

RESEARCH ARTICLE

The Effect of Moringa Oleifera. Lam Leaf Extract on Bcl2 and Bax Expression in Paracetamol-induced Renal Tubular Apoptosis in Rats

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ABSTRAK

LATAR BELAKANG: Parasetamol dapat menyebabkan apoptosis pada tubulus ginjal. Daun kelor (*Moringa oleifera*.Lam) mempunyai efek antioksidan karena banyak mengandung flavonoid. Selain itu, flavonoid juga memiliki efek biologis untuk memodulasi jalur transduksi signal. **TUJUAN:** Untuk mengetahui pengaruh ekstrak daun kelor (*Moringa oleifera*.Lam) terhadap peningkatan ekspresi Bcl-2 dan penurunan ekspresi Bax pada kerusakan sel tubulus ginjal tikus yang diinduksi parasetamol.

METODE: Penelitian dengan rancangan "post tes only control group design" menggunakan 24 ekor tikus dibagi menjadi 4 kelompok. AqC-G diberi aquades, EtC-G diberi etanol 70%, MoL1-G dan MoL2-G diberi ekstrak daun kelor masing-masing 1,075g/KgBW dan 2,150g/KgBW. Ekspresi Bcl2 dan Bax diukur dengan menggunakan pengecatan immunohistokimia. Jumlah ekspresi Bcl2 dan Bax diuji dengan *One Way Anova* dilanjutkan dengan uji *Post Hoc LSD*.

HASIL: Hasil uji *Post Hoc LSD* menunjukkan bahwa ekspresi Bcl-2 pada MoL1-G (9,1) dan MoL2-G (13,683) lebih tinggi bermakna, $p < 0.001$. Di sisi lain, ekspresi Bax pada MoL1-G (6,85) dan MoL2-G (2,633) lebih rendah bermakna, $p < 0.001$.

KESIMPULAN: Pemberian ekstrak daun kelor dengan dosis 1.075 dan 2.150 meningkatkan ekspresi Bcl-2 dan menurunkan ekspresi Bax pada sel tubulus ginjal tikus Wistar yang diinduksi parasetamol.

Kata kunci: Daun kelor, ekspresi Bcl2, ekspresi Bax

ABSTRACT

BACKGROUND: Paracetamol may cause apoptosis in the renal tubules. Moringa leaf (*Moringa oleifera*. Lam) has been shown to have antioxidant effects due to its flavonoids. In addition, flavonoids also have biological effects to modulate the signal-transduction pathway. **OBJECTIVE:** to investigate the effect of Moringa oleifera Lam extract on Bcl-2 and Bax expression on paracetamol-induced renal tubular injury.

METHODS: in this post-test only control group design study, 24 rats were divided into 4 groups: AqC-G (aquades), EtC-G (70% ethanol), MoL1-G and MoL2-G (moringa leaf extract at the dose of 1.075 g/Kg BW and 2.150 g/Kg BW respectively). Expression of Bcl2 and Bax was determined using immunohistochemical staining. The data on the number of Bcl2 and Bax expressions tested with *One Way Anova* followed by the *Post Hoc LSD* test.

RESULTS: *Post Hoc LSD* test showed that the expression of Bcl-2 in the experimental groups ((MoL1-G (9.1) and MoL2-G (13,683)) was significantly higher compared to that of controls ($p < 0.001$). Bax expression in the experimental groups (MoL1-G (6,85) and MoL2-G (2,633)) was significantly lower compared to that of controls, $p < 0.001$.

CONCLUSION: The administration of Moringa leaf extract at the dose of 1,075 and 2,150 increases Bcl-2 expression and decreases Bax expression in paracetamol-induced renal tubular injury.

