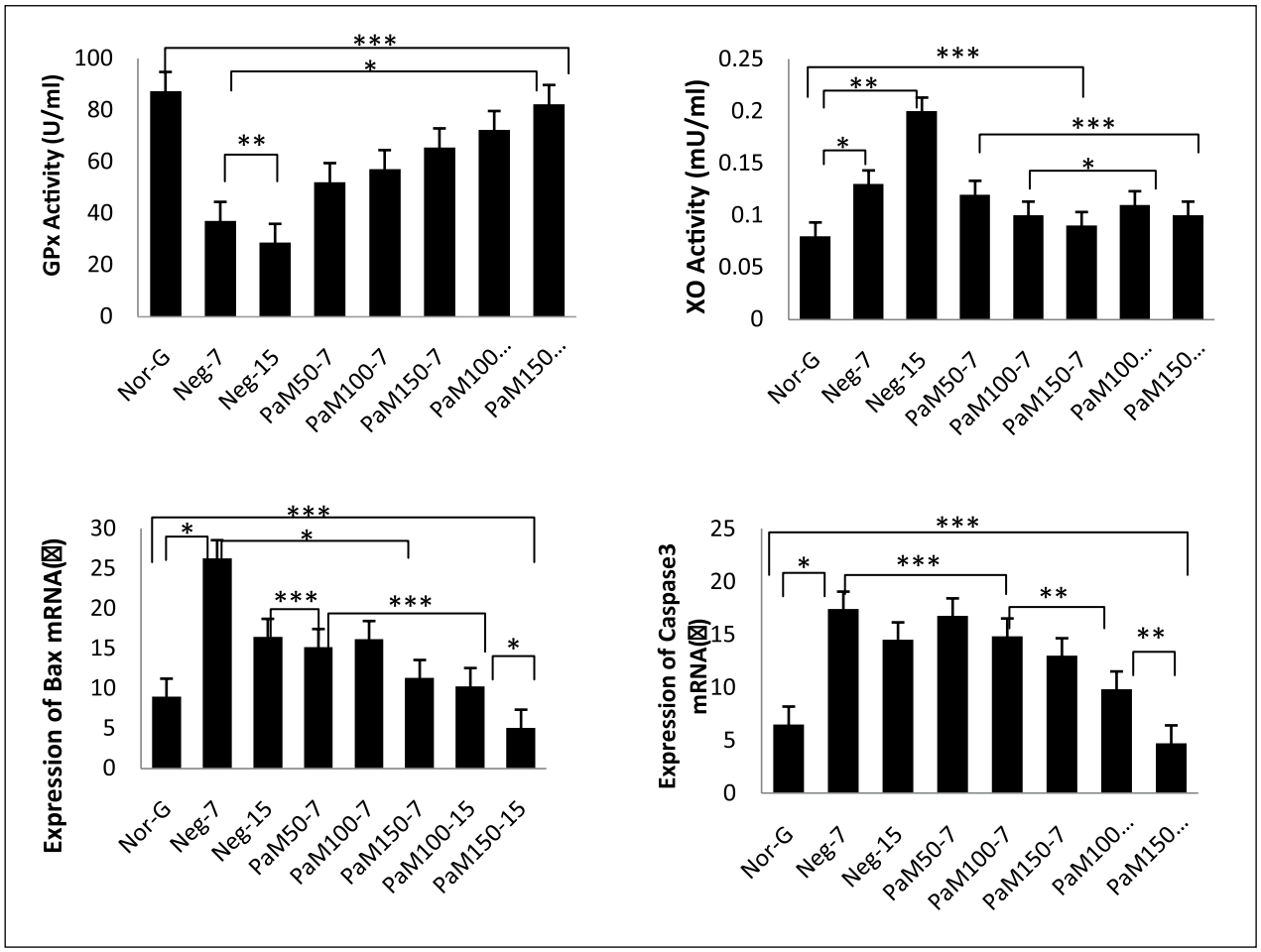

Figure 1. Glutathion and Xanthin Oxidase activities, and Expression of Caspase3 and Bax mRNA. Post Hoc: * $p < 0.01$; ** $p < 0.05$; *** $p > 0.05$

The Expression of Bax and Caspase$_3$ mRNA

Post Hoc analysis indicated that the expression of Bax and caspase$_3$ mRNA in Neg-7 and Neg-15 was significant higher compared to that of Nor-G, $p < 0.001$. Whilst the expression of Bax mRNA in Neg-15 was significant lower compared to that of Neg-7, $p < 0.05$. Conversely, there was no significant difference expression of caspase3 mRNA in Neg-7 and Neg-15, $p > 0.05$. The expression of Bax mRNA in PaM50-7, PaM100-7, PaM150-7, PaM100-15, and PaM150-15 was significant lower compared to that of Neg-7, $p < 0.001$. Interestingly, the expression of Bax mRNA and caspase3 in all PaM groups when compared to that of Neg-15, the results were varies.

