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ULTRASOUND-ASSISTED EXTRACTION AS EFFICIENT METHOD FOR OBTAINING OPTIMUM ANTIOXIDANT FROM MANGROVE LEAVES OF *RHIZOPHORA MUCRONATA*

AUDAH KA[1]*, MANUELLA K[1], AMSYIR J[1], HAPSARI AM[1]‡, SUTANTO H[2]

[1]*Department of Biomedical Engineering,* [2]*Department of Chemical Engineering Swiss German University, Prominence Tower, West Sutera Road, South Tangerang 15143, Banten Province, Indonesia*

‡*Waterco Indonesia, Jl. Raya Boulevard Barat, Ruko Inkopal Blok B No. 31 – 32, DKI Jakarta 14240, Indonesia*

*Corresponding author: kholis.audah@sgu.ac.id

ABSTRACT

Extraction method plays a critical role in the study of medicinal plants. Nowadays, a wide range of technologies is available to promote different methods of extraction with the better extraction output. The aim of the study was conducted to compare two extraction method, maceration and ultrasound-assisted extraction (UAE), for obtaining optimum antioxidant. The mangrove leaves of *Rhizophora mucronata* was chosen as a natural source of antioxidant. The leaves were extracted by employing 24 hours maceration and UAE in five different extraction times (5, 15, 30, 45 and 60 minutes). The experiments were conducted using water and 70% ethanol as solvents. The total phenolic content was determined by Folin-Ciocalteau assay and flavonoid content by Aluminium chloride assay. While the antioxidant activity was determined by the DPPH method. As a result, the phenolic compounds yielded by UAE with ethanol was considerably higher and need shorter time than maceration. The IC_{50} value was obtained by UAE with ethanol is 52.86 ppm. All these data showed that UAE with ethanol was the efficient method and mangrove leaves *Rhizophora mucronata* is a very potent natural antioxidant source.

KEYWORDS:
antioxidant, phenolic compounds, Rhizophora mucronata, ultrasound-assisted extraction (UAE),

INTRODUCTION:

Free radicals are generated during metabolism and other biological system activities beyond the antioxidant capacity gave rise to oxidative stress which plays a role in heart diseases, neurodegenerative diseases, cancer and aging process (Huda-Faujan et al., 2009; Birben et al., 2012, Sies, 2015). As an answer, the dietary antioxidants can lower the risk of those diseases (Ghasemzadeh et al., 2010; Peng et al., 2014). Antioxidants are substances that at low concentration functioned to delay the oxidation of proteins, carbohydrates, lipids and DNA (Hamid *et al*., 2010; Sindhi *et al*., 2013).

Nowadays, most of the antioxidants used are manufactured synthetically. The synthetic antioxidants commonly used are Butylated hydroxyl anisole (BHA), Butylated hydroxyrotoluene (BHT), Propyl gallate (PG), Tertiary butyl hydroquinone (TBHQ) and Nordihydro guaretic acid (NDGA) (Hamid *et al*., 2010; Dolatabadi and Kashanian, 2010; Thorat *et al*., 2013). However, they have been under scrutiny since the potent hazardous effects to human health (Dolatabadi and Kashanian, 2010; Schilaci *et al*., 2014; Eskandani *et al*., 2014). Therefore, many of researchers have been interested more in exploring potent antioxidant from natural sources (Brewer, 2011, Celıkyurt, 2011; Shebis *et al*., 2013; Abourashed, 2013).

Indonesia as an archipelago country which possesses of an estimate of 95,181 km of coastline which bears the largest mangrove vegetated area in the world of about 3,244,018 ha (Bakosurtanal, 2009). Mangrove

forests have become the essential support of human lives along the coastlines for centuries, providing economic and environmental benefits (Mangkay et al., 2013; Duncan et al., 2016). In the past decade, extracts from mangroves and mangrove associates have been scientifically studied for their effectiveness against human, animals and plant pathogens, as well as for their antioxidant properties (Bandaranayake, 2002, Abeysinghe, 2010; Krishnaiah et al., 2011).

R. mucronata is a widespread species of mangrove in Indonesian coastline that has been described as a potent antioxidant agent (Banerjee et al., 2008; Rege and Chowdary, 2014; Wahyuni et al., 2015). The species is robust and even can survive harsh living condition in the contaminated water by producing their own antioxidant in the form of phenolic compounds (Michalak, 2006). Accordingly, the extraction of phenolic compounds from medicinal plants includes mangrove have become a hotspot.