Keywords: Moringa leaf, Bcl2 expression, Bax expression

INTRODUCTION

According to research on in 2008, 18-27% of total acute kidney disease in the United States was caused by drug abuse. Paracetamol is one of the most commonly abused drug due to its affordable price, and fewer side effect. The overdosage of paracetamol can cause increased reactive paracetamol metabolite, N-acetyl p-benzoquinoneimine (NAPQI), which is free a radical substance (Katzung, 2012). Toxic dose of Paracetamol

(15 grams) can cause liver and kidney damage (hepatonephrotoxicity) i.e. acute tubular necrosis and apoptosis (Rini et al., 2013). Moringa leaf (*Moringa oleifera*.Lam; MoL) contains bioactive compound such as flavonoids, vitamin A, vitamin C, iron, zinc, and selenium (Singh et al., 2009). Moringa leaf prevents apoptosis due to its high antioxidant activity. Leaf Extract has been shown to improve renal cell damage. However, the inhibitory activity of Moringa against apoptosis in renal tubular

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cells has not been established.

In Indonesia, the incidence of kidney disease is estimated at 100 per million populations or about 20.000 new cases every year. Hemodialysis has been a major problem due to its expensiveness when it is not covered by health insurance. Most of the patients with kidney diseases seek medication in the late state because it showed no symptoms (asymptomatic) at first. Stage 2 kidney disease indicated by polyuria due to the renal tubular disorder. Patient with kidney diseases is less likely to recover (Widiana, 2007).

Several studies showed that moringa has a nephroprotective effect in mice (Reddy, 2013). The administration of Moringa leaf extract also showed improvement in telomerase and apoptotic activity in carcinogenesis in rats (Rassjad, 2011). Other studies found that ethanolic extract of moringa lowered damage in renal proximal tubular epithelial cells at the dose of 4 mg, 8 mg, and 16 mg/20 grams in mice (Rahman, 2015). However, the study did not determine whether the renal tubular repair was mediated by apoptosis (Reddy, 2013; Rassjad, 2011; Rahman, 2015).

Moringa leaves have been shown to have antioxidant activity due to its flavonoid compounds including quercetin (Singh et al., 2009). Besides functioning as antioxidants, flavonoids can also promote cell signaling by modulating cell signal transduction, regulating the growth process, proliferation, and apoptosis in various tissues. Various studies showed that the high levels of ROS can induce apoptosis in a variety of cell type.

There is a negative correlation between levels of ROS and Bcl-2 in cells. Decreased levels of ROS are followed by increased Bcl-2 expression and vice versa (Hildeman et al., 2013). Bcl-2 has been shown to prevent cell death or apoptosis, possibly through intrinsic apoptosis mechanism (Sasi, et al., 2009). Bcl-2 Protein represents the characteristics of the first gene involved in programmed cell death by inhibiting apoptosis (anti-apoptosis) and improving cell survival. Whilst the Bax protein, is a pro-apoptotic family that can induce apoptosis (Cox & Hampton, 2007; Anderson, et al., 2009).

Toxic dose of paracetamol could cause apoptosis on renal proximal tubulas. In the study using moringa leaf extract dose of 4 mg/BW, 8 mg/BW and 16 mg/BW in mice, proved capable of reducing paracetamol-induced renal tubular damage (Rahman, 2015). Therefore, the purpose of this study was to determine whether moringa leaves affect Bcl-2 and Bax expression in paracetamol induced renal tubular damage in rats.

METHODS

Study Design

This research was designed as the post-test control group design. Twenty four wistar rats were randomly divided into 4 groups of 6 rats each: Group-1 (AqC-G) induced paracetamol and given 2 ml aquadest; group-2 (EtC-G), paracetamol-induced rats and 0.01 ml ethanol; group-3 (MoL1-G), paracetamol-induced rats and Moringa leaf extract at a dose of 1.075 g/kg BW; group-4 (MoL2-G), paracetamol-induced rats and Moringa leaf extract at a dose of 2.150 g/kg BW. Paracetamol induction was given for one week at a dose of 240 mg/rat. All treatments were given daily for 7 days.

