Ni Putu Ermi Hikmawanti-The Effect of Ethanol Concentrations as The Extraction Solvent on Antioxidant Activity of Katuk (Sauropus androgynus (L.) Merr.) Leaves Extracts

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The Effect of Ethanol Concentrations as The Extraction Solvent on Antioxidant Activity of Katuk (Sauropus androgynus (L.) Merr.) Leaves Extracts

Ni Putu Ermi Hikmawanti*, Sofia Fatmawati, Anindita Wulan Asri

Faculty of Pharmacy and Science, Universitas Muhammadiyah Prof. DR. HAMKA Klender, East Jakarta, DKI Jakarta, 13460, Indonesia

*ermy0907@uhamka.ac.id

Abstract. Katuk is widely popular with its benefits for breastfeeding mothers. Katuk is also known as a plant with a high antioxidant content. This study aims to determine the effect of using variations in the ethanol concentration as an extracting solvent in producing Total Phenolics Content (TPC) and Total Flavonoids Content (TFC) and their activities in reducing DPPH free radicals. The dried katuk leaves were extracted by cold maceration method. The solvent used for extraction is ethanol with 3 variations in concentration: 50%, 70%, and 96% (absolute ethanol). TPC and TFC were determined by colorimetric method using a UV-Vis spectrophotometer. TPC was stated to be equivalent to gallic acid, while TFC was stated to be equivalent to quercetin. DPPH free radical scavenging activity was measured based on the IC50 value. The results showed that Katuk leaf extract produced from 50% ethanol solvent was able to produce TPC (42.18 ± 0.30 mgGAE / g), TFC (11.18 ± 0.38 mgQE / g) and reduction activity against DPPH radicals (IC50 = 88.33 ± 3.53 ppm). These were higher than ethanol with other concentrations. However, various things need to be considered when using this solvent given the high water content in the solvent.

1. Introduction

Sauropus androgynus (L.) Merr in Indonesia is known as katuk (family Phyllanthaceae). Katuk contains high nutrients in the form of vitamins, minerals, fiber, lipids, carbohydrates, as well as bioactive compounds such as phenolics, tannins, flavonoids, anthocyanins, phytosterols, and so on. Katuk is included in the group of green vegetables with ethnomedicine potential, including as an antitussive, tonic, antipyretic, breast milk facilitator, and others (Petrus, 2013). Katuk was also reported to have aphrodisiac activity (Rusdi et al., 2018) and fertility enhancers in male rats given orally (Hikmawanti et al., 2020). The antioxidant capacity of katuk leaves associated with various pharmacological activities has also been discovered such as antimicrobial, cytotoxic against cancer cells, anti-inflammatory and wound healing (Petrus, 2013; Khoo et al., 2015).

Antioxidants are substances that are used in small concentrations to inhibit and/or to reduce oxidation caused by an oxidant. Plants are a source of natural ingredients with great natural antioxidant potentials, such as phenolic compounds (phenolic acids, flavonoids, tannins, anthocyanins, liginins), carotenoids, vitamins (vitamins A, C, E) and so on. To obtain these antioxidant compounds, an extraction process is necessary (Altemimi et al., 2017). Extraction is a procedure performed to obtain metabolites in plants such as alkaloids, phenolics, flavonoids, glycosides, and others using selective solvents. Solvent

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selection is one of the stages of preparation for extraction (Azwanida, 2015). The selectivity of the solvent to extract the target compound from a plant material is related to the polarity compatibility of the two. Ethanol is a solvent that is safe for human consumption as a solvent for natural substances for both food and natural medicinal purposes. Absolute ethanol and aqueous ethanol have been used successfully to extract phenolic derivative antioxidant compounds from natural ingredients with good results (Sultana et al., 2009).

The purpose of this study is to determine the total phenolic content (TPC), total flavonoid levels (TFC), and the antioxidant activity against DPPH radicals from katuk leaf extract produced from three types of variations in the ethanol concentrations as an extracting solvent (50%, 70% and 96%). It is important to discover ethanol concentrations to produce katuk leaf extract with a high content of antioxidant compounds. In addition, with the right solvent, extracts with guaranteed safety and quality can be produced and applied widely.

2. Methodology

Materials

Fresh leaves of katuk were obtained from the Biopharmaca Cultivation Conservation Unit (UKBB), Center for Tropical Biopharmaca Study, LPPM IPB, Bogor, West Java, Indonesia. Plants were determined in the same place. Ethanol as a solvent extraction in analytical grade was obtained from Merck in Darmstadt, Germany. Gallic acid, quercetin, and DPPH (2,2-diphenyl-1-picryl-hydrazyl) were obtained from Sigma-Aldrich Co. in St. Louis, the US.