Extraction method plays a critical role in the study of medicinal plants (Vagashiya et al., 2011; Azmir et al., 2013; Azwanida, 2015). The conventional extraction methods which have been employed for decades, maceration and soxhlet extraction, can be applied only at the small research setting and they are also time consuming (Dhayanithi et al., 2012; Yompakdee et al., 2012). Therefore, significant advances have been developed in the modern extraction methods. One of them is ultrasound-assisted extraction (UAE), in which this advance is aimed to the better extraction output (Azwanida, 2015; Sutanto et al., 2015). The purpose of this study was to compare two extraction methods, maceration and ultrasound- assisted extraction (UAE), for obtaining optimum antioxidant. So that antioxidant agents contained in the sample can be extracted better and the duration of extraction becomes shorter. So that an efficient extraction process can be obtained.

MATERIAL AND METHODS:

Plant material

The *R. mucronata* leaves *were* harvested from *Kawasan Ekowisata Mangrove*, Pantai Indah Kapuk, North Jakarta, Indonesia with the permission from *Dinas Kelautan, Pertanian, dan Ketahanan Pangan DKI Jakarta*. *R. mucronata* was identified by LIPI (Indonesian Institute of Sciences) taxonomist at Cibinong Science Center. Before use, the leaves were firstly cleaned with tap water to remove dirt and then rinsed with water. The leaves were cut into transverse sections of approximately 0.02 m width and dried using oven (Memmert, Germany) at the temperature of 45 ºC for 12 hours. The dried leaves were crushed using blender (Cucina Philips, Indonesia) and screened using mesh number 35 (CISA Cedaceria Industrial, Spain). The leaf powder was transferred into glass bottles with a rubber stopper, wrapped in aluminium foil and stored in the freezer -20 ºC prior to extraction.

Extraction

For the maceration method, five grams of leaf powder was transferred into erlenmeyer flask and immersed in the solvent (1:10) for 24 hours in a shaker set at 125 rpm, 25 ^{o}C. The erlenmeyer flask opening was sealed by using aluminium foil and parafilm, and the whole flask was covered by aluminium foil to protect the content from the light as described by Dhayanithi et al. (2012).

The UAE experiments were adapted from Molyneux (2004). The experiments were carried out with five different extraction times (5, 15, 30, 45 and 60 minutes) with using water and 70% ethanol as solvents. The ultrasonic device used was Bandelin SONOREX SUPER 10P ultrasonic bath (Germany). The frequency used was 35 kHz for 400 W ultrasonic devices and enabled transient cavitations with bubbles implosion effect. The ultrasonic probe was immersed in the mixture directly.

Five grams of leaf powder was transferred into erlenmeyer flask and 50 mL of solvent was added. A glass rod was used to evenly immerse the powder in the solvent. The opening of the flask was covered with aluminium foil and sealed with Parafilm. The erlenmeyer flask was then submerged inside a beaker glass filled with tap water, to ensure stability during extraction in the water bath shaker (Edmund Bühler SM 25 Shaker, Germany) and placed into the ultrasonic bath. The water level of the ultrasonic bath was adjusted so that it is the same or slightly higher than in the beaker glass and erlenmeyer flask. To prevent an excessive

increase in temperature, the power setting in the ultrasonic bath was adjusted to 50%. In the case of temperature increase, the water in the water bath was cycled with fresh cold water.

After extraction (maceration and UAE), the extracts were filtrated through cotton fine-meshed cloth (Triqtex, Indonesia) in order to remove most of the raw material and the filtrates were then centrifuged (Hettich Rotina 35R centrifuge, Germany) for 10 minutes at 10,000 rpm, 25 ºC. The supernatants were collected using a graduated pipette and used for determination of total phenols, flavonoids, antioxidant capacity spectrophotometrically. All treatments were carried out in quadruplicate.

Determination of total phenolic content (TPC)

The determination of TPC of extracts obtained was adapted from Banerjee et al. (2008) with slightly modification and calibrated against gallic acid as the reference standard. A 0.3 ml sample was mixed with 1.5 ml of Folin-Ciocalteu reagent and 1.2 ml of sodium carbonate (7.5%), consecutively. The mixture of each step should be mixed well by using vortex (Genie 2 mixer, Scientific Industries, USA) and allowed to stand for one hour in a dark chamber. Absorption was measured by using UV-Vis spectrophotometer (Genesys 10 UV-Vis spectrophotometer, Thermo Electron Corporation, USA) at 765 nm. The standard curve gallic acid was prepared by diluting the stock standard with the extraction solvents to yield 50 to 200 ppm TPC. The results were calculated according to the calibration curve for gallic acid and mass fraction of TPC, derives from quadruplicate analyses and expressed as gallic acid equivalents (GAE mg/ g) of dry material (DM).