Moringa Leaf Extraction

Moringa leaf used was separated from the small branches then washed and dried under the sunlight before kept in the drying cupboard. After it dried, it was blended, weighed to obtain 100 grams and mounted to an Erlenmeyer glass. After soaked with 96% ethanol up to 900 ml volume, it was mixed by stirring. After sitting overnight, the top layer of the solvent part was taken and then filtered using filter paper. The top layer of ethanol mixture with active ingredients was taken and loaded into the evaporating tube installed in the evaporator. The water bath was filled with water. The tools including rotatory evaporator, water bath heater installed and connected to the power supply. The ethanol solution was allowed to separate with the active substance already present in the tube. After 1.5-2 hours, the result obtained approximately ½ of the materials used. The extract was inserted into plastic bottles and kept in freezer.

Examination of Bcl2 and Bax Expressions with Immunohistochemical Methods

The dissected tissue was in piece of the paraffin block placed on the slide, then washed with xylol 2 times each for 3 min, then rehydrated with stratified alcohols (absolute 95%, 90%, 80%, 70%) respectively for 5 minutes. The preparation was washed with PBS for 10 minutes. Then it is incubated with 100 micro trypsin for 20 minutes. Then it was washed with PBS 2 times each for 10 minutes. Furthermore, it was incubated with H₂O₂ (Hydrogen Peroxide) let to sit for 10 minutes before washed under tap water and PBS for 2-5 minutes. The preparations were incubated in prediluted blocking serum for 10 minutes at 25 °C. Each of the Bcl-2 and Bax antibodies was added to a 100-ul preparation. Incubate 1 hour at room temperature or

overnight with a temperature of 4 °C. Preparations were washed with PBS for 5 minutes. The preparations were incubated with ready to use streptavidin/peroxidase complex reagents for 10 minutes and washed with PBS for 5 minutes. The preparations were incubated in 15-100 micro biotin for 20 minutes before it was washed with PBS for 2 minutes 2 times each. The preparations were incubated in 100 peroxidase substrate solution (DAB) per preparation for 7-10 minutes. Preparations were washed with tap water. Mayer hematoxylin (counterstain) was then incubated 1-3 minutes, and then washed under tap water. The next slide is immersed in the alcohol and then cleansed. The mounting media then covered with glass tube and was observed using a light microscope.

Cells that express Bcl2 or Bax proteins are brownish cells in immunohistochemical imaging. The calculation of cells expressing Bcl2 and Bax is by calculating the brown cells in the cytoplasm in the 10 rectangular tubular field by using the Olympus BX 41 light microscope 400 times magnification of each preparation.

STATISTICAL ANALYSIS

The data on Bcl2 and Bax expression in rat renal tubular cells were statistically analyzed using ANOVA, with a significance level of 0.05 ($p = 0.05$). The analysis was performed after normality and homogeneity test

of the variants for the two study parameters. This study was conducted after obtaining approval from the Commission of Ethics, Medical Faculty of UNISSULA Semarang.

RESULTS

The study was conducted between November and December 2016 in the Parasitology Laboratory, Faculty of Medicine of Gadjah Mada University, Yogyakarta, and Pathology Anatomy Laboratory of Medical Faculty of UNISSULA Semarang/Islamic Hospital of Sultan Agung, Semarang.

The histopathologic features of the Bcl-2 and Bax expression in renal tubulus in rats after staining is shown in figure 1.

The highest mean of Bcl-2 expression was found in the MoL2-G group, followed by the MoL1-G group, EtC-G group, and the lowest was the AqC-G group. In contrast, the highest mean of Bax expression was found in the EtC-G group, followed by the AqC-G group, the MoL1-G group, and the lowest being the MoL2-G group. The normality and homogeneity test of Bcl-2 and Bax expression data were analyzed by Shapiro Wilk and Levene test showed the distribution of data was normal and homogeneous meeting the requirement of the parametric test One Way Anova. One way Anova showed that there was a significant difference in Bcl-2 and Bax expression among the groups, $p < 0.001$.