In PaM50-7, PaM1007, and PaM150-7, there was no significant difference expression of Bax mRNA compared to that of Neg-15, $p > 0.05$. On the other hand, in PaM100-15 and PaM150-15, the expression of Bax mRNA was significant lower compared to that of Neg-15, $p < 0.05$. Moreover, the expression of Bax mRNA in PaM150-7, PaM100-15, and PaM150-15 was no difference compared to that of Nor-G, $p > 0.005$. There was no significant difference expression of caspase3 mRNA in PaM50-7 and PaM100-7 when compared to that of Neg-7, $p > 0.05$. Meanwhile, the expression of caspase3 mRNA in PaM150-7, PaM100-15, and PaM150-15 was significant lower compared to that of Neg-7, $p < 0.001$. On the other hand, there was no significant difference expression of caspase3 mRNA in PaM50-7, PaM100-7, and PaM150-7 compared to that of Neg-15, $p > 0.05$. Whereas, the expression of caspase3 in PaM100-15 and PaM150-15 was significant lower compared to that of Neg-15, $p < 0.05$. The expression number of caspase3 in PaM100-15 and PaM150-15 was lower compared to that of Nor-G, however the difference was not significant, $p > 0.05$. (Figure 1)

Correlation among Variables

Based on the Pearson correlation analysis there were strong correlation among variables. The strong negative correlation (- 795, $p < 0.01$) was occur between GPx and XO. Similarly, there were strong negative correlations (-697 and -715, $p < 0.001$) between GPx in one hand and the expression of Bax and caspase3 mRNA on other hand. In contrary, there was positive correlation (+610, $p < 0.01$) between the expression of Bax and caspase3 mRNA. (Figure 2)

Discussion

At present, it is well known that UVB irradiation capable of inducing DNA damage directly by absorption of energized UVB photons or indirectly 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 or named as photodynamic action, leading to ROS production.[11,39] The production of ROS by photodynamic action give rise several type of DNA damage including strand breakage, base modifications, and DNA-protein cross-linkage. [39,40] There is growing evidence that the ROS which is generated by UVB radiation comprise OH•, O-, and H2O2,[39] were able to induce oxidative stress characterized by increase in MDA and 8-oxo-2'-deoxyguanosine (8OHdG), otherwise decrease in total antioxidant capacity (TAC) and GPx activity,[7] and subsequently apoptosis.

The result of the present study was in line with those previous studies. In this result indicated that following UVB irradiation GPx activity in Neg-7 and Neg-15 was significant lower compared to that of normal. In contrary, XO activity in Neg-7 and Neg-15 was significant higher compared to that of normal. This result suggested that decrease in GPx activity and followed by increase in XO activity demonstrated that cells were suffering from oxidative stress. It was confirmed by the study of Higuchi and co workers indicated that increase in ROS capable of inducing oxidative stress and apoptosis mediated by XO and caspase$_3$ activity.[22] On the other hand, in vitro study was reported by Li and co workers showed that 6-CySeCD, a novel GPx also proven capable of improving DNA damage induced by UVB.[41] It was plausible since GPx serve as an antioxidant protector of lipid membrane and other cellular components.[42] In addition, in the present study also demonstrated that UVB irradiation during 7 days and seven days aftermath (day 15) was blank without UVB radiation, however the UVB radiation effect remain occur. It was demonstrated by the increased in XO activity and decreased in GPx activity significantly in Neg-15 compared to those of Neg-7. This result suggested that, although the UVB irradiation was halted, however the destruction effect of ROS triggered by UVB remain work continuously as well as oxidative stress. It was possibly caused by oxidant or oxidative stress memory of cells, however further research is needed. The similar result was reported by numerous studies, demonstrating that activities of SOD, Catalase, and Glutathione reductase were reduced after a single UVB irradiation. However, when exposure to UVB was prolonged for more than 12 weeks, the activity of SOD was also raised.[43].

