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Antioxidant activity of ethanolic extract of three *Selaginella* species from Java Island, Indonesia

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Abstract. *Miftahudin, Hasibuan RS, Chikmawati T. 2019. Antioxidant activity of ethanolic extract of three* Selaginella *species from Java Island, Indonesia. Biodiversitas 20: 3715-3722.* Three *Selaginella* species, *S. ornata, S. plana,* and *S. willdenowii,* from Java Island, Indonesia, have been known to have antioxidant properties; however, *in vivo* antioxidant activities of these species have not been reported. This research aimed to evaluate the *in vivo* antioxidant activity of ethanolic extract of three *Selaginella* species. The 70% ethanol extract of three *Selaginella* species at four different doses was administered to mice one day before being treated with oxidative stress. The liver tissue of mice treated with or without oxidative stress was analyzed their lipid peroxidation by measuring MDA concentration and Superoxide Dismutase (SOD) activities. The results showed that there were variations in antioxidant activity among the three *Selaginella* species. In general, the dose of 0.3 g extract kg^{-1} BW has been able to reduce lipid peroxidation and increase SOD activity. The administration of *S. ornata* extract to the mice at 1.2 g extract kg^{-1} BW reduced the MDA concentration to the lowest level, but the same dose of two other *Selaginella* extracts caused toxic effects in mice. The antioxidant activities of *S. ornata* and *S. plana* were better than that of *S. willdenowii* extract, and among those species, *S. ornata* has the best antioxidant activity.

Keywords: Antioxidant, MDA, *Selaginella,* SOD

INTRODUCTION

The declining quality of the environment causes humans to be continuously exposed to polluted environments, high ultraviolet radiation, and other free radicals that cause oxidative stress, which subsequently causes premature aging and various degenerative diseases. Consumption of antioxidants is needed to reduce the level of oxidative stress. Antioxidants are compounds that can delay, slow down, or prevent free radicals from causing lipid peroxidation. Antioxidant compounds play an important role in reducing oxidative damage of cells and tissues caused by Reactive Oxygen Species (ROS). Free radicals include superoxide anion radicals, hydroxyl radicals, oxygen singlets, and non-free radical compounds such as hydrogen peroxide (Kumar et al. 2010; Nimse and Pal 2015).

Antioxidants are widely used in the food industry to prevent food deterioration and extend food shelf life (Ghasemzadeh et al. 2012). In recent years, there has been a rapidly growing interest in natural antioxidants since synthetic antioxidants are suspected to increase the risk of human cancer and liver damage (Mavundza et al. 2010). Some medicinal plants have been found to possess antioxidant activity due to the presence of diterpenes, flavonoids, tannins, and phenolic acids (Dawidowicz et al. 2006; Irudayaraj 2010).

Selaginella, the genus of Pterydophyte, is characterized by scale-like leaves (microphylls), mostly growing on organically-rich, moist, well-drained soils in shade or half shade, often near streams, beside trails, and at the edge of clearings in lowland to mid-montane primary and secondary forests (Mukhopadhyay 2001). *Selaginella* is a potential source of natural antioxidant since it is rich in active biflavonoid compounds, secondary metabolites in the dimer form of flavon and flavanone with 5,7-4'-oxygenation pattern (Seigler 1998). Previous studies showed that several species of *Selaginella* exhibit good antioxidant activities. The extract of *S. bryopteris* contributes favorably to the memory enhancement effect (Garg et al. 2012), possess anti-stress and antioxidant activities that may help relieve stress-induced complications including those caused by heat shock (Sah et al. 2005). The study by Woo et al. (2005) showed that the extract of *S. tamariscina* inhibits the production of oxide nitrate, and the expression of nitrate oxide synthase (iNOS) induced lipopolysaccharides (LPS). Chai and Wong (2012) reported that aqueous extract of *Selaginella willdenowii* contains phenolic dan flavonoids and showed the free radical scavenging and ferric reducing activities so that *S. willdenowii* was a good source of dietary antioxidant as well as a medicinal herb. Furthermor, Chai and Wong (2012) reported that the antioxidant activity of *S. willdenowii* was correlated to the total phenolic content. Ethanolic extracts of *S. involvens*, *S. intermedia*, *S. inaequalifolia,* and *S. tenera* have been reported to possess antioxidants activity (Sivaraman et al. 2013). Yao et al. (2017) reported that *S. doederleinii* contains eight biflavonoid types which enhanced the antitumor immune response in the mouse lung cancer model. Syaefudin et al.