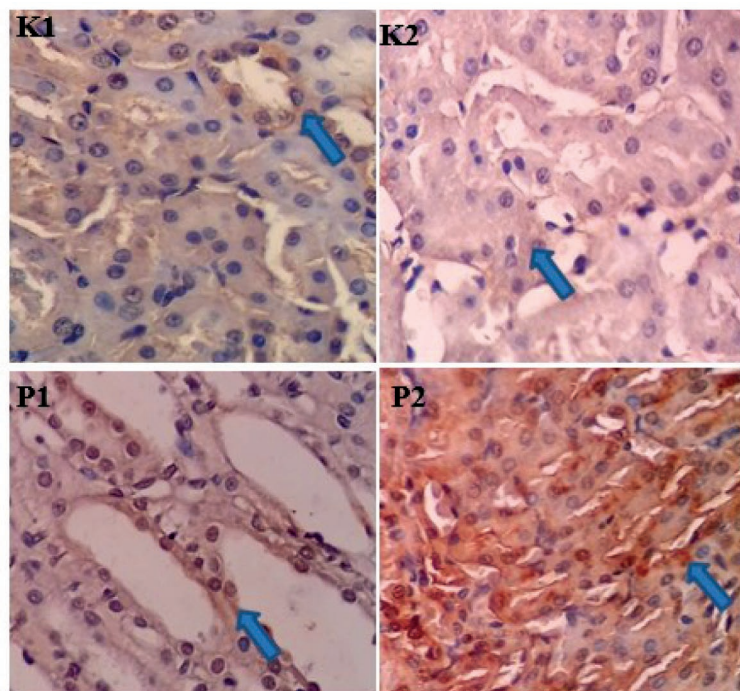


Figure 1. Bcl2 expression counts in the renal tubulus of control group and the experimental group, IHC staining, using the Olympus BX Light Microscope at 41 times magnification showed a positive immunostaining expression against Bcl2.

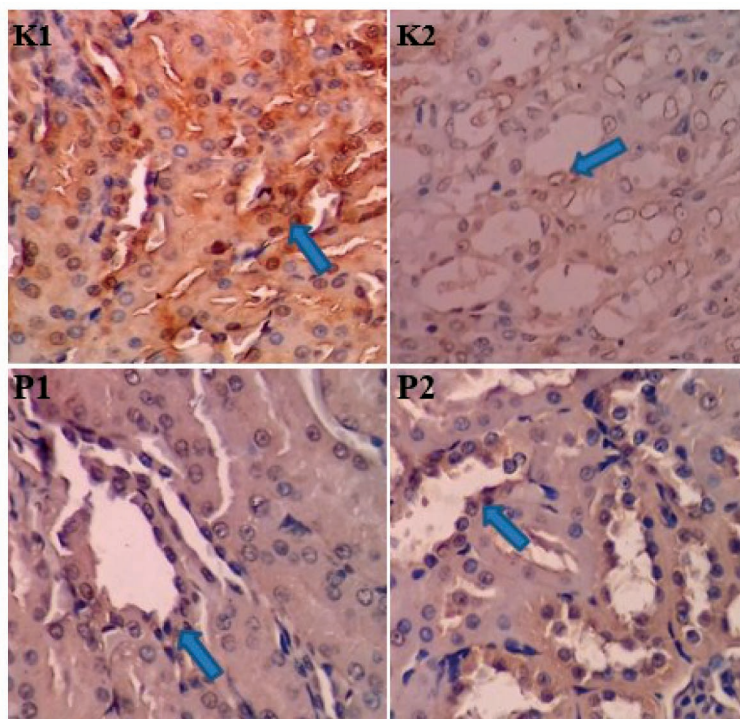


Figure2. Bax expression counts in the renal tubulus of control group and the experimental group, IHC staining, using the Olympus BX Light Microscope at 41 times magnification showed a positive immunostaining expression against Bax.