Extraction

The extraction of dried katuk leaf powder (150.0 g) was carried out by cold maceration method following the Indonesian Herb Pharmacopoeia. The solvent used was ethanol with various concentrations, namely 50%, 70% and 96% (absolute ethanol). Each extraction process was carried out for 24 hours. The filtrate was separated from the residue using Whatmann filter paper. The residue was remacerated 3 times. The filtrate from each solvent was collected and evaporated using a rotary evaporator N-1200 BS series from EYELA (Shanghai, China) at 50 ° C and using a water bath at 50 ° C until a thick extract was obtained (Ministry of Health Republic of Indonesia, 2008).

Determination of the Physico-Chemical Properties of the Extract

The physico-chemical parameters of katuk leaf extract including organoleptic, percentage of extract yield, drying loss, and total ash content were determined according to the procedure stated in the Indonesian Herb Pharmacopoeia (Ministry of Health Republic of Indonesia, 2008).

Phytochemical screening

Phytochemical screening was carried out qualitatively using detection reagents based on the procedures explained in Hanani (2015) and the Indonesian Herb Pharmacopoeia (Ministry of Health Republic of Indonesia, 2008). The classes of compounds identified in the extract included phenolics, flavonoids, tannins, saponins, alkaloids, steroids and triterpenoids.

Determination of Total Phenolics Content (TPC)

TPC determination followed Yang et al., (2007)'s procedure with modification using Folin-Ciocalteu Reagent (FCR) and gallic acid as standard. The calibration curve was made using gallic acid solution with a concentration range of 18-66 ppm. The relationship between the concentration of gallic acid (x) and its absorbance (y) was plotted to produce a linear line equation (y = bx ± a). Each 1000 ppm katuk leaf ethanol extract in ethanol was pipetted as much as 0.3 mL. It was then added 1.5 mL FCR reagent (which has been diluted 1:10) and homogenized. The solution was incubated for 3 minutes, then 1.2 mL of 7.5% Na_2CO_3 solution and water were added to obtain a total volume of 10 mL. The mixture was incubated again for 60 minutes at a room temperature. The absorbance of the extract solution was measured using a UV-Vis Shimadzu UV-1601 Series (Kyoto, Japan) spectrophotometer with a maximum wavelength of 756.5 nm at room temperature. The absorbance obtained was plotted into a linear line equation and then the TPC was calculated using the formula: phenolic content in solution (µg / mL) multiplied by the dilution factor and the solution volume (mL) and divided by the extract weight (g). TPC was expressed in mg which is equivalent to gallic acid per gram of extract. Each extract was tested with 5 repetitions and reported as mean ± SD.

Determination of Total Flavonoids Content (TFC)

TFC determination followed the procedure in Chang et al., (2002) with modifications. The standard used was quercetin. Quercetin solutions with a concentration range of 33-129 ppm were used to create a calibration curve. The relationship between the concentration of quercetin (x) and its absorbance (y) was plotted to produce a linear line equation (y = bx ± a). Each ethanol extract of katuk leaves with a concentration of 7500 ppm in methanol was pipetted 0.5 mL. It was then added 1.5 mL of methanol and 0.1 mL of 10% $AlCl_3$. 0.1 mL of sodium acetate 1M was added and made sufficient with aquadest up to 5 mL. The solution was incubated for 60 minutes at room temperature. The absorbance of the extract solution was measured with a UV-Vis Shimadzu UV-1601 Series (Kyoto, Japan) spectrophotometer at a wavelength of 434 nm. The absorbance obtained was plotted into a linear line equation and then the TFC was calculated using the formula: the level of flavonoids in solution (µg / mL) multiplied by the dilution factor and the solution volume (mL) and divided by the extract weight (g). TFC was expressed in mg which is equivalent to quercetin per gram of extract. Each extract was tested with 5 repetitions and reported as mean ± SD.

Antioxidant Activity Testing

The antioxidant activity test of katuk leaf extract was carried out using the DPPH method following the procedure of Wan et al., (2011) with modifications. Quercetin was used as a comparison. The extract was dissolved in methanol and diluted into 5 variations of concentration: 20, 40, 60, 80, and 100 ppm. Quercetin solution was dissolved in methanol and diluted to 2, 4, 6, 8 and 10 ppm. Each variation of the concentration extract solution or quercetin was pipetted 1 mL and was added 1 mL of fresh 0.5 mM DPPH in methanol. The solution was made sufficient with methanol to a total volume of 5 mL. The mixture in a tube lined with aluminum foil paper was incubated for 30 minutes at a room temperature in dark conditions. Blank solution was made from 1 mL DPPH mixed with 4 mL methanol. Furthermore, the absorption was measured at a wavelength of 515.5 nm. Each concentration series of each sample was tested triplo. Results were reported as mean ± SD. The calculation of the percentage of DPPH radical inhibition used the formula:

$$Inhibition\ of\ DPPH\ (\%) = \frac{Ab - As}{Ab}\ x\ 100$$

where Ab is the absorbance of the blank and As is the absorbance of the sample.