The result of the present study also implied that UVB irradiation with 1.6 kJ per M^2 or equivalent to minimal erythematic dose (MED) for 30 minutes per day during seven days worked appropriately. Study was reported by Nasihun as to UVC irradiation on rats have similarity result to this finding. Because of UVC irradiation between 190 and 280 nm (peak 254 nm) at a distance of 20 cm for 30 minutes daily was able to increase level of MDA and 8OHdG, conversely decrease GPx activity and TAC concentration as markers of oxidative stress.[7] The result of the present study also supported by a lot of previous studies that irradiation of UVB capable of decreasing antioxidant and invariably followed by increasing oxidant activities, oxidative stress, cellular damages, and

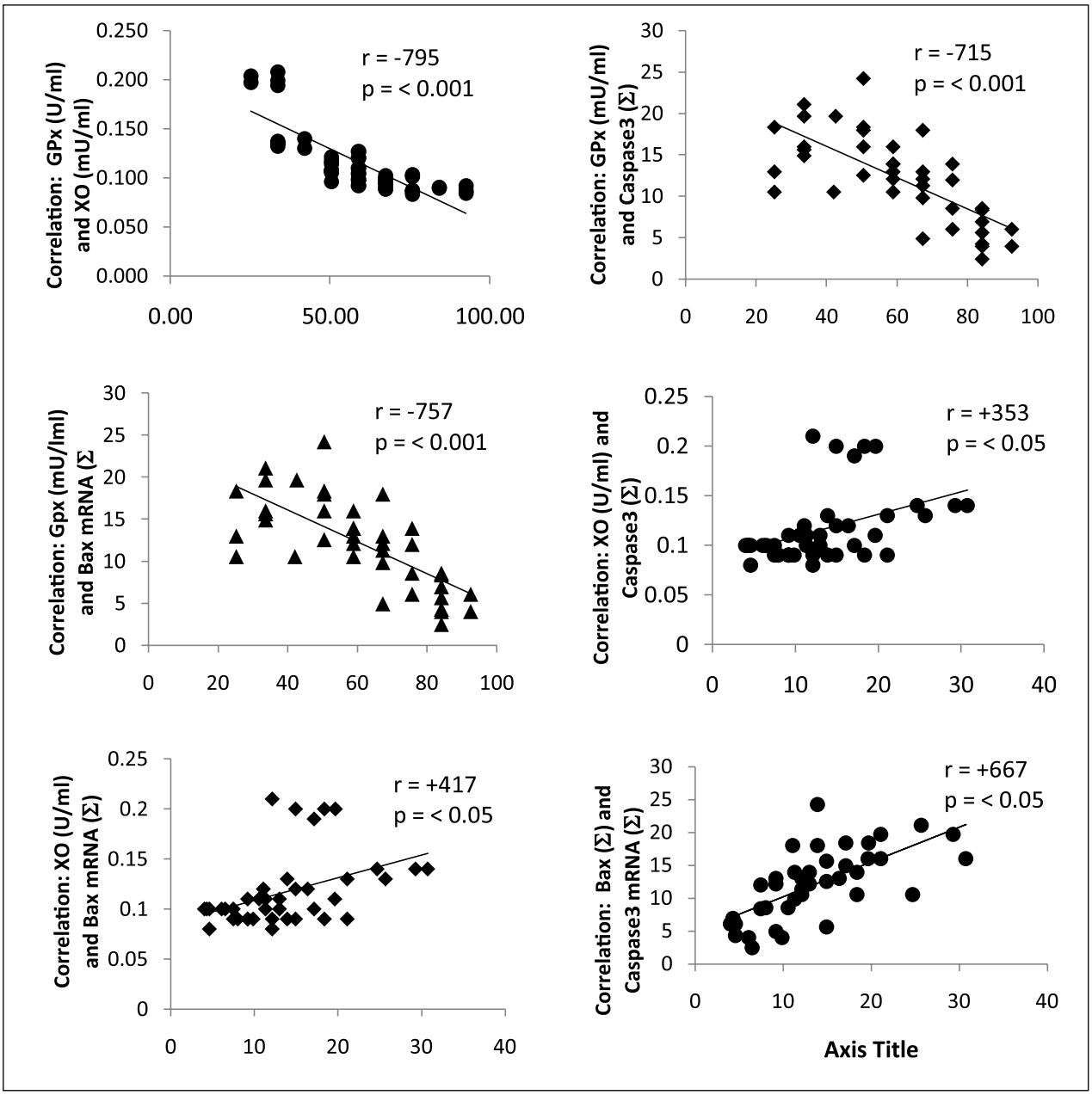

Figure 2. Pearson Correlation Analysis between GPx dan XO activities and Expression of Bax and Caspase3 mRNA on Sprague Dawley male rats after UVB irradiation