Table 1. Analysis of Bcl-2 and Bax Expression on different dose of Moringa Leaf Extracts

Variables	GROUPS				P
	AqC-G	EtC-G	MoL extracts		
	(n=6) (Mean ± SD)	(n=6) (Mean ± SD)	MoL1-G (n=6) (Mean ± SD)	MoL2-G (n=6) (Mean ± SD)	
Bcl2 expression	0,320 ± 0,0837	0,650 ± 0,2429	9,1 ± 0,7155	13,683 ± 0,3312	
<i>Shapiro wilk</i>	0,314	1,000	0,314	0,229	>0,05*
<i>Levene test</i>					0,162**
<i>One way anova</i>					0,000***
Bax expression	9,940 ± 0,4506	13,017 ± 0,3817	6,85 ± 0,2258	2,633 ± 0,2066	
<i>Shapiro wilk</i>	0,074	0,532	0,6	0,473	>0,05*
<i>Levene test</i>					0,476**
<i>One way anova</i>					0,000***

To determine the expression of Bcl-2 and Bax in each group, an LSD post hoc assay was applied, and the results are presented as follows:

The result of post hoc test showed that Bcl-2 expression in group AqC-G was lower compared with that of in EtC-G group but the difference was not significant (p=0.215). Bcl-2 expression of the MoL1-G group was significantly higher compared with that of in AqC-G group, p <0.001. Bcl-2 expression of the MoL2-G group was significantly higher compared with the AqC-G group (p <0.001) (figure 2).

The results of this study showed that moringa leaf extract could increase Bcl-2 expression.

The result of post hoc test showed that that of Bax expression in group AqC-G was significantly lower compared to that of EtC-G (p<0.001). Bax expression of the MoL1-G was significantly lower compared with that of AQC-G group (p<0.001). Bax expression of MoL2-G was significantly lower compared with that of group Aqc-G (p<0.001). Bax expression in the EtC-G group was significantly compared to that of the MoL1-G group (p <0.001). Bax expression in the EtC-G group