Determination of flavonoid content (FC)

The determination of FC was adapted from Do et al. (2014) with slight modification and calibrated against quercetin as the reference standard. The Leaf powder was first diluted with its solvent to reach dilution factor of 25 for test the FC in the samples. A 1.5 ml of methanol was prepared in the test tube. A 0.5 ml sample was mixed with 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml distilled water, consecutively. The mixture of each step should be mixed well by using vortex. The mixtures were incubated in a dark chamber for 30 minutes and absorbance was measured by using a UV-Vis spectrophotometer at 415 nm. The standard curve quercetin was prepared by diluting the stock standard with the extraction solvents to yield 20 - 200 ppm of FC. The results were calculated according to the calibration curve for quercetin and mass fraction of FC, derives from quadruplicate analyses and expressed as quercetin equivalents (QE mg/ g) of dry material (DM).

Determination of antioxidant activity (AAT)

The higher the consumption of DPPH in a sample, the more the inhibitory concentration (IC_{50}) is reduced. A 500 µL of sample in a test tube covered with aluminium foil with various dilutions were prepared. A 500 µL of 250 µM DPPH solution was added into each of the samples in test tubes. A control was prepared by adding 500 µL of DPPH stock solution into 500 µL extraction solvent (water or 70% ethanol). The tubes were mixed and allowed to stand for 30 minutes in a dark chamber before transferred into a cuvette and subjected to the measurement of absorbance by using UV-Vis spectrophotometer at 517 nm according to the methodology adapted from Rege and Chowdary (2014). All determinations were performed in quadruplicate.

For each extract, at least four dilutions were made in order to be able to plot a graph of DPPH scavenging activity (%) versus concentration of sample (ppm). The IC value was defined as the concentration in mg of dry material per ml that inhibits the formation of DPPH radicals by 50%. Each value was determined by the following regression equation:

$$\mathbf{IC_{50}\ DM = IC_{50} \times (100\% - P_m)}$$

Equation 1. Calculation formula of IC_{50}

Where IC_{50} DM was the IC_{50} in dry mass base with the unit still in mg/L, IC_{50e} was the IC_{50} of extract in wet based raw material, while P_m was the percentage of moisture in *R. mucronata* powder measured using a moisture content analyser (MA35, Sartorius, Germany) for 30 minutes at 105ºC.

Statistical analysis

The statistical data analysis performed in this research was the simple linear regression analysis and ANOVA using Microsoft Excel. In addition, Tukey HSD post-hoc test and one sample t-test were performed in the OpenStat application. All analyses were conducted at 95% confidence level where $p<0.05$ show a significant difference. In regression analysis, the correlation coefficient r was range from -1 to 1. After the Tukey HSD post-hoc test, letters were assigned to the data. Data with the same letter were not significantly different from each other.

RESULTS AND DISCUSSION:

In this study, two methods (maceration and ultrasound-assisted extraction) were used to extract TPC or FC from *R. mucronata* leaves under previously described condition. Table 1 shows the comparison of extraction times, moisture content, TPC, FC, and IC_{50} in relation to extraction methods. Two variables influencing the extraction were investigated which are solvents (water and 70% ethanol) and time (5, 15, 30, 45 and 60 minutes, and 24 hours maceration).

The ultrasonic-assisted extraction has been widely used for obtaining phenolic compounds from plants using ethanol, water, a mixture of ethanol/water and acetone (Khan et al., 2010; Zlabur et al., 2015; Rosello-Soto et al., 2015). Based on the experiments, it was observed that TPC or FC obtained by ethanol considerably higher at any method and time of extraction. The choice of solvents depends on the chemical properties of the components which would be extracted from a matrix. Therefore, ethanol is possibly the most suitable solvent system for the extraction of *R. mucronata* leaves due to the different polarities of the active constituents (Boeing et al., 2014; Do et al., 2014; Iloki-Assanga et al., 2015).

In term of extraction times, the yield of TPC reached a maximum at 45 minutes UAE with ethanol, 156.20 ± 12.08 GAE mg/ g DM, as table 1 showed. It was indicated that long period of extraction time favors the TPC production. But further increased the extraction time (60 minutes) in UAE showed decreasing in the TPC. Accordingly, 45 minutes was chosen as the TPC extraction time in succeeding experiments. For the maceration, the longer extraction time exposed the extract to the more environmental factors such as oxygen, heat and UV radiation which may lead degradation of TPC (Murugesan et al., 2012; Xu et al., 2017). The UAE yield of FC slightly increased within 30 minutes (16.23 ± 0.28 QE mg/ g DM), but after 30 minutes, its yield lower. According to the Noyes-Whitney theory, the dissolution is fast at firstly and changed little when the active ingredient concentration between inner and outer diffusion layer reach equilibrium after a period of extraction (Wang et al., 2012). Therefore, 30 minutes was chosen as the FC extraction time.