culminating in apoptosis.[39-41] It was plausible, since the increase in lipid peroxidation, MDA, and 8OHdG level, followed by decrease in SOD, Catalase, and GPx activities are prerequisite for cellular damages insulted by UVB.[11]

As mentioned above, H_2O_2, $OH^{\bullet}$, and O^{-}, were ROS resulted from UVB irradiation, by which several cellular components such as cellular membrane, mitochondrial and nuclear DNA suffers from damages.[30,42] The DNA damages were generated from reaction between ROS particularly radical $OH^{\bullet}$ and guanine residues of DNA producing 8-OHdG.[7,11] Moreover, the DNA damages is invariably followed by either DNA repair or apoptosis dependent on severity of DNA damages. If the enormity of DNA damages exceeding the capacity of DNA repair, consisting of mismatch repair, base excision repair, nucleotide excision repair, and double strands break repair, cells lead to undergo apoptosis instead of DNA repair, thus the defect of cell progeny can be prevented. However, high rate apoptosis can stimulate tissue's degeneration; subsequently decrease in strength and vitality. In contrary, slow rate apoptosis result in dysfunctional cells and tissues, potentially induce malignancy and aging. It is plausible owing to the DNA damages triggered by ROS encompasses

a large modification consisting of strand breakage, base modifications, and DNA-protein cross-linkage, resulting in mutagenesis, cellular senescence, and carcinogenesis.[39] Considering, apoptosis is resulted from enormous and unrepaired DNA damages and DNA damages is attributable to excessive oxidative stress, hence modify the degree of oxidative stress with antioxidant flavonoids in particular may inhibit apoptosis.[7,41]

Glutathion peroxydase is an enzyme dependent on the micronutrient selenium (Se), plays a critical role in the reduction of lipid and hydrogen peroxides.[44] If GPx activity is decreased, more hydrogen peroxide is present, which leads to direct tissue damage and activation of nuclear factor-κB–related inflammatory pathways. On the other hand, XO is an enzyme catalyzes the reaction of hypoxanthine to xanthine and xanthine to uric acid. In both steps, molecular oxygen is reduced, forming the superoxide anion followed by the generation of hydrogen peroxide.[45] In addition, various studies have also proven that intake exogenic antioxidant particularly from plants were able to counteract oxidative stress. It were attributable to phenolic compounds contained in plants.[46]

In the present study following treatment of PaM the GPx activity in PaM50-7, PaM100-7, and PaM150-7 was higher significantly compared to that of Neg-7 and Neg-15. In contrary, the activity of XO in PaM150-15, PaM100-15, PaM150-7, PaM100-7, and PaM50-7 was lowering significantly compared to that of Neg-7 and Neg-15. This result suggested that administration of PaM with 50 - 150 mg during 7 – 15 days were able to elevate antioxidant activity and lowering pro-oxidant activity, therefore ameliorate the oxidative stress in rats after UVB radiation. Interestingly, GPx activity in PaM150-15 and XO activity in PaM150-7 was not significant lower and higher respectively compared to those of Nor-G. This result suggested that dosing of PaM with 150 mg for 7 – 15 days was capable of increasing GPx activity and decreasing XO activity comparable to those of normal condition. This result corroborated by the study was reported by Nasihun et al. that supplementation of PaM was capable of increasing GPx activity and total antioxidant capacity, otherwise decrease MDA and 8OHdG levels in rats following UVC radiation.[7] Albeit UVB and UVC posses difference wavelength, however they have a common target on DNA to damage. The DNA damaged was induced by UVB and UVC maid occur both directly by cyclobutane pyrimidine dimers (CPD) and indirectly by ROS production.[7,11] Vast evidences pointed out that both CPD and ROS were able to trigger cellular apoptosis.[11]