(2016) reported that nanoparticle of *S. doederleinii* leaves extract could inhibit A549 cancer cell growth. The extract of *S. convoluta* also showed good antioxidant activities that correlated with the presence of phenolic compounds in its active fractions (de Oliveira Macêdo 2018).

In Indonesia, *Selaginella* is found on several islands including Sumatera, Java, Lesser Sunda Island, and the Moluccas. *Selaginella ornata, S. plana*, and *S. willdenowii* are abundantly found in Java Island. Local people in West Java use those three species of *Selaginella* as medicinal plants. Extracts of those three species were reported to contain alkaloid, flavonoid, saponin, tannin, and steroid (Chikmawati et al. 2012). The previous study by Chikmawati et al. (2009) showed that the extracts of 3 *Selaginella* species were able to scavenge radical hydroxyl and inhibit lipid peroxidation in the liver cell of mice. Besides, *S. plana* extract has the best ability to reduce lipid peroxidation (40%) (Chikmawati et al. 2009). To the best of our knowledge the *in vivo* antioxidant activity of *S. ornata*, *S. plana*, and *S. willdenowii* collected from Java Island has not been reported. The objective of this study was to evaluate the *in vivo* antioxidant activity of three *Selaginella* species (*S. ornata, S. plana*, and *S. willdenowii*) collected from Java Island in mice.

MATERIALS AND METHODS

Plant materials and plant extract preparation

Three species of *Selaginella*, i.e., *S. ornata*, *S. plana*, and *S. willdenowii*, were used in this experiment. Crowns (leaves and stems) of three *Selaginella* species were cleaned under tap water, drained and dried in an oven at 50ºC for three days. Dried samples were ground into powder using a grinder. Five grams of *Selaginella* powder of each species were macerated with 100 mL of 70% ethanol at room temperature for 24 h and stirred with a stirrer bar at 300 rpm for four hours (Gayathri et al. 2005). The filtrate was filtered using Whatman filter paper no. 42 and evaporated using a rotary vacuum evaporator at 60ºC for 3 to 4 h at 200 rpm. The remaining water was removed by drying the concentrated extract with a freeze dryer to form a paste and stored in a refrigerator at 4ºC.

Experimental animals

Male DDY (Deutch Danken Yolken) mice, 2.5-3-month-old, weighing 21.7-40.8 g were used to evaluate the antioxidant activity of the *Selaginella* extract. The experimental procedure on animals was carried out following the Experimental Rules of Bogor Agricultural University, Indonesia. The experiment was carried out in Pharmacology Laboratory, Faculty of Veterinary, Bogor Agricultural University, Indonesia with room temperature condition and lighting with 12 hours white light each day.

Acute toxicity

The toxic effect of extract of three *Selaginella* species was tested on male DDY mice. A preliminary study of the toxic effect using a dose of 15 g extract kg^{-1} BW (bodyweight). *Selaginella* extract is administered orally once to mice. Each extract of *Selaginella* was tested on five mice. Bodyweight and mortality were observed at 24 h and the seven days after treatment (Harmita and Radji 2008).

Acute toxicity test to determine the LD_{50} value of three *Selaginella* extracts was carried out using the Weil method (Weil 1952). Four doses of *Selaginella* extract (1, 3, 9, dan 27 g extract kg^{-1} BW) were administered orally to mice. Each experimental unit consisted of four mice. The dosage selection was carried out based on geometric progression (Harmita and Radji 2008) using the following formula:

$$Y_N = Y_1 R^{N-1}$$

Where:
Y_N : the N^{th} dose
Y_1 : the first dose
R : multiplication factor
N : dosage series

The toxic effects of the *Selaginella* extract were evaluated at 24 h and seven days after treatment (Harmita and Radji 2008) and the median lethal dose (LD_{50}) was calculated based on the Weil method (Weil 1952) using the following formula:

$$\text{Log } m = \log D + d(f+1)$$

Where:
m : LD_{50} value
D : the smallest dose given
d : log of dose multiplication (log R)
f : a factor in Weil table

Experimental design for in-vivo antioxidant assay

The experiment was designed in a Completely Randomized Design with two factors. The first factor was three species of *Selaginella* (*S. ornata*, *S. plana* and *S. willdenowii*), and the second factor was the dose of *Selaginella* extract (0, 0.3, 0.6, dan 1.2 g extract kg^{-1} BW). Mice were divided into four treatment groups, i.e.: (i) Mice were neither administered with *Selaginella* extract nor treated with oxidative stress, (ii) Mice were not administered with *Selaginella* extract but treated with oxidative stress, (iii) Mice were administered with *Selaginella* extract but not treated with oxidative stress, (iv) Mice were both administered with *Selaginella* extract and treated with oxidative stress. The *Selaginella* extracts were administered orally using a syringe (oral sonde). Each experimental unit has consisted of 3 mice.