Table 1
Comparison of extraction times, moisture content, TPC, FC, and IC_{50} in relation to extraction methods, UAE and maceration

Time	Moist (%)		Freq	TPC (GAE mg/ g DM)		FC (QE mg/ g DM)		IC_{50} (ppm) Eth	
	water	(KHz)	eth	water		eth	water	eth	Water
5 min	7.77	10.89	35	133.47	108.12	15.75	13.63	59.12	64.79
				135.71	111.25	15.53	13.07	55.51	65.60
				139.06	109.91	15.86	13.40	50.59	53.94
				142.52	108.57	15.41	13.74	46.20	58.35
			Avg	137.69	109.46	15.64	13.46	52.86	60.67
			SD	3.96	1.41	0.20	0.30	5.65	5.54
15 min	13.64	9.00	35	124.65	107.11	15.86	13.63	61.56	66.14
				124.76	104.88	15.97	12.84	65.42	75.22
				121.30	108.23	15.64	13.40	62.68	60.79
				122.31	107.56	14.97	12.84	61.69	59.79
			avg	123.26	106.95	15.61	13.18	62.84	65.49
			SD	1.72	1.45	0.45	0.40	1.79	7.06
30 min	10.71	7.48	35	140.40	108.79	16.53	13.63	57.00	74.58
				135.60	109.68	16.31	13.07	55.06	74.63
				142.19	106.89	16.20	14.74	55.28	71.84
				146.10	103.54	15.86	14.63	48.48	75.52
			avg	141.07	107.23	16.23	14.02	53.96	74.14
			SD	4.36	2.72	0.28	0.81	3.75	1.59
45 min	13.08	6.31	35	145.95	108.01	15.41	14.07	55.53	50.57
				145.54	104.88	15.30	14.07	60.14	62.13
				167.32	95.72	15.41	13.52	57.16	64.18
				165.98	97.84	16.08	13.52	52.70	57.14
			Avg	156.20	101.61	15.55	13.80	56.38	58.51
			SD	12.08	5.79	0.36	0.32	3.11	6.06
60 Min	13.73	11.43	35	111.25	96.84	15.86	15.86	61.12	68.63
				106.78	96.28	15.30	15.19	68.99	75.99
				119.07	101.53	15.75	15.53	60.79	69.53
				118.06	101.53	15.64	16.20	59.52	72.74
			avg	113.79	99.05	15.64	15.70	62.61	71.72
			SD	5.82	2.88	0.24	0.43	4.31	3.35
24 h	8.41	13.2		133.70	89.52	19.66	15.59	55.29	116.62
				136.61	88.80	19.77	14.58	58.41	104.84
				133.48	86.96	18.04	13.96	52.10	102.49
				130.18	87.96	18.93	14.41	41.15	102.19

*Moist (%): moisture content in %, Freq: frequency, eth: 70% ethanol, avg: average, SD: standard deviation.

When compared with maceration, UAE was produced a significantly higher TPC and FC. With only five minute extraction time, the TPC obtained by UAE with ethanol was comparable with the yield obtained by using 24 hours maceration (137.69 ± 3.96: 133.49 ± 2.63 GAE mg/ g DM). The FC obtained by 30 minutes UAE with ethanol was also efficient in terms of time compared with the yield obtained by using 24 hours maceration (16.23 ± 0.28: 19.10 ± 0.80 QE mg/ g DM). This indicated that this method was time efficient since it greatly reduced extraction time.

The mechanism of ultrasound in liquids relies on the mechanical effect caused by the implosion of cavitational bubbles. During implosion of micro-sized cavitational bubbles, strong shear forces are created, while both high pressures and temperatures generated as a consequence of the bursting bubbles, cause rapid plant tissue disruption or cell wall breakage allowing cellular material release and improved mass transfer as well that lead to mass transfer of phenolic compounds to the solvent (Saleh et al., 2016; Chemat et al., 2017). In addition, ultrasound-assisted extraction can provide the opportunity for enhanced extraction of

heat-sensitive bioactive components at lower processing temperatures and is a more effective technique than conventional thermal extraction with most of the plants extracted within 15 minutes (Vilkhu et al., 2008; Cares et al., 2010; Dent et al., 2015).

