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Potential In Vitro and In Vivo Antioxidant Activities from *Piper crocatum* and *Persea americana* Leaf Extracts

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***Piper crocatum* and *Persea americana* Mill leaves are commonly used in traditional medicinal remedies, such as antidiabetes, antitumors and Alzheimer treatment. However, the extensive use of plant extracts over worldwide becomes significant concerns including its safety, efficacy, and quality. Therefore, the accurate scientific evaluation has become a precondition for acceptance of herbal health claims. The aim of this study was to determine the antioxidant activities of *Piper crocatum* Ruiz & Pav and *Persea americana* Mill leaf extracts. Isolation of antioxidant fractions were conducted using organic solvent extraction techniques. Antioxidant assays were conducted by using in vitro and in vivo methods involving DPPH and MDA methods. In this study, in vitro assays of *Persea americana* Mill and *Piper crocatum* Ruiz & Pav leaf extracts showed the best activity in water fraction. Moreover, in vivo assays of both plant leaf extracts showed the best dose at 8 mg. *Persea americana* Mill and *Piper crocatum* Ruiz & Pav leaf extracts have been successfully determined in antioxidant actions in vitro and in vivo. Persea americana Mill in water and ethanol solvents exhibit strong antioxidant properties. Meanwile, Piper crocatum Ruiz & Pav exhibit moderate activity in water and weak antioxidant activity in ethanol. Both plant leaves showed that 8 mg dose was better than the dose of 4 mg and 16 mg in vivo.**

Keywords: Antioxidant, *Piper crocatum*, *Persea americana*, in vivo, in vitro.

Many medicinal plants family member from Indonesia known as potential candidate for natural antioxidant, such as mengkudu (*Moringa citrifolia*) [1], rambutan (*Nephelium lappaceum* sp.) [2], betel (*Piper betle*) [3, 4] and avocado (*Persea americana* Mill) [5]. Red betel is commonly found in tropical area and its leaves are conventionally used by the surrounding natives as medical treatment to overcome several diseases such as diabetes, inflammation [6], and wound healing [3]. Beside its unique odor from its essential content, red betel leaves also possess several functional active compounds including flavonoid, alkaloid, saponins, and tannins [7]. Many recent studies have reported that active compounds of medicinal plants exhibit hypoglycemic activity and antioxidant activity [8-10] whereas comparable to which shown by Butylated hydroxytoluene (BHT) and vitamin E [11].

Several studies have been carried out to evaluate antioxidant activities of avocado fruit and its part. Antioxidant activity of avocado peel extract had been evalutaed using radical scavenging assay including Ferric Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorbance Capacity (ORAC) [12]. Daiuto et al. also evaluated antioxidant activities of seed and peel parts of avocado [13]. Avocado leaves were enriched with phenolic bioactive compounds which potential as a natural antioxidants[14] and positively contains alkaloids, flavonoids, saponins, tannins and steroids under methanolic solution to extract avocado leaves[15]. Avocado leaves have been empirically used as a diuretic, analgesic, anti-inflammatory, hypertensive, hypoglycemic, diarrhea, sore throat and hemorrhage cure [16, 17]. Meanwhile, avocado fruit is nutritious as a preservative and antioxidant [18, 19]. Avocado flesh can be used as an anti-hyperlipidemia and has the potential to reduce the risk of metabolic síndrome [20-22].

The potentials use of red betel (*Piper crocatum* Ruiz & Pav) and avocado (*Persea americana* Mill) leaf extracts as natural source of antioxidant must be completed by the understanding of its safety and possible side effects [23]. Moreover, we also carried out antioxidant activity assays of red betel and avocado leaf extracts to shed light on the safety of its potential application as natural source of antioxidant without any further negative side effects. We have employed 1,1-diphenyl-2-picrylhidrazyl (DPPH) in so called cytotoxicity assay. The specimens' resistance on DPPH will provide the insight of their capability to overcome free radicals without necessarily distinguish the radical's type [24]. The aim of this research was to determine the antioxidant activities of *Persea americana* Mill and *Piper crocatum* Ruiz & Pav leaf extracts in vitro and in vivo by using DPPH and MDA methods in mice models.

