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Original Article

Phenolic contents and antioxidant activities of leaf extracts from *Elaeocarpus submonoceras* Miq.

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Abstract

The phenolic contents and antioxidant activities of leaf extracts from *Elaeocarpus submonoceras* Miq. were determined for two alternative extractions, namely maceration with ethanol and successive extraction by Soxhlet, with various solvents. Ethanol extract showed the highest phenolic content and strong antioxidant activities against DPPH and ABTS, followed in rank order by methanol and ethyl acetate extracts. The ethyl acetate extract showed no cytotoxic effect towards HaCaT, a human skin non-carcinoma cell. Based on thin layer chromatography, cytotoxic and antioxidant activities, ethyl acetate extract was chosen for isolation. The structure of an isolated compound was identified as gallic acid from ^{1}H-NMR. This study provides the first scientific information regarding the phenolic content and antioxidant activities of *E. submonoceras* Miq.

Keywords: *Elaeocarpus submonoceras*, phenolic content; antioxidant, cytotoxicity, gallic acid

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1. Introduction

Elaeocarpus submonoceras Miq., a member of the Elaeocarpaceae family, is a native tree from Kalimantan and West Java, Indonesia, with edible fruits, and is found in primary or secondary forests, swamps, or dipterocarp forests (Coode, 2001; Uji, 2004). This plant is a sub-canopy tree with alternate, simple, and penni-veined leaves, and white flowers (Figure 1). The seed is oval with a cone shape at the end and has a dark brown color (Lailati & Ekasari, 2015). *E. submonoceras* is also called "Katulampa" by local people in West Java and "Nkodoi" by the Dayak tribe, a local people in Temula Village, West Kutai, East Kalimantan, Indonesia. The leaf has long been used by the Dayak tribe for the treatment of skin diseases, wound healing, and face washing for cleansing and skin brightening purposes. Genus *Elaeocarpus* has approximately 200 species, some of which have been investigated for their chemical constituents (Chand, Dasgupta, Chattopadhyay, & Ray, 1977). Phenolic compounds, such as myricetin and gallic acid, have also been found in this genus (Chand *et al.*, 1977). Phenolic compounds are well known to be related to many biological activities, including antioxidant activities (Fawole, Makunga, & Opara, 2012).

The demand for cosmetic and skin care products has grown significantly in recent years due to the increasing interests in health and beauty, including for maintaining healthy skin. People are also demanding the use of natural ingredients (i.e., organic matter or derivatives, mainly plants) in cosmetic products (Fonseca-Santos, Corrêa, & Chorilli, 2015). Natural products displayed significant scavenging activities against reactive oxygen species, which can cause many skin care problems, such as photoaging, dyschromia, rhytides, and actinic damage (Bowe & Pugliese, 2014). In addition, synthetic ingredients, for example the whitening agent named hydroquinone, can cause ochronosis in long-term use and this is also a carcinogen (Sarkar, Arora, & Garg, 2013).

Consequently, this research aimed to investigate the phenolic content, antioxidant activities and cytotoxic effects of *E. submonoceras* Miq. leaf extracts. Moreover, isolation of the key compound and its structural identification were carried out.

Figure 1. Leaves and flowers of *Elaeocarpus submonoceras* Miq.

2. Materials and Methods

2.1 Plant material

Fresh leaves of *E. submonoceras* Miq. were collected in the rainy season (January to May) from Temula village, West Kutai, East Kalimantan, Indonesia. Mature leaves were collected from 3 to 6 years old trees. A reference specimen was deposited in Wood Chemistry Laboratory, Faculty of Forestry, Mulawarman University, East Kalimantan, Indonesia.

2.2 Leaf extraction

The leaves were air-dried under the shade and then ground. Ten grams of leaf powder were extracted with ethanol (95% v/v, 3x100 mL) for 48 h with constant shaking. The solution was then filtered through Whatman No. 1 filter paper (Sigma-Aldrich, USA) and evaporated at 40°C in a rotary evaporator to obtain ethanol crude extract.

Five-hundred grams of dried ground leaves were extracted successively using Soxhlet (4 L, 12 h) by hexane, dichloromethane, ethyl acetate and methanol, respectively. The solution was evaporated at 40°C in a rotary evaporator to obtain four different extracts.