The AAT data showed that there was a significant difference in IC_{50} between the two different solvents (water and 70% ethanol). The lowest IC_{50} value was showed by five minutes UAE with ethanol and 24 hours maceration with ethanol, 52.86 and 51.74 ppm, respectively. Although IC_{50} of maceration is lower than UAE, UAE is more efficient in terms of time. The extracts produced by both UAE and maceration methods had considerably high antioxidant activity, indicated by the IC_{50} DM value lower than 200 ppm (Molyneux, 2004).

The IC_{50} analysis result was consistent with the proposed relation between TPC and AAT. Further analysis showed that there was no significant difference among the extracts with the same solvent regardless the difference in extraction times. There was a strong correlation between DPPH scavenging activity with TPC with R^2 value 0.911 which showed in Figure 1. It suggested that TPC contributed as much as 91.1% of AAT for each extract and 8.9% contribution came from other compounds. The results also showed that percentage of scavenging activity increased linearly to TPC of each extract. This result was consistent with the conclusion of a study on the correlation between TPC of several mangrove extracts and their AAT (Agati et al., 2013). Flavonoids have also been contributed to the AAT of the plants due to its role in protecting leaves against the UV radiation in sunlight (Agati et al., 2007). The higher phenolic compounds the lower IC_{50} value since the extract with higher phenolic compounds would be able to scavenge more of free radicals at a given extract concentration (Stanković, 2011; Gao et al., 2014; Pamulaparthi et al., 2016). The compounds that function as antioxidant determined by the presence of free -OH functional group, such as flavons, flavonons, squalens, β-carotene, tocopherol and vitamin C (Parwata et al., 2009).

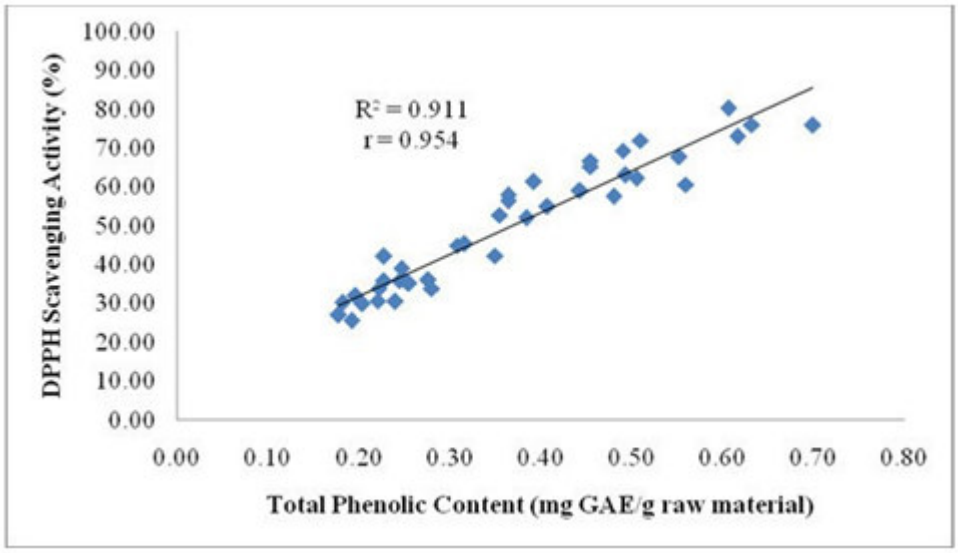

Figure 1
Correlation between phenolic content (mg GAE/g DM) with DPPH scavenging activity

Taken altogether, ultrasound-assisted extraction is an effective extraction technique that can offer high reproducibility in shorter time, higher yields of bioactive compounds, simplified manipulation, decreased temperature during processing, reduced solvent consumption, and lower energy input (Virot et al., 2010; Klen and Vodopivec, 2012; Dent et al., 2015).

CONCLUSION:

The results of this study showed that UAE with ethanol was a suitable and efficient method for the extraction of TPC and FC. Ethanol is possibly the most suitable solvent for the extraction of *R. mucronata* leaves due to the different polarities of the active constituents. The yield of TPC had reached a maximum at

45 minutes UAE with ethanol, 156.20 ± 12.08 GAE mg/ g DM. The UAE with ethanol yield of FC slightly increased within 30 minutes, 16.23 ± 0.28 QE mg/ g DM. The phenolic compound yielded by UAE with ethanol was considerably higher and need shorter time than maceration. The higher phenolic compounds obtained, the lower IC_{50} value since the extract with higher phenolic compounds would be able to scavenge more of free radicals. The IC_{50} value UAE with ethanol is 52.86 ppm. All this data showed that UAE with ethanol was the efficient method and *R. mucronata* is a very potent for the natural antioxidant source.

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