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Cite as: AIP Conference Proceedings **1854**, 020019 (2017); https://doi.org/10.1063/1.4985410
Published Online: 26 June 2017

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Antioxidant Activities of Different Solvent Extracts of *Piper retrofractum* Vahl. using DPPH Assay

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Abstract. *Piper retrofractum* Vahl., which belongs to the family Piperaceae, is geographically dispersed in tropical region including Indonesia. They are well-known spice possessing high medicinal properties. This study aimed to determine the antioxidant activity of *P. retrofractum* fruit, extracted with different solvents (methanol, ethyl acetate, n-hexane) using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. This research was carried out using different concentrations of methanol, ethyl acetate, and n-hexane extracts, (0, 5, 15, 30, 45, 60 ppm). Ascorbic acid was also used as positive antioxidant control. The percentage of inhibition and IC_{50} were measured. The results showed that the DPPH free radicals were scavenged by all plant extracts in a concentration dependent manner. Moreover, the IC_{50} values for DPPH radicals with methanol, ethyl acetate and n-hexane extract of the *P. retrofractum* Vahl. were found to be 101.74; 66.12 and 57.66 ppm, respectively. Interestingly, the IC_{50} value of n-hexane extract (57.66 ppm) was lower than ascorbic acid (66.12 ppm), indicating that n-hexane extract was a more potent scavenger of free radicals than methanol and ethyl acetate extracts. Taken together, our results suggested that n-hexane extract of *P. Retrofractum* Vahl. might contain potential antioxidant compounds.

Keywords: antioxidant, *Piper retrofractum* Vahl, DPPH assay

INTRODUCTION

Piper retrofractum Vahl. or traditionally known as Javanese Chili is widely distributed and cultivated in tropical region including Indonesia for their medicinal properties. In addition, Indonesian people usually use *P. retrofractum* fruit as a traditional beverage, mixed with other source of medicinal plant. Like other Piper species, the medicinal properties of this plant are related to their phytochemical contents including alkaloids, saponins, tannins, flavonoids, steroids, terpenoids and glycosides[1]. Flavonoid compound isolated from other *Piper* plant such as *Piper crocatum* possess antidiabetic, anticancer, antiseptic and anti-inflammatory activities. While the alkaloid compounds have been also reported to act as a potent antineoplastic properties, which inhibits the uncontrolled growth of cancer cells[2]. In term of its biological function, *P. retrofractum* have been used to unleash blood circulation, treat influenza and hypertension[3]. *P. retrofractum* have been also used for their anti-obesity activity[4], expectorant, antifungal and appetizing[5]. Recent studies have also demonstrated that *P. retrofractum* functions as anti-inflammatory agent[4].

Proceeding of International Biology Conference 2016
AIP Conf. Proc. 1854, 020019-1–020019-6; doi: 10.1063/1.4985410
Published by AIP Publishing. 978-0-7354-1528-7/$30.00

Many research have reported that oxidative stress, which could generate the production of free radicals, might give a significant contribution to certain inflammatory diseases, ischemic diseases, respiratory diseases and some degenerative disorders[6]. Free radicals can behave as oxidants or reductants which donate or accept an electron from biological molecules[7]. They include hydroxyl radical, superoxide, hydrogen peroxide, oxygen singlet nitric oxide radicals[8]. Human body develop unique strategy for counteracting the presence of these free radicals by synthesizing endogenous antioxidants. The later include glutathione, ubiquinol and uric acid[9]. In addition, human diet provide exogenous antioxidants such as vitamin E (α-tocopherol), ascorbic acid and β-carotene, a vitamin A precursor[10]. The imbalance between free radicals production and antioxidants might accelerate molecular damage within the cells including proteins, lipids and nucleic acids[11]. Nowadays, natural antioxidants produced from plant sources has attracted considerable public attention compared to synthetic antioxidants due to their side effects such as toxicity and carcinogenicity[12]. Some experimental evidences have suggested that these compounds could inhibit cellular damage by scavenging the free radicals. The present work aimed to determine the antioxidant activity of *P. retrofractum* fruit, extracted with different solvents (methanol, ethyl acetate, n-hexane) using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