Selaginella extract application and oxidative stress treatment

The administration of *Selaginella* extracts and oxidative stress treatment was conducted based on the method by Gayathri et al. (2005) and Wresdiyati et al. (2007) with a modification. An amount of 0.5 ml *Selaginella* extract with the appropriate dose of treatment was administered orally to the mice once a day before oxidative stress treatment. Oxidative stress was induced by fasting, but mice have free access to drinking water and the animals subjected to the

forced swimming activity for five minutes a day for three days. On the fifth day, mice were euthanized with cervical dislocation procedure, and the liver samples were dissected from each mouse, soaked in 0.9% NaCl and then stored in a 1.15% KCl solution (Okhawa et al. 1979) for further use.

Liver homogenate preparation

The liver was washed with aquabidest and weighed to make a final concentration of 25% homogenate in aquabidest (Gayathri et al. 2005). Liver tissue was ground with a mortar and filtered with cheesecloth. The filtrate was either directly used for analysis or stored at-20ºC for further use (Hasani et al. 2007).

Lipid peroxidation assay

The ability of *Selaginella* extract to inhibit membrane lipid peroxidation was tested using the Fe^{2+}/ascorbate system (Gayatri et al. 2005). Lipid peroxidation measurement by Thiobarbituric acid (TBA) assay was carried out according to the method by Okhawa et al. (1979) and Mihara et al. (1980). 0.1 mL liver homogenate (25% w/v) added with 0.1 mL 1M Tris-HCl (pH 7), 0.1 mL 1.5 mM ascorbic acid, 0.1 mL 4 mM Fe-sulfate ammonium, and 0.1 mL distilled water in a total volume of 0.5 mL. The mixture was incubated at 37ºC for one h and then added with 0.5 mL Trichloroacetic Acid (TCA, 0.1%) solution containing 1mM Butylated Hydroxytoluene (BHT) at 4ºC. The homogenate was added with 3 mL of 2% (vv^{-1}) H3PO4 solution and 1 mL of 0.6% (w v^{-1}) TBA in a 20% TCA (w v^{-1}). The mixture was incubated at 100ºC for 30 min, then cooled to room temperature. After reaching room temperature, 4 mL of 100% (v v^{-1}) n-butanol was added to the mixture and then vortex vigorously. The butanol and the solution phases were separated by centrifugation at 3000 rpm for 30 min (Labofuge 400R). The absorbance of the TBA-MDA complex in the butanol phase was measured with a spectrophotometer at λ 532 nm, while the non-specific absorbance value was measured at λ 520 nm. MDA concentration as the product of lipid peroxidation calculated by reducing the absorbance value at λ 532 nm with the absorbance value at λ 520 nm.

The concentration of MDA was calculated using the following formula:

$$[\text{MDA}] = \frac{A}{\varepsilon \text{ x d}} \times V$$

Where:
[MDA] : MDA concentration formed (nmol)
A : Different in absorbance value
Ɛ : MDA extinction coefficient (155 $mM^{-1}cm^{-1}$)
d : Cuvette width (cm)
v : Volume sample (mL)

Superoxide dismutase (SOD) analysis

Superoxide dismutase (SOD) was analyzed based on the method of Kubo et al. (2002) and Wijeratne et al. (2005) with slight modification. The method measures the activity of superoxide anion radicals produced enzymatically by the xanthine-xanthine oxidase system. A volume of 0.06 ml liver homogenate was reacted with a solution consisted of 2.7 mL of 40 mM Sodium Carbonate buffer containing 0.1 mM EDTA (pH 10), 0.06 mL of 10 mM Xantin, 0.03 mL of 0.5% BSA and 0.03 mL of 2.5 mM NBT (nitroblue tetrazolium). Subsequently, the solution was added with 0.1 mL Xanthine Oxidase (0.04 units) and then incubated at room temperature for 30 min. The absorbance was measured at λ 560 nm. The SOD activity (%) was calculated using the following equation:

$$\left[1 - \frac{A}{B}\right] \times 100$$

Where:
A: absorbance of sample solution;
B: absorbance of control

Data analysis

Data were analyzed using Analysis of Variance (ANOVA) at a confidence level of 95 %. If the treatments have a significant effect on the response, it was then followed by Duncan Multi Range Test (DMRT) with α = 0.05.