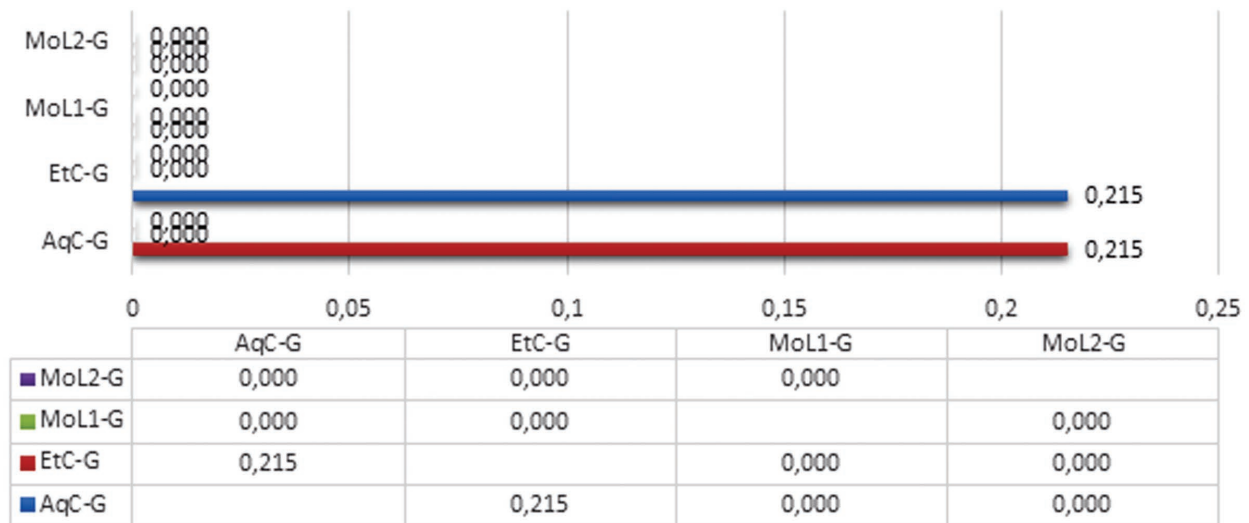


Figure 3. Mean Differences of Bcl2 expressions between two groups

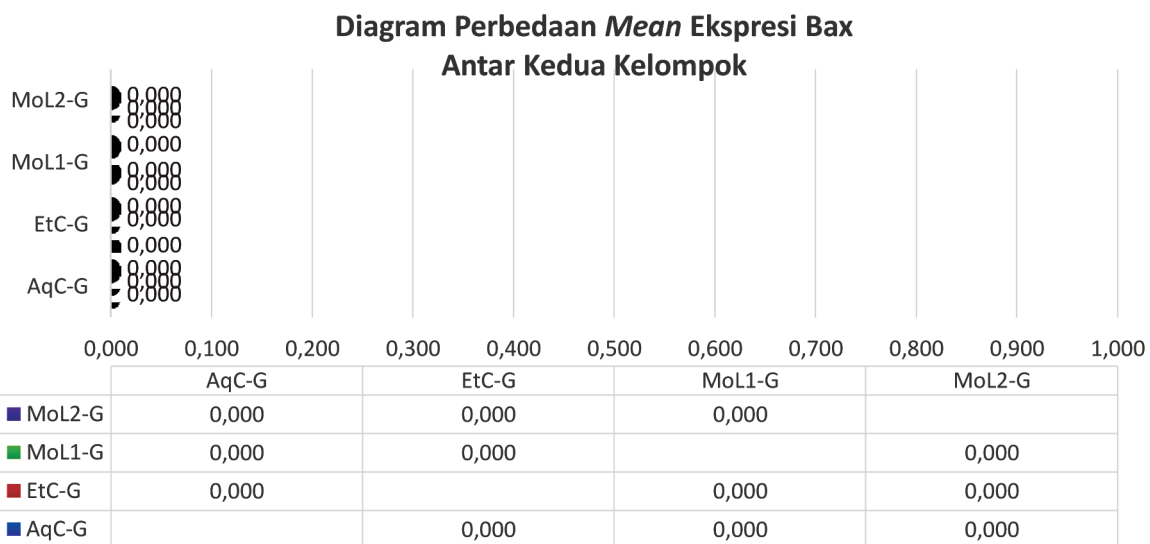


Figure 4. Mean Differences of Bax expressions between two groups

was significantly higher compared with that of MoL2-G group, ($p < 0.001$). Bax expression was significantly higher in the MoL1-G group compared with that of in the MoL2-G group ($p < 0.001$) (figure 3).

The results of this study suggested that that moringa leaf (*Moringa Oleifera* Lam) extract can decrease Bax expression.

DISCUSSION

The finding showed that the induction of paracetamol were able to cause kidney damage. The study resulted in the lower Bcl-2 and higher Bax expression in negative control, compared to the group treated with moringa leaf extract. This finding supports the study by Rahman (Rahman, 2015). Paracetamol toxic doses may induce renal tubular damage due to reactive and toxic

NAPQI. The highly reactive metabolite of paracetamol is N-acetyl-p-benzoquinimine (NAPQI), formed through the cytochrome P450 dependent pathway. NAPQI will react with nucleophilic groups in proteins, DNA, and mitochondria, cause oxidative stress that can lead to cell death or apoptosis (Katzung, 2012). A high concentration of NAPQI metabolite can cause damage to the liver and kidneys (Rahman, 2015). In the use of therapeutic dose of paracetamol, side effects can be minimized as long as glutathione is present. Glutathione conjugate form non-toxic mercapturic acids. Overuse of paracetamol will cause a reduction in glutathione reserves, leading to the failure to convert to NAPQI into mercapturic acid (Katzung, 2012). Bax expression in the ethanol group was significantly higher than that of in the aquadest groups. This demonstrated