MATERIAL AND METHOD

Plant Material and Extraction

P. retrofractum fruits were collected from Sumenep Region, Indonesia. The plant was firstly identified following dichotomous key based on "Atlas Tumbuhan Obat"[13] and Flora[14]. The fruits were washed with tap water and dried under shade for a week[15]. Subsequently, samples were ground into fine powder and then subjected to maceration process using different solvents such as methanol (BRATACO Chemical) as polar solvent, ethyl acetate (BRATACO Chemical) as semipolar solvent, and n-hexane (BRATACO Chemical) as nonpolar solvents for 48 hours at ambient temperature. Each extract was then placed and agitated in a flask for 1 h. Subsequently, the extract was filtered using Whatman No. 1 filter paper. The extraction process was repeated three times. Finaly, filtrates were concentrated at 50°C by rotary evaporator in order to obtain a crude extract of n-hexane (E1), ethyl acetate (E2) and methanol (E3). 10 mgr crude extract of E1, E2 and E3 were diluted using methanol (BRATACO Chemical) to obtain 100 ppm. Finaly, each extracts were prepared into several final concentrations of 0, 5, 15, 30, 45 and 60 ppm for being used subsequently in antioxidant activity assay.

Preparation of Ascorbic Acid Standard

10 mg of ascorbic acid (SAP Chemical) were dissolved in 100 mL methanol (BRATACO Chemical) to obtain a solution with a concentration of 100 ppm. Solution was then prepared into several final concentrations of 0, 5, 15, 30, 45 and 60 ppm for being used subsequently in antioxidant activity assay.

2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The free radical scavenging activity of the three extracts (methanol, ethyl acetate and n-hexane) of *P. retrofractum* fruit was analyzed using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) according to[15]. The DPPH solution was prepared in methanol and subsequently added to various concentrations of the extracts (5, 15, 30, 45 and 60 ppm). The absorbance changes were measured at 517 nm. Ascorbic acid was used as standart. These measurements were performed in duplicate and percentage of inhibition (*Pi*) was calculated using the following equation :

$$Pi = \frac{Ab - As}{Ab} \times 100\%$$

Ab is the absorbance of control and *As* is the absorbance of the extract. The IC_{50} values were calculated using linear regression analysis and used to indicate antioxidant capacity.

Statistical Analysis

The data obtained in this study were expressed as mean ± SD. All tested samples were statistically analyzed using one-way analyses of variance followed by Post-Hoc Tukey test. The *P* values of less than 0.05 were adopted as statistically significant.

RESULT AND DISCUSSION

Radical Scavenging Activity Measurement

Plants have been used long ago across human history of life for their usage in food and medicinal purposes. In modern life, natural products have been extracted and isolated from various kind of plants for the development of new drugs. Plant natural products are mainly divided into three major compounds including terpenoids, alkaloiods and phenolic compounds[16].Therefore, the study on preparation of plant extracts would be useful in the isolation and bioassay of the bioactive compounds[17]. Extraction methodology is important in the antioxidant assay. The yield of the extract should depends on the polarity of solvent used during preparation[18]. Moreover, solubility of the natural products and the choice of solvent could also determine the yield. For instance, lipophilic compounds, including some terpenoids and alkaloids should use non polar solvent such as n-hexane[19]. Some alkaloid, flavonoids and terpenoid compounds are usually extracted using ethyl acetate. Meanwhile, polar solvents including methanol, ethanol and acetone are major solvents used to extract some flavonols, alkaloids, polyphenols and saponins[19]. We determined the antioxidant activity using DPPH (diphenylpicrylhydrazyl) assay. DPPH is a stable free radical compounds and has an absorbance in its oxidized form around 515-520 nm[15, 20]. DPPH assay is relatively rapid and efficient method to evaluate free radical scavenging activity. DPPH is able to accept an electron or hydrogen radical to form a stable diamagnetic molecule. Changes in color, from purple to yellow indicates a decrease in absorbance of DPPH radical. This demonstrates that the antioxidant found in a mixture solution interact with the free radicals[21]. In the present study, percentage of inhibition (Table 1) was measured to determine the antioxidant activity of the extracts which is able to inhibit free radicals.