Data Analysis

Data were analyzed with a one-way analysis of variance (ANOVA) followed by Tukey's test (α ≤ 0.05).

3. Result and Discussion

The ethanol extract of katuk leaves in this study had organoleptic characteristics with a distinctive odor, thick shape, slightly sweet taste and dark brown color (Table 1). The three extracts had an ash content value in the range 5.74-8.23%, w / w. Ash content describes the amount of inorganic material contained in the katuk leaf extract, both obtained internally by plants and during the extraction process. The percentage of ash content of the katuk leaf ethanol extract obtained in this study (<10%) indicated that the extract had a fairly good quality. Ash content is a contaminant parameter that is difficult to remove in extracts but can still be controlled by proper post-harvest handling of plants and a maintained extraction process (Kunle et al., 2012). The drying shrinkage value describes the amount of compound lost during the 105°C heating process for several hours. The drying shrinkage value of the three katuk leaf extracts was in the range of 1.96-4.96%, w / w (<5%) (Table 1.). This value range showed that the ethanol extract of katuk leaves had a good quality.

The characteristic parameter of the extract determined was the chemical content of the ethanol extract of katuk leaves. The results of phytochemical screening of the three ethanol extracts of katuk leaves showed the presence of alkaloids, phenolics, flavonoids, tannins, saponins and steroids (Table 2.). Although the ethanol solvent used for extraction was from different concentrations, it was still able to attract the same type of compound class to the katuk leaves. Ethanol has the ability to attract glycosides (Houghton & Raman, 1998), polyacetylenes, sterols (Tiwari et al., 2011), polyphenols, tannins, flavonols, terpenoids, and alkaloids (Azmir et al., 2013).

The choice of ethanol as the extraction solvent was considered to provide many advantages over other organic solvents, which is relatively safer (less toxic). Ethanol is a protic organic solvent with a polarity index value of 5.2 (Synder, 1974; Abarca-Vargas et al., 2016) and a dielectric constant of 24.55. Absolute ethanol contains 0.01% by volume of water (Covington & Dickinson, 1973). The 50% ethanol solvent in this study was able to extract more metabolites from the dry powder of katuk leaves (37.77%) than other ethanol solvents (70% ethanol> 96% ethanol). The ethanol solvent polarity of the three concentrations used in this study was influenced by the high concentration of water contained in ethanol. The more water contained in it, the higher its polarity compared to absolute ethanol (Tiwari et al., 2011). Solvents with high polarity had the ability to extract a class of compounds with a wider polarity. This allowed non-phenolic polar compounds such as carbohydrates and proteins to be dissolved during the extraction process which resulted in increased extraction yields (Do et al., 2014).

Phenolic is a plant-produced metabolite with a distinctive structure in the form of an aromatic that binds to one or more -OH rings. Meanwhile, flavonoids are phenolic derivative pigment metabolites which are widely found mainly in higher plants. One of the quantitative determinations of phenolics and flavonoids can be done colorimetrically using the UV-Vis spectrophotometer technique (Khoddami et al., 2013). This technique is considered relatively simple, fast, and is able to provide satisfactory quantitative results (Cong-cong et al., 2017). Quantitative determination of phenolics was carried out using the Folin-Ciocalteu Reagent (FCR) containing tungsten and molybdenum. The product of this reaction was a blue solution $((PM_oW_{11}O_{40})^{4-})$ which was absorbed at a wavelength of about 760-765 nm (Prior et al., 2005; Khoddami et al., 2013). FCR has a weakness, namely that this reagent is also able to react with other compounds such as vitamin C, aromatic amines and saccharides (Cong-cong et al., 2017). Meanwhile, the quantitative determination of flavonoids was carried out using 10% $AlCl_3$ reagent which was carried out under alkaline conditions such as if 7.5-20% Na_2CO_3 was added. The uptake of this reaction product can be measured at a wavelength of about 410-423 nm (Khoddami et al., 2013). Based on the results in Table 3., the best TPC (42.18 mgGAE / g) and TFC (11.18 mgQE / g) were obtained in katuk leaf extract extracted with 50% ethanol (70% ethanol> 96% ethanol) solvent. These results indicated that 50% ethanol solvent is the best solvent for extracting antioxidant compounds from katuk leaves. Petrus (2013) explained that dried katuk leaves had TPC and TFC of 1150.95-2300.00 mgGAE / 100 g and 1040 mgRE / 100 g, respectively.