RESULTS AND DISCUSSION

LD_{50} (Median Lethal Dose) value

The preliminary study of acute toxicity test using a dose of 15 g extract kg^{-1} BW was carried out to determine the LD_{50} of *Selaginella* extracts. The results showed that the mortality rate of three species of *Selaginella* extract reached 80% at the 24 hours after administering with *Selaginella* extracts, and the rest were still alive until the 7th after administering *Selaginella* extract. The LD_{50} value could not be obtained from the preliminary study. On the last day of observation, showed that the bodyweight of mice received *S. ornata* and *S. plana* extracts were increased 1.1 and 0.5 g, respectively, while mice received *S. willdenowii* extract experienced weight loss of 1.2 g (Figure 1).

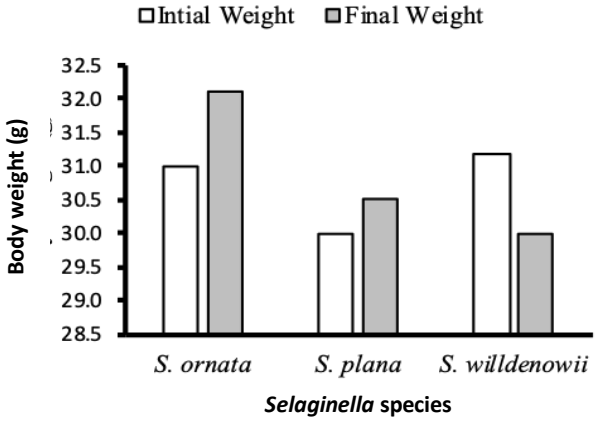

Figure 1. The initial and final body weight of mice received *Selaginella* extracts (*S. ornata*, *S. plana*, and *S. willdenowii*) in a preliminary test of acute toxicity using a dose of 15 g extract kg^{-1} BW.

Table 1. Mortality rate (%) of mice at 24 h after receiving *Selaginella* extract

Species	Doses (g extract kg^{-1} BW)			
	1	3	9	27
	Mortality (%)			
S. ornata	0	0	50	100
S. plana	0	0	100	100
S. willdenowii	0	75	75	100

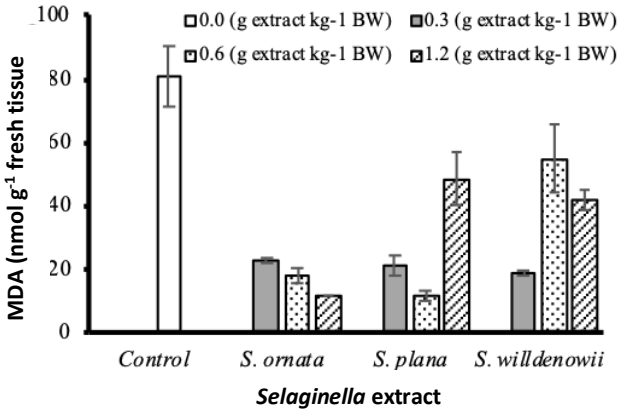

Figure 2. MDA concent of mice receiving an extract of *S. ornata*, *S. plana*, and *S. willdenowii* after being exposed to oxidative stress. The control treatment was a group of mice that did not receive *Selaginella* extracts but exposed to oxidative stress. Bars are standard errors of the mean

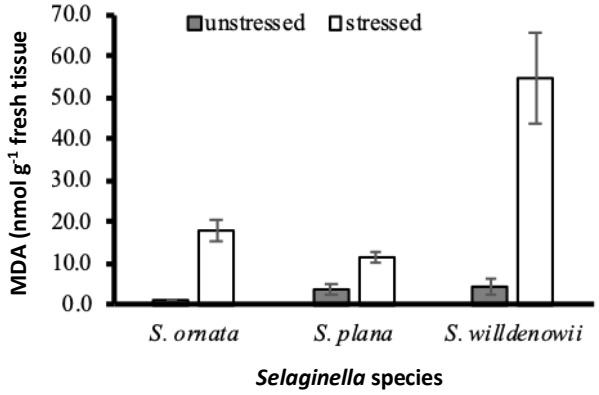

Figure 3. MDA concentration of stressed and unstressed mice receiving 0.6 g extract kg^{-1} BW of three species of *Selaginella* extract. Unstressed mice were the mice that received the extract but not treated with oxidative stress. Bars are standard errors of the mean

Acute toxicity test to determine the LD_{50} value of the three *Selaginella* extracts was carried out in four different doses, i.e., 1, 3, 9, and 27 g extract kg^{-1} BW. The results showed changes in body weight on the 7th after treatment. In general, the mortality rates increased with increasing dose of *Selaginella* extract. The mortality rate of mice that received *S. ornata* and *S. plana* extracts increased in the treatment after receiving a dose of ≥ 9 g extract kg^{-1} BW, while the mortality rate of mice receiving *S. willdenowii* extract increased in the treatment of ≥ 3 g extract kg^{-1} BW (Table 1). LD_{50} values of three species *Selaginella* extract was determined based on the percentage of mice mortality by Weil method (Weil 1952) were 9, 5.2, and 3 g extract kg^{-1} BW respectively for *S. ornata*, *S. plana*, and *S. willdenowii* extracts. Based on the LD_{50} and the differences with Median Effective Dose (ED_{50}), so that three doses of extract (0.3, 0.6, and 1.2 g extract kg^{-1} BW) were applied to evaluate the antioxidant activity of three species of *Selaginella* extracts.

The effect of oxidative stress treatment on MDA content

Oxidative stress was applied to the starved mice with forced-swimming for five minutes a day. The effect of oxidative stress treatment in mice was measured as the content of MDA and SOD in stressed and unstressed mice. The results showed that the MDA concentration of liver in stressed mice (80.65 ± 9.68 nmol g^{-1} fresh liver tissue) was higher than that of in unstressed mice (MDA = 7.74 ± 3.87 nmol g^{-1} fresh liver tissue); while SOD content of stressed mice (32.00 ± 0.127 %) was lower than that of unstressed (52.60 ± 0.48%). The results showed that stressed mice that were not administered with *Selaginella* extract had higher lipid peroxidation and lower protection to oxidative stress.

The results showed that species of *Selaginella* and the extract dose administration affected the level of lipid peroxidation as indicated by MDA concentration (Figure 2). An increasing dose of extract resulted in increased inhibition of lipid peroxidation as indicated by lowering MDA concentration in *S. ornata* administration. At a level of 1.2 g, *S. ornata* extracts kg^{-1} BW reduced 8x of MDA content ((11.61 nmol g-1 fresh tissue) compared to negative control treatment. *S. plana* extract has a similar inhibitory effect on lipid peroxidation with that of *S. ornata* extract up to 8 times (11.61 nmol g^{-1} fresh tissue) at the dose of 0.6 g extract kg^{-1} BW. However, the administration of 1.2 g extract kg^{-1} BW results in increased MDA content (48.39 nmol g^{-1} fresh tissue). The administration of *S. willdenowii* also reduced MDA content at the dose of 0.3 g extract kg^{-1} BW. Administration of 0.6 g extract kg^{-1} BW increased MDA content up to 54.84 nmol g^{-1} fresh tissue.

To evaluate the effect of *Selaginella* extract on lipid peroxidation of stressed and unstressed mice, a 0.6 g extract kg^{-1}BW of each *Selaginella* species was administered to the stressed and unstressed mice. Figure 3 showed the MDA contents in unstressed mice were not significantly different despite receiving *Selaginella* extracts from different species. However, the administration of different species of *Selaginella* extracts affected MDA contents significantly. The results showed that administration of *S. ornata* and *S.plana* extracts were able to reduce MDA content significantly compared to that of *S. willdenowii* extract.

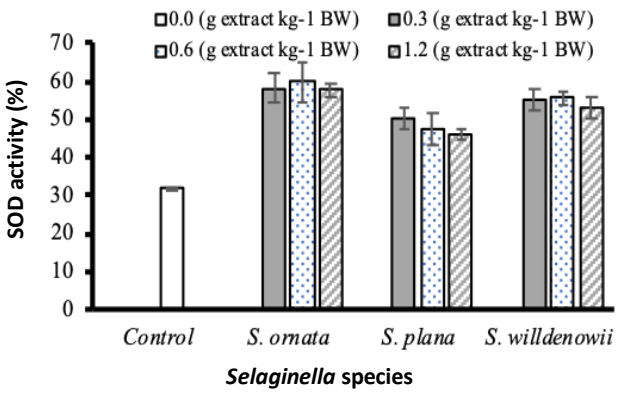

Figure 4. SOD activity (%) of control and oxidative stressed mice treated with extract of *S. ornata*, *S. plana*, and *S. willdenowii*. Control was a group of mice that did not receive *Selaginella* extracts but exposed to oxidative stress. Bars are standard errors of the mean

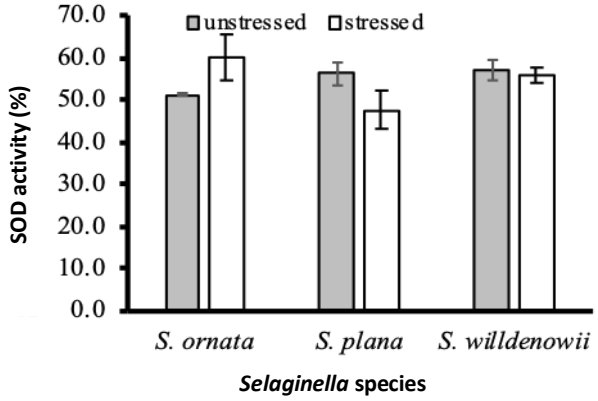

Figure 5. SOD activity of stressed and unstressed mice receiving 0.6 g extract kg^{-1} BW of three species of *Selaginella* extract. Unstressed mice were the mice that received the extract but not treated with oxidative stress. Bars are standard errors of the mean

Superoxide Dismutase (SOD) Activity

Superoxide dismutase analysis used the xanthin-xanthin oxidase system that measured superoxide radicals produced by the reaction between xanthine and xanthine oxidase. Superoxide radicals oxidize yellow tetrazolium salts to form blue formazan. When there is an activity of SOD in the system, superoxide radicals will be neutralized, and as a consequence the higher SOD activity, the lower superoxide radicals, and less formazan formed.

The administration of different species of *Selaginella* extract in oxidatively stressed mice results in different effects on SOD activity. The average SOD activity of stressed mice treated with 0.3-1.2 g extract kg-1 BW of *S.ornata* and *S. willdenowii* was 51.9% and 49.0%, respectively which was higher compared to SOD activity in the mice treated with *S. plana* extract (43.8%). It means that extract of *S.ornata* and *S.willdenowii* were potentially able to neutralize superoxide radicals better than that of *S. plana* (Figure 4). However, there were no significant differences in SOD activity among the doses of extract within the same *Selaginella* extract.

The SOD activity in stressed and unstressed mice responded differently to different *Selaginella* extract when 0.6 g extract kg^{-1} BW applied (Figure 5). SOD activity was significantly increased in stressed mice compared to that of unstressed mice when *S. ornata* extract was administered. Conversely, the SOD activity declined in stressed mice when treated with *S. plana* extract. The level of SOD activity in mice treated with *S. willdenowii* extract was not significantly different between stressed and unstressed mice. It means *S. ornata* extract at concentration of 0.6 g kg^{-1} BW could increase antioxidant activity in the stressed mice.

Discussion

Selaginella ornata, *S. plana*, and *S. willdenowii* have been used as herbal medicine to treat several diseases, especially in West Java community (Chikmawati et al. 2009). A scientific study on ethanol extract of *Selaginella* species could be useful to prove that the plant extract has potential benefits as antioxidant. A preliminary test to determine the level of toxicity based on the median lethal dose (LD_{50}) was crucial to be done before testing the extract on experimental animals. An acute toxicity test was performed to get information about the toxicity categories of substances being tested (Weil 1952; Harmita and Radji 2008). The results of the acute toxicity test showed that *S. ornata* and S. *plana* extracts were categorized as slightly toxic, while the *S. willdenowii* extract was categorized as quite toxic. The higher the LD_{50} value, the lower the toxicity. *S. ornata* extract has the highest LD_{50} value (9 g extract kg^{-1} BW) compared to the other two extracts, so the *S. ornata* extract is less toxic and expected to be safer than the other two species of *Selaginella.*

The differences in toxic categories of three species of *Selaginella* may affect changes in body weight at the end of the experiment (Figure 1). The changes in the bodyweight of mice may be due to differences in the level of toxicity related to chemical compounds of each *Selaginella* extract that was administered at the same concentration. *S. ornata* and *S. plana* extracts categorized as slightly toxic were able to increase body weight of mice, while *S. willdenowii* extract categorized as quite toxic caused weight loss in mice. The toxicity level of plant extract is related to the secondary metabolites content of the extract (Hutapea 1999). The main secondary metabolite compound in *Selaginella* is biflavonoids (Seigler 1998). Previous phytochemical screening showed that ethanol extract of *S. ornata*, *S. plana*, and *S. willdenowii* from Java Island contain alkaloids, tannins, saponins, and steroids (Chikmawati et al. 2012). *S. willdenowii* was more toxic might be due to higher saponin content than that of *S. ornata* and *S. plana*. As it has been reported by Diwan et al. (2000), saponin in high concentration could be toxic for mice.

Evaluation of the antioxidant activity of *Selaginella* extract in mice was done by administering the extract before being treated with oxidative stress. The results showed that administration of *Selaginella* extract in mice

with and without oxidative stress had a different effect on lipid peroxidation that indicated by different MDA concentrations. During stress conditions, the body needs energy-producing compounds. Physiologically, in conditions of food shortage, the body has to maintain blood glucose levels. Liver glycogen can only provide glucose for a few hours, and after that, the process of gluconeogenesis in the liver requires a substrate from other tissues that derived from glycogenic amino acids and fats (Montgomery et al. 1983). Neutral fat is catabolized to fatty acids and glycerol. Catabolism of fatty acids during normal conditions is different from catabolism during starvation conditions. Catabolism of fatty acids under normal conditions occurs in the mitochondria through β-oxidation processes. However, under starvation condition, there is an increase in the β-oxidation process in the peroxisomes (a minor pathway in the β-oxidation process). The increase of β-oxidation activity in the peroxisomes increases the number of free radicals (oxidants) which are metabolic byproducts (Orellana et al. 1992; Wresdiyati & Makita 1995). It was reported that free radicals, such as reactive oxygen species (ROS), that were produced from biochemical processes in the body could increase lipid peroxidation of unsaturated lipids in cell membranes (Alfarabi et al. 2010). Lipids that contain unsaturated fatty acids are easily attacked by free radicals in their double bonds and form lipid peroxidation that causes structural damage. Free radicals attack subsequently results in the emergence of various diseases such as heart disease, atherosclerosis, stroke, and cancer (Hariyatmi 2004; Alfarabi et al. 2010).

Lipid peroxidation assay by measuring MDA concentration in biological materials has been widely used as an indicator of oxidative damages, especially the damage of unsaturated fat (Okhawa et al. 1979). The administration of *S. ornata* extract in mice caused the lowest lipid peroxidation compared to the two other *Selaginella* species. Under stress conditions, administration of *S. ornata* extracts with a dose of 0.6 g extract kg^{-1} BW to mice was able to suppress lipid peroxidation level 14.4% lower than mice without the administration of *Selaginella* extract (Figure 2). The inhibitory activity of *Selaginella* extracts to lipid peroxidation in mice due to biflavonoid content. Biflavonoid has the potential as an antioxidant (Gayathri et al. 2005; Chikmawati et al. 2009). Biflavonoids have a series of hydroxyl donors (OH), so they are capable of suppressing lipid peroxidation at the initiation step, inhibiting free radicals from developing new free radicals (Rahman et al. 2007). Flavonoid compounds of *Selaginella* extract may be able to protect cell membranes from free radical attack (Saija et al. 1995).

The administration of *S. ornata*, *S. plana*, and *S. willdenowii* extracts in mice reduced the level of lipid peroxidation to a relatively similar level at a dose of 0.3 g extract kg^{-1} BW. However, the administration of *S. plana* (1.2 g extract kg^{-1} BW) and *S. willdenowii* (0.6 and 1.2 g extract kg^{-1} BW) caused a higher increase in lipid peroxidation activity compared to that of 0.3 g extract kg^{-1} BW. The high increase in lipid peroxidation in both *Selaginella* extracts might be due to the toxic effects at high doses (Figure 2). The toxic effect of the extract might be due to other chemical compounds in the *Selaginella* extract, including saponins and alkaloids. Previous phytochemical tests by Chikmawati et al. (2012) showed that *S. willdenowii* extract contains more saponins than the other two *Selaginella* extracts. Nio (1989) reported that saponins have a bitter taste and strong toxic effects on fish and amphibians. Saponin was also showed toxic effects in mice on concentration basis. The higher concentration of saponin applied to mice, the higher toxicity as represented by higher percentage of mice mortality (Diwan et al. 2000). Other bioactive materials, such as alkaloids, are also suggested to have toxic effects (Zulak et al. 2006).