TABLE 1. Percentage of inhibition in some of *P. retrofractum* Vahl. extracts

Concentration (ppm)	% Inhibition			
	Metanol Extract	Ethyl acetate Extract	N-Hexane Extract	Ascorbic Acid
0	0,000^{d}	0,000^{d}	0,000^{d}	0,000^{d}
5	2,298^{d}	1,278^{d}	6,568^{d}	3,119^{d}
15	6,239^{cd}	8,702^{cd}	16,091^{cd}	3,448^{cd}
30	14,449^{bc}	16,748^{bc}	30,706^{bc}	21,346^{bc}
45	18,719^{b}	27,257^{b}	34,646^{b}	37,931^{b}
60	31,527^{a}	51,559^{a}	53,366^{a}	43,185^{a}

Six varying concentrations (0, 5, 15, 30, 45 and 60 ppm) of different solvent extract of *P. retrofractum* demonstrated different percentage of inhibition. Interestingly, scavenging activity of each extracts was increased in a concentration dependent manner. The 60 ppm extract showed the best antioxidant activity, where among them, the n-hexane extract was the highest (53,366 %), followed by ethyl acetate and methanol extract (51,559 and 31,527%), respectively. The radical scavenging activity of those three extracts, at their highest concentration (60 ppm), were higher than the antioxidant capacity of ascorbic acid at the same concentration.

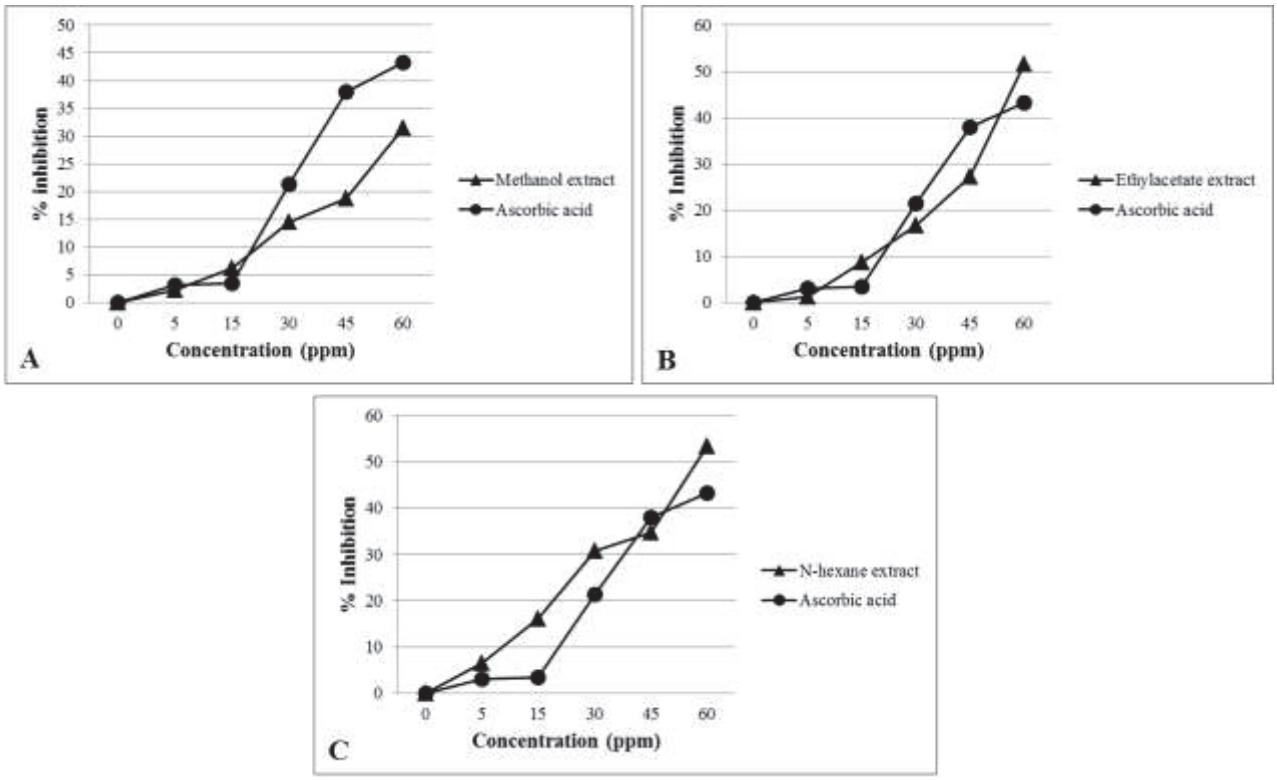

FIGURE 3. The radical scavenging activity, represented by percentage of inhibition, of the three different solvent extract compared to ascorbic acid. A, percentage of inhibition of methanol extract; B, percentage of inhibition of ethyl acetate extract; C, percentage of inhibition of n-hexane extract.