Antioxidant activity testing in this study was carried out using DPPH radicals. This method is considered relatively inexpensive, effective, efficient and has good sensitivity of a UV-Vis spectrophotometer. DPPH is a free radical with a dark blue color. With the presence of an antioxidant, the antioxidant would donate its Hydrogen atom to the DPPH radical (becoming DPPH-H). This reaction is characterized by a decrease in absorbance due to DPPH loses its reactivity (Alam et al., 2013; Huyut et al., 2017). The range of quercetin concentrations as a comparison used in this test was 2-10 ppm. Meanwhile, the extract concentration used was 20-100 ppm. The concentration range used was adjusted to the reactivity of each material against the DPPH radical. In addition, other considerations were based on the limitations of the minimum and maximum sample absorption values that can still be read by the UV-Vis spectrophotometer instrument at a wavelength of 515-517 nm (Maesaroh et al., 2018). The antioxidant activity in this study was determined based on the IC_{50} value of the sample. The IC_{50} value showed the sample concentration needed to inhibit 50% of DPPH radicals. This value was obtained from the results of calculations using linear regression analysis. The smaller the IC_{50} value of an antioxidant, the higher its power to inhibit free radicals (Do et al., 2014). Based on the results in Table 3., the ethanol extract of 50% katuk leaves had the highest antioxidant activity with an IC_{50} value of 88.33 ppm (70% ethanol> 96% ethanol). This showed that the ethanol extract of 50% katuk leaves had a strong DPPH radical scavenging activity (<100 ppm).

The phenolic and flavonoid contents in katuk leaves play an important role in its activity as a source of antioxidants. The number of hydroxyl groups in a phenolic molecule affects its capacity as an antioxidant. Phenolic has a tendency to donate hydrogen atoms or electrons from its hydroxyl groups to free radicals (Dai & Mumper, 2010). As for flavonoids, the number and location of the aromatic hydroxyl groups in their structure affect their antioxidant capacity (Fernandez-Panchon et al., 2008).

4. Conclusion

Based on this study, the variation in the concentration of ethanol used as the extraction solvent was able to have an effect on the acquisition of phenolic and flavonoid compounds as well as the DPPH free radical scavenging activity from the katuk leaf extract. The 50% ethanol solvent was able to extract high amounts of phenolic and flavonoids from katuk leaf powder using the cold maceration method compared to ethanol solvent with a concentration of 70% and 96%. The best antioxidant activity was also obtained from katuk leaf extract which was extracted using 50% ethanol solvent. Thus, the selection of this solvent was proven to be easy and efficient to use to produce katuk leaf extract which is rich in antioxidants, both as a raw material for functional food and for natural medicine. However, it is necessary to consider again the amount of time and high energy required in the evaporation process, as well as the stability associated with the safety of the extract produced from the solvent so that the quality and quality of the extract are guaranteed.

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Table 1. Characteristics of Katuk Leaf Ethanol Extract

No	Parameter	Katuk Leaf Extract		
		Ethanol 50%	**Ethanol 70%**	**Ethanol 96%**
1	**Organoleptis Organoleptic**			
	Smell	Typical	Typical	Typical
	Shape	Thick extract	Thick extract	Thick extract
	Taste	Little sweet	Little sweet	Little sweet
	Color	Dark brown	Dark brown	Dark brown
2	**Extraction Yield (%, w/w)**	37.77 ± 0,93	36.26 ± 3,38	33.55 ± 2.77
3	**Ash content (%, w/w)**	5.74 ± 0.48	7.13 ± 1.08	8.23 ± 0.61
4	**Shrink drying (%, w/w)**	4.96 ± 1.19	1.96 ± 0.75	4.06 ± 0.82

Note: Data presented are mean (n = 5) ± SD.

Table 2. Results of Phytochemical Screening of Ethanol Extract of Katuk Leaves

No	Compound Group	Katuk Leaf Extract		
		Ethanol 50%	**Ethanol 70%**	**Ethanol 96%**
1	Alkaloids	+	+	+
2	Phenolic	+	+	+
3	Flavonoids	+	+	+
4	Tannin	+	+	+
5	Saponins	+	+	+
6	Steroids	+	+	+
7	Terpenoids	-	-	-

Description: (+) = detected compound identified; (-) = no identified compound was detected

Table 3. Total Phenolic and Flavonoid Levels of Katuk Leaf Ethanol Extract

Parameter	Katuk Leaf Extract		
	Ethanol 50%	**Ethanol 70%**	**Ethanol 96%**
TPC (mgGAE/g)	42.18 ± 0.30*	25.33 ± 0.13	16.25 ±0.16
TFC (mgQE/g)	11.18 ± 0.38*	8.87 ± 0.14	5.68 ± 0.34
IC_{50} (ppm)	88.33 ± 3.53	90.04 ± 1.00	95.73 ± 2.95*

Note: Data presented are mean (n = 5) ± SD. * It differs significantly from other extracts (α = 0.05).

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