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that in the ethanol group there is more damage to renal tubular cells than in aquadest group. This supports the theory that ethanol is destructive, where the cell damage caused by ethanol is due to its interaction with the membrane which will affect membrane function in transmitting intracellular signals. It is suspected that ethanol stimulates the formation of acetaldehyde and decreases the ratio of NAD⁺/NADH. The ethanol poisoning cause increased Ca⁺ concentration, decreased cytoskeleton and ATP, resulting in blebs, leading to the activation of the caspase system and the lysosomal enzyme to stimulate apoptosis and necrosis (Hernawati, 2011)

Moringa leaf extract at doses of 1.075 g/Kg BW was significantly higher than that of aquades and ethanol, indicating that administration of Moringa leaf extract had an effect on Bcl-2 expression, while 2.150 g/Kg BW dose resulted in higher increment effect. This suggested that the higher dose of Moringa leaf cause a higher Bcl-2 expression, which has been shown to reduce cell death or apoptosis through intrinsic pathway. On the other hand, high dose of moringa leaf extract can decrease Bax expression leading to renal tubular damage.

Moringa leaf is rich in polyphenolic compounds including flavonoid that is found in plants with antioxidant activation. One of the flavonoids contained in the moringa leaf is quercetin. In addition to its function as an antioxidant, flavonoids can also help signaling cells, which modulate signal transfers such as growth, poliferation, and cell death (apoptosis). In addition to quercetin, moringa leaves contain antioxidants, such as gallic acid, cholesterolic acid, ellagic acid, ferulic acid, kaempferol, proantosianidin, vinilin, vitamin C, vitamin E, β-carotene, zinc, and selenium (Singh, et al., 2009). β-carotene is one of the fat soluble carotenoids which is a pro-vitamin A essential for vision function. In addition, β-carotene has a strong antioxidant function and is a singlet oxygen (oxygen with high reactivity) breaker. β-carotene may protect fat-rich tissue against lipid peroxidation (Chakraborty, et al., 2007). Vitamin C (ascorbic acid) is one of the antioxidants (Sasi, et al., 2009) that can prevent lipid peroxidation and assist regeneration of vitamin E by reacting with a radical form of vitamin E (Lu, et al., 2012). Vitamin C can also help regeneration from glutathione. Glutathione is an antioxidant playing an important role in glutathione peroxidase (GPX) enzyme activity. Moringa leaves also contain minerals that act as antioxidants such as iron (Fe) and zinc (Zn) (Landete, 2012).

The administration of leaf extract increased expression of Bcl-2 in a dose dependent manner, in

which the highest dose showed the highest effect. It is associated with antioxidant compounds (flavonoids) in moringa leaf. On the other hand, with the addition of a dose of moringa leaf extract there was a significant decrease in Bax expression in renal tubular cells when compared with aquadest and ethanol. This also happens because the antioxidants in moringa leaf are able to inhibit the increase of ROS (Reactive Oxygen Species) due to paracetamol induction, so that cell and cell damage can be prevented, through decreased Bax expression.

The dose of moringa leaf extract used in this study was determined from the conversion value from previous research. Previous research with the administration of leaf-ethanol extract of moringa with the dose of 4 mg, 8 mg, and 16 mg was performed on paracetamol-induced mice showed a nephro-protective effect on proximal renal epithelial cell damage.

CONCLUSION

The results of this study showed that the administration of moringa leaf extract at 1.075 g/Kg BW, and 2.150 g/Kg BW for one week increased Bcl2 expression and decreased Bax expression in parasetamol-induced tubular injury(damage). The administration of leaf extract of Moringa at the dose of 2.150 g/Kg BW was more effective than 1.075 g/Kg BW.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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