2.3 Total phenolic content (TPC)

TPC was determined using a method reported by Pientaweeratch, Panapisal, and Tansirikongkol (2016) with modification. The extracts were diluted in 1% dimethyl sulfoxide (DMSO) in distilled water. The final concentration of samples was 50 µg/mL, while gallic acid as a reference was used in the range of 0.195-50 µg/mL. Twenty microliters of extracts and 100 µL of 0.2 N Folin-Ciocalteu reagent (Loba Chemie Pvt. Ltd., India) were added into 96-well plate (#3599, Corning®, NY, USA), then incubated for 5 min. After incubation, 80 µL of sodium carbonate (BDH Chemicals, Toronto, Canada) (75 g/L) was added, slightly shaken and reincubated for 120 min in dark, at room temperature. The absorbance of the mixture reaction was measured at 760 nm by SpectraMax M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA) with Softmax software (SoftMax, San Diego, CA, USA). Blank solution contained 1% (v/v) DMSO in distilled water. A standard curve to calculate phenolic contents was made by using linear regression. The results are reported as mg gallic acid equivalent (GAE)/g of dried weight (DW) of extract.

2.4 Antioxidant assays

2.4.1 Total antioxidant capacity (TAC)

The method for determining TAC was modified from Prieto, Pineda, and Aguilar (1999). Final concentrations of ascorbic acid were varied from 5 to 100 µg/mL, while the extract concentration was 50 µg/mL. Forty microliters of each extract were pipetted into 1.5 mL tube, and 400 µL of TAC reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate and 4 mM ammonium molybdate) were added then incubated for 90 min at 95°C. Two-hundred microliters from each reaction tube were transferred into a 96-well plate, and allowed to cool

to room temperature. The absorbance of reaction mixture was measured at 695 nm against a blank (contained 1% DMSO in distilled water only). The ascorbic acid reference standard results were plotted to make a standard curve by linear regression. TAC values are expressed as mg ascorbic acid equivalent (AAE)/g of dried weight (DW) of extract.

2.4.2 DPPH (2.2-diphenyl-1-picrylhydrazyl) inhibition

DPPH inhibition assay was modified from Lin *et al.* (2014). Reaction was made by mixing fifty microliters of sample (various concentrations of extracts or the positive control, ascorbic acid (from zero to 1000 µg/mL)) dissolved in 1% DMSO-methanol with 150 µL of 100 µM DPPH reagent in methanol, then incubated for 30 min in the dark. The inhibition of DPPH in the samples caused discoloration from purple to pale yellow and was measured at 512 nm using SpectraMax M3 reader.

An equation to estimate the percentage of DPPH free radical inhibition was adopted from Benmehdi, Behilil, Memmou, and Amrouche (2017):

$$\frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \quad (1)$$

where A_{sample} is the absorbance from the reaction of DPPH with the sample and $A_{control}$ is the absorbance of DPPH and solution only (without sample). Results from this equation were plotted and normalized using linear regression to get IC_{50} (concentration of the sample when inhibiting 50% of DPPH). Results of percent inhibition were calculated for 100 µg/mL sample concentration.

2.4.3 ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) inhibition

The ability of the sample to scavenge ABTS radical was assayed based on Fu, Zhang, Guo, and Chen (2014) with slight modification in a solution used to dilute ABTS•+ and potassium persulfate reagents, by change to be distilled water. The absorbance was measured within three minutes. Equation from linear regression of percent inhibition of sample was used to calculate IC_{50}. Concentrations of sample and ascorbic acid varied from 0.78 to 1000 µg/mL. Result of percent inhibition was obtained for 100 µg/mL sample concentration.

2.5 Cytotoxicity effect

Cytotoxicity of the plant extracts was determined by the method modified from Ritto *et al.* (2017). HaCaT human keratinocyte cell was maintained in DMEM (Gibco, NY, USA) and supplemented with 1% antibiotic-antimycotic 100x (Gibco, NY, USA), 10% heat-inactivated fetal bovine serum (HIMEDIA, Mumbai, India), and 1% 200 mM glutamine (PAA, Pasching, Austria). The cells were grown at 37°C and 5% CO_2 in a cell culture incubator. In 96-well plates, 10,000 cells/well in 100 µL of media were seeded and incubated for 48 h. Samples were prepared in 1% DMSO-complete media. After adding the samples, the cells were reincubated for another 48 h and then changed to 10% PrestoBlue (Invitrogen, CA, USA)-complete media. After incubating for 2 h, fluorescence units were measured at 570 (excitation) and 600 nm (emission) based on the manufacturer's reference. The cytotoxicity effect was determined from percentage of viable cells when treated with various final concentrations of extracts (10 to 1000 µg/mL). The IC_{50} value was obtained for 50% of cell viability by plotting the percentages from logarithmic regression in Excel.

2.6 Isolation of compound and structural identification

Ethyl acetate extract was isolated based on its thin layer chromatography, antioxidant activities and cytotoxic effect. The ethyl acetate extract (5.28 g) was eluted using silica gel 60 column chromatography with solvent system *n*-hexane-ethyl acetate (3:1, 2:1, 1:1, 1:3 v/v), ethyl acetate 100% followed by ethyl acetate-methanol (98:2, 95:5, 9:1, 7:3, 6:4, 5:5, 3:7, 2:8, 1:9 v/v) and methanol 100% to yield