Methanol has been known as potential polar solvent to extract phenolic compounds[15, 22]. The antioxidant activity measurement has revealed that methanol extract of *P. retrofractum* has greater activity than ascorbic acid (Figure 3A). Some reports have shown that methanol extract of medicinal plants possessed good pharmaceutical activity. Recent finding has reported that methanol extract of *P. retrofractum* fruit potentially mediates mast cell stabilization[23]. In accordance with the result of methanol extract, antioxidant activity of ethyl acetate extract has also increased in a concentration dependent manner and even higher than that found in ascorbic acid. Ethyl acetate extract has been used for extracting some phenolic and nitrogenous compounds[24]. These compounds are known to scavange the free radicals and reactive oxygen species (ROS) including superoxide anion, hydroxyl radicals and singlet oxygen[20]. Ethyl acetate extract of *Podophyllum hexandrum* showed a significant dose-dependent inhibition of DPPH activity. This bio-potential activity was correlated with its function to prevent liver damage[25]. Finaly, among the two previous solvent extracts, n-hexane extract of *P. retrofractum* fruit exhibited highest potential antioxidant in a concentration dependent manner. Similar with this finding, n-hexane extract from leaves of *Piper auritum* possesses high antioxidant activity. In addition, biological assay of this extract suggest that *P. auritum* prevents oxidative stress and acting as a suppressor of liver cell damage[26].

The IC_{50} Value of DPPH Radical Scavenging Activity

The IC_{50} value was calculated to determine the concentration of the sample required to inhibit 50% of radical. The lower the IC_{50} value, the higher the antioxidant activity of samples[27]. The observed IC_{50} value showed that n-hexane extract exhibited highest antioxidant activity (57.66 ppm) followed by ethyl acetate extract (66.12 ppm) and methanol extract (101.74 ppm), respectively (Table 2) . Interestingly, the IC_{50} value of the n –hexane extract was also lower than ascorbic acid (66.12 ppm).

TABLE 2. IC_{50} value of DPPH radical scavenging activity

Sampel	IC_{50} (ppm)
Methanol Extract	101,74
Ethyl acetate Extract	66,12
N-Hexane Extract	57,66
Ascorbic Acid	66,12

According to[28], extracts which possess IC_{50} values ranging from 50 to 100 mg / mL is considered to exhibit intermediate antioxidant activity. Meanwhile, extracts with IC_{50} value ranging between 10 to 50 mg / mL is considered to possess strong antioxidant acitivity (Table 3). In this case, both ethyl acetate and n-hexane extraxts possessed intermediate antioxidant activity. N-hexane solvents are usually chosen to extract terpenoid-derived molecules[29].

TABLE 3. Antioxidant Activity according to Phongpaichit, 2007

IC_{50} (µg/mL)	**Mark**
10-50 µg/mL	Strong Antioxidant Activity
50-100 µg/mL	Intermediate Antioxidant Activity
>100 µg/mL	Weak Antioxidant Activity

Terpenoids have been defined as substances that are characterized by its isoprene backbones. Terpenoid-derived compounds have been known as potential bioactive compounds. They are acting as pigment for photosynthesis, ensuring membrane integrity, attracting po llinators, involved in the protein N-glycosylation[16, 30]. In addition, terpenoids play important roles in human health. They are providing the provitamin A, influencing the human immune function and also act as potential antioxidant[31]. Nevertheless, terpenoid-derived substances are also known as toxic subtances such as phorbol esters[32]. Some alkaloids are also extracted using n-hexane including piperidine from some piperaceae plants[33]. Some reports have demonstrated that piperidin can act as potential antioxidant to reduce high-fat induced oxidative stress to the cell[34, 35].

CONCLUSION

Methanol, ethyl acetate and n-hexane extracts of *P. retrofractum* Vahl fruit exhibited potential antioxidant activity. They actin a concentration-dependent manner. The result showed that ethyl acetate and n-hexane extracts possessed intermediate antioxidant activity. Meanwhile, methanol extract of *P. retrofractum* Vahl showed weak antioxidant activity.

ACKNOWLEDGMENT

This research was supported by Contract No. 01653/IT2.11/PN.08/2016 from Directorate General of Higher Education of the Ministry of Research and Technology, Republic of Indonesia.

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