The administration of the *Selaginella* extract at a dose of 0.3 g extract kg^{-1} BW has been shown to lower MDA concentration in all mice receiving *Selaginella* extracts (Figure 2). It means that the administration of 0.3 g extract kg^{-1} BW is sufficient to inhibit lipid peroxidation. Results of this research showed that the administration of *S. ornata* and *S. plana* extracts at doses of 1.2 and 0.6 g extract kg^{-1} BW, was the best dose of each *Selaginella* because it was able to suppress MDA at the lowest levels (11.61 nmol g^{-1} fresh tissue) (Figure 2).

MDA levels of mice without oxidative stress, but received *Selaginella* extracts were lower compared to mice without oxidative stress and without administration of *Selaginella* extract. It proves that *Selaginella* extract can suppress the level of lipid peroxidation not only in the stressed mice but also in the unstressed mice. Based on the results, it can be concluded that *Selaginella* extract has the potential as an antioxidant, and it can be used to overcome the adverse effects of oxidative stress.

Superoxide dismutase is an enzyme that participates in the process of degradation of intracellular free radical compounds, such as superoxide anions, hydrogen peroxide, and oxygen radical. The enzyme inhibits the simultaneous presence of superoxide and hydrogen peroxide anions derived from the formation of hydroxyl radicals (Wresdiyati et al. 2007). The administration of *S. ornata* extract (0.3 g extract kg^{-1} BW) to mice showed the highest antioxidant activity indicated by 51.9% of SOD activity which was 20% higher than the SOD activity in negative control mice (32%). The increasing SOD activity due to the administration of *Selaginella* extract can inhibit the production of superoxide ions (O_2^-) and peroxynitrite (ONOO-) (Menvielle-Bourg 2005).

High antioxidant activity is indicated from the inhibition of lipid peroxidation (low MDA concentration) and increased SOD activity. SOD enzymes are a group of protective enzymes that function as a defense system capable of protecting cells from the influence of oxygen metabolites (Hariyatmi 2004). Consumption of antioxidants originated from plants can increase the resistance to oxidative stress (Sanchez-Moreno et al. 1999). *Selaginella* was reported to contain bioactive compounds so that *Selaginella* has a potential source of active ingredients (secondary metabolites), especially biflavonoids. Amentoflavone biflavonoid has been detected in specific *Selaginella* extract, including *S. willdenowii* extract (2.46 ppm), but it was not detected in *S. ornata* and *S. plana*.

Other types of biflavonoids, such as robustaflavone may be present in both *Selaginella* species (Chikmawati et al. 2012). Yang et al. (2006) reported that amentoflavone in *S. tamariscina* could inhibit the production of NO (nitric oxide) in macrophages through inactivation of nuclear factor-κB (NF-κB), but it is not for robustaflavone. Wang et al. (2015) reported that *S. doederleinii* contains nine biflavone compounds that are potential as antioxidants. Biflavones were divided into three types, i.e., amentoflavone-type, robustaflavone-type, and hinokiflavone-type.

Testing of antioxidant activity by measuring MDA and SOD levels of mice liver homogenate showed differences among the three *Selaginella* species. The method to evaluate the antioxidant activity of *Selaginella* extract using lipid peroxidation analysis seems to be more sensitive than using SOD analysis. The lipid peroxidation data tells more about the ability of *Selaginella* extract to overcome oxidative stress due to all types of ROS than the SOD data does. The results showed that *Selaginella* extract had the potential to be further developed into standardized herbs and phytopharmaca.

In conclusion

, the extract of *S. ornata*, *S. plana*, and *S. willdenowii* have different antioxidant activities. *S. ornata* has the highest antioxidant activity with the lowest MDA concentration (11.61 nmol g-1 fresh tissue) and the highest average of SOD activity (51.9%). An extract dose of 0.3 g kg-1 BW of *Selaginella* extract was sufficient to suppress lipid peroxidation as low as 23.2 to 28% and increase SOD activity up to 54.6%. Testing the antioxidant activity of *Selaginella* extract using lipid peroxidation analysis yields better results than using SOD analysis.

The median lethal dose (LD_{50}) of *S. ornata* and *S. plana* extract were categorized as slightly toxic (9 and 5.2 g extract kg^{-1} BW), whereas *S. willdenowii* extract was categorized quite toxic (3 g extract kg^{-1} BW). Among the three *Selaginella* species, *S. ornata* extract is less toxic than other extracts. *Selaginella* extract had the potential to be further developed into standardized herbs and phytopharmaca.

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