Liver apoptosis may be induced by viral infection, excessive alcohol consumption, exposure any type of chemical carsinogenic, and DNA damage caused by high concentration of intra cellular ROS. In addition, the liver resident cells such as hepatocytes, cholangiocytes, activated stellate cells, and Kupffer cells express high levels of cell-death-associated receptors. Fas/FasL signalling has been largely implicated in liver pathophysiology but the mitochondrial intrinsic pathways are also involved in liver homeostasis.[47]

In the present study UVB irradiation have been proven induced liver cells (hepatocytes) apoptosis in rats characterized by increase in Bax and caspase3 mRNA expression in Neg-7 and Neg-15 compared to those of normal and PaM groups. Cellular apoptosis occurring after UVB irradiation may through both intrinsic and extrinsic pathways. The intrinsic apoptosis pathway can be triggered by both excessive ROS and DNA damages, by which mitochondrial DNA release Cytochrome c and Growth arrest DNA damage-inducible gene 45β (GADD45beta) regulates apoptotic cell death in response to DNA damage is upregulated. Moreover, Cytochrome c holoform along with apaf-1, procaspase$_9$, and ATP form apoptosome death complex (ADD), which proteolitically activate and convert procaspase$_9$ to caspase$_9$ and then activate caspase7 6, and caspase3, acting as apoptosis executor.[48] The activities of these caspases result in DNA fragmentation and cells death. Regulation of apoptosis was controlled by various pro-apoptosis protein such as Bax and Bak and anti apoptosis protein such as Bcl2 and Bcl-xL. Accordingly, in this context, occurrence of apoptosis is fully determined by the ratio between these pro and anti apoptosis protein which generated by severe oxidative stress triggerred by XO. A study was reported by Higuchi et al. showed that apoptosis can be induced by xanthin and XO. In the low level, XO may induce apoptosis mediated by protein like caspase3. In the high level, XO may induce necrosis also mediated by protein like caspase3. Conclusion was taken by the author that albeit apoptosis and necrosis could be induced by ROS, however, enzymes were engaged in this process is protein like caspse3 instead of caspase3.[22] The result of the present study is in disagreement with the Higuchi study, since in this study expression of Bax and caspase3 mRNA increased following UVB

irradiation. Considering, both Bax and caspase3 mRNA constitute pro-apoptosis protein, hence can be implied that increase in ROS after UVB radiation result in apoptosis mediated by Bax and caspase3. The available literatures have also confirmed that apoptosis triggered by UVB was mediated by caspase3 in intrinsically and extrinsically fashion. In the extrinsic apoptosis, UVB trigger and activate FAS/CD95 death-receptor pathway and in turn recruits caspase-8 to form the CD95 death inducing signaling complex (DISC). Oligomerization of caspase-8 upon DISC formation drives its activation through self-cleavage. Caspase-8 then eventually activates downstream effector caspases such as caspase3. Similarly, in intrinsic apoptosis, ROS which is produced by UVB is able to damage membrane mitochondrial and activate its protein effectors downstream and activation of caspase3 later on.[11,49,50]

The result of the present study also indicated that activities of GPx and XO were increased and decreased respectively following PaM treatment with 100 and 150 mg doses for 15 days. This result demonstrated that PaM containing flavonoid have been proven capable of increasing GPx activity and decrease XO activity. This circumstance bring about cells may able to circumvent from oxidative stress and prevent cell undergo apoptosis and death. Administration of PaM with 100 and 150 mg dose daily during 15 days was able to decreased expression of Bax mRNA and caspase3. There are mounting evidences that Bax/Bak and caspase3 are protein pro-apoptosis. The release of Cytochrome c from mitochondria following UVB radiation is dependent on JNK activation.[51-53] Protein pro-apoptotic Bax/Bak are the target of JNK induced apoptotic signalling pathway. On the other hand, protein anti apoptotic such as Bcl2/BclxL are phosphorilated and inhibited by JNK.[54] Therefore decrease in expression Bax and caspase3 mRNA going to prevent cellular apoptosis. Analysis correlation of Pearson also indicated that there is a negative correlation between GPx and XO activities. In addition, there was a positive correlation between XO and mRNA and Caspase3 (figure 2). Taken together the results of the present study imply that increase in oxidant invariably followed by decrease in antioxidant and apoptosis after UVB irradiation.

Conclusion

In conclusion, treatment of PaM with 100 and 150 mg dose per day during 15 days was capable of improving cellular oxidative stress and inhibits apoptosis.

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Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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