METHOD

Preparation of extract simple

Red betel and avocado leaves were obtained from Aromatic Research Center (Balitro) in Bogor. Samples were cleaned, dried and ground into fine powder. To obtain the extract, the maceration process is done by soaking the fine powder as much as 200 g in 500 mL of solvents. Four different solvents were used in this study, including ethanol, ethyl acetate, hexane and water. The sample solution was stirred every two hours at room temperature to extract the bioactive compounds. After two days, the soaking result was filtered. The residue was again soaked in a fresh volume of solvents and the soaking process was repeated three times until clear filtrates were obtained. The resulting extracts were then evaporated using rotary evaporator at 35°C, 50 rpm. The concentrated extracts were dried by oven at 40°C until reached constant dry weight.

Phytochemical analysis

Phytochemical analysis was evaluated qualitatively to determine its bioactive compounds in plant leaf extracts of *Persea americana* Mill and *Piper crocatum* Ruiz & Pav leaf at various solvent fractions. Phytochemical analyses were caried out including glycoside, saponins, flavonoids, alkaloids, triterpenoid, steroids, essential oils, and tannins [25].

In vitro antioxidant assay with DPPH method

In this study, antioxidants were determined by DPPH method. Vitamin C was used as standard solution with concentrations of 2.5, 5.0, 10, and 20 ìg/mL. Moreover, vitamin C were reacted with 0.5 ml DPPH (1 mM in methanol solvent). The solution was homogenized by vortex and was allowed to complete at room temperature for 30 min., then the absorbance of solution was measured using spectrophotometer at 515 nm. The results obtained were given as percentage inhibition. Furthermore, all extract was done following the same method above, at various concentration of 10, 50, 100, 200 ìg/mL, respectively. The IC_{50} value represented as the concentration required for 50% inhibition of DPPH was calculated using the graph of inhibition percentage versus the extract concentration in mg/g (w/w), by following equation below.

Table 1. Category of antioxidant activity strength in vitro against DPPH

Intensity of IC_{50}	Value (µg/mL)
Very active	<50
Active	50-100
Medium	101-250
Weak	250-500
Inactive	>500

% DPPH Inhibition = [Abs Vit C – Abs Sample / Abs Control]x 100%

The intensity of IC_{50} of antioxidant activity may vary in different medicinal plant as categorized on Table 1.

In vivo antioxidant assay with Malondialdehyde (MDA) method

Plasma MDA levels measured by means of a thibabituric acid (TBA) reaction in mice groups before and after treatment. The in vivo test was began by weighing a mouse (190 g - 200 g) and labeled. The extract was administered to the mice daily for 10 days with the dose of 4 mg/200 g body weight (BW) for the first group, 8 mg/200 g BW for the second group, and 16 mg/200g BW for the third group, and a dose of vitamin C was 2.08 mg/200 g BW for positive control group and 1 ml of water for negative control group. On the tenth day, mice were given maximum physical activity that was in the form of swimming for 20 minutes. Observation was done on all mice activities in water. Furthermore, 2 mL blood sample was taken from each mouse through its tail after centrifugation to separate the blood plasma. Measurement of MDA levels in blood plasma was done by reacting 250 ìl of blood plasma with 100 ìl of 8.1% Sodium Duodecyl Sulfate (SDS) and 750 ìl of 0.5 M HCl, 750 ìl TBA and 125 ìl aquabidest. All substance was vortexed subsequently become homogeneous. The solution was then heated at 90°C for 15 min., then cooled for 10 min. After cooling in solution, 2.5 ml n-Butanol and 500 ìl aquabidest was added. The absorbance of MDA levels in plasma were measured by fluorometer at 520 nm excitation and 550 nm emission.

Sample size use in this research was following Federer's formula:

(k-1) (n-1) > 15

(8-1) (n-1) > 15

n = 3

k = number of group

n = number of mice in group

RESULTS

Phytochemical analysis and in vitro antioxidant activities

The results of phytochemistry analysis of *Persea americana* Mill *Piper crocatum* Ruiz and Puf are shown in Table 3. The in vitro antioxidant activity of *Percea americana* Mill and *Piper crocatum* Ruiz & Pav are showed in Table 4 and Table 5, respectively. Potential antioxidant activity of *Persea americana* Mill leaves extract was tested by comparison with antioxidant activity of vitamin C. The parameter used for antioxidant activity against DPPH radical was IC_{50} which indicated that

Table 2. In vivo treatment of antioxidant activity with plant extract

Mice group	Fraction	Dose (mg sample/ g mice body wight)
A1	*Persea americana* Mill	4 mg/200 g
A2	*Persea americana* Mill	8 mg/200 g
A3	*Persea americana* Mill	16 mg/200 g
B1	*Piper crocatum* Ruiz & Pav	4 mg/200 g
B2	*Piper crocatum* Ruiz & Pav	8 mg/200 g
B3	*Piper crocatum* Ruiz & Pav	16 mg/200 g
D	Vitamin C (positive control)	2.08 mg/200 g
E	Water (negative control)	-

Table 3. Phytochemistry analysis ethanol extract and ethyl acetat of *Percea americana* Mill and *Piper crocatum* Ruiz and Puf

Phytochemical Constituent	*Percea americana* Mill extract				*Piper crocatum* Ruiz and Puf extract			
	ethanol	water	ethyl acetate	hexane	ethanol	water	ethyl acetate	hexane
Glycosides	+	+	+	-	+	+	+	-
Saponin	+	+	+	-	+	+	-	-
Flavonoid	+	+	+	-	+	+	-	-
Alkaloid	+	+	+	-	+	+	+	-
Triterpenoid/Steroid	+	-	+	-	+	-	-	-
Essential oil	+	+	+	-	+	+	+	-
Tannin	+	+	+	-	+	+	-	-

concentration of compound is required to reduce DPPH radical by 50%. The smaller the value of IC_{50}, the more effective the function of the assayed extracts as antioxidant agents.

The results of in vivo assay of *Persea americana* Mill leaf extracts showed MDA levels before treatment was higher than MDA levels after treatment at 4 mg extract concentration (Fig 1). Meanwhile, *Piper crocatum* Ruiz & Pav leaf extracts also showed similar MDA levels before treatment was higher than MDA levels after treatment at 4 mg extract concentration (Fig 2).

DISCUSSIONS

For both studies of in vitro and in vivo assays, we used water as a negative control and vitamin C as a positive control. These methods were carried out to evaluate the antioxidant potential of *Persea americana* Mill and *Piper crocatum* Ruiz & Pav extracts against free radicals. In vitro antioxidant assays are very beneficial, cost-effective and time saving to investigate the antioxidant potential of *Persea americana* Mill and *Piper crocatum* Ruiz & Pav extracts before getting the extract to the in vivo mouse model for the antioxidant activity by free radical scavenging. Vitamin C as positive control is a water-soluble vitamin with the mechanism works as an antioxidant by stopping the propagation stage (chain-breaking antioxidant) and provides rapid electron transfer inhibiting lipid peroxidation, thus making the potent antioxidant of vitamin C[24]. The differences of IC_{50} value of *Persea americana* Mill extract in water, ethanol, ethyl acetate and hexane are caused by the solvent polarity. Water and ethanol are polar solvents, ethyl acetate is semi polar and hexane is non-polar solvents[26,27]. Identification of major and minor compounds in *Persea americana* Mill extract, ethanol, water and ethyl acetate were found to be most effective in the extraction of different compounds. Meanwhile *Piper crocatum* Ruiz & Pav extract pattern most effective in ethanol and water, but it was moderate in ethyl acetate. However, both *Persea americana* Mill and *Piper crocatum* Ruiz & Pav extracts were found none of constituent compounds in hexane.

Aqueous fraction of *Persea americana* Mill leaf extract can neutralize free radicals maximally, with IC_{50} value was 29.7, but not as good as antioxidant vitamin C (IC_{50} = 7.03). This is due to the content of vitamin C is pure antioxidant

Table 4. In vitro antioxidant activities of leaf extracts of *Persea americana* Mill in various extraction fractions

Extract Fraction	Concentration (µg/mL)	% Inhibition	IC_{50} (µ/mL)
Vitamin C (Positive control)	5	42.5	7.03
Ethanol	50	66.7	35.90
Ethyl Acetate	50	28.9	157.30
Hexane	50	8.0	440.80
Water	50	91.6	29.70

Table 5. In vitro antioxidant activities of leaf extracts of *Piper crocatum Ruiz* & Pav in various extraction fractions

Extract Fraction	Concentration (µg/mL)	% Inhibition	IC_{50} (µ/mL)
Vitamin C (Positive control)	5	42.5	7.03
Ethanol	100	33	185.29
Ethyl Acetate	10	0.8	202.78
Hexane	10	0.1	552.2
Water	10	6.2	81.24

compound that can potentially neutralize the free radicals better than plant extracts. The aqueous fraction of *Piper crocatum* Ruiz & Pav leaf extracts also showed the the best IC_{50} value compared to other solvent extracts. However, it still higher than vitamin C as positive control (IC_{50} = 7.03). Both aqueous plant extracts contained bioactive compounds such as flavonoid, alkoloid polyphenols, glucosides and terpenoids. These compounds contribute to antioxidant activities, but they are not supposed to be pure compounds and still bound to one another with the glycoside group[28].

In vivo assay of *Persea americana* Mill and *Piper crocatum* Ruiz & Pav leaf extracts, there were decreases of MDA levels in all groups, except negative control as shown on Figure 1 and Figure 2, respectively. This is because both leaf test extracts potentially as exogenous antioxidants, so it can suppress the free radicals that are formed, after the mice are given the physical burden of swimming for 15 minutes. In the positive control of decreased MDA levels was much better than with MDA level reduction at concentrations of 4 mg, 8 mg and 16 mg groups. The increased plasma MDA levels were elevated in the negative control group after the mice treatment through swimming, suggesting that the endogenous antioxidants in the body of mice were unable to neutralize free radicals. Endogenous and physiological reactive oxygen species (ROS) are largely generated within mitochondria as by-products of respiratory electron transport chain of normal cellular metabolism. Excessive exposure to ROS may interfere the redox homeostasis causes the cell vulnerable to ROS, and ultimately can induce cell damage.

CONCLUSION

Persea americana Mill and *Piper crocatum* Ruiz & Pav leaf extracts have been successfully determined as antioxidant agents in vitro and in vivo. *Persea americana* Mill in water and ethanol solvents exhibit strong antioxidant

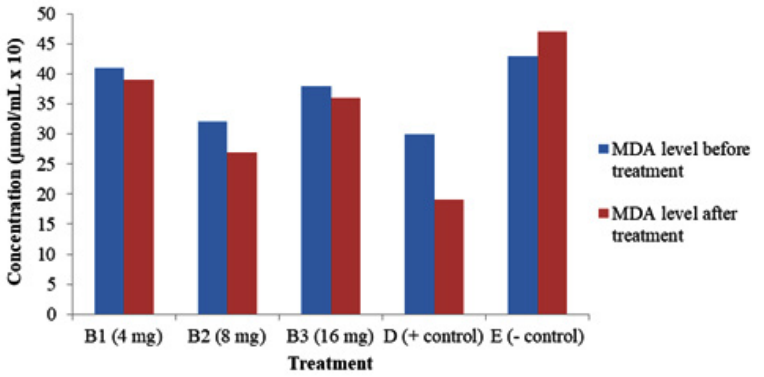

Fig. 1. In vivo antioxidant activity of *Persea americana* Mill leaf extracts

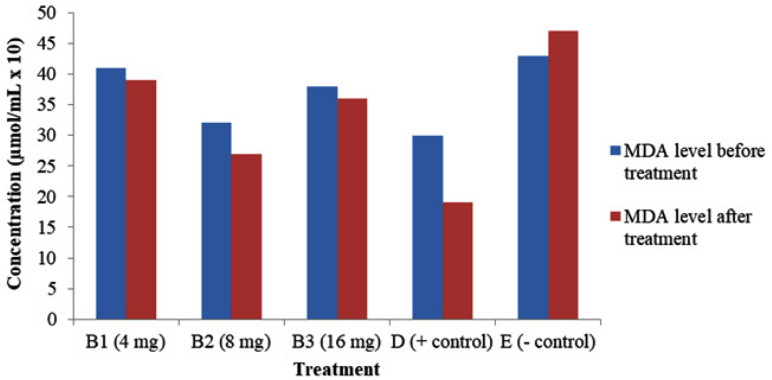

Fig. 2. In vivo antioxidant activity of *Piper crocatum* Ruiz & Pav leaf extracts

properties. Meanwhile, *Piper crocatum* Ruiz & Pav exhibit moderate activity in water and weak antioxidant activity in ethanol. Both plant leaves showed that 8 mg dose was better than the dose of 4 mg and 16 mg, in vivo. The antioxidant systems of exogenous antioxidants could repress the ROS level by regulating the genes expression and related metabolic networks to maintain the redox balance and support cellular component for stress adaption. Thus, the antioxidant remedies using *Persea americana* Mill and *Piper crocatum* Ruiz & Pav leaf extracts offer a promising strategy to prevent and treat the diseases caused by the excessive ROS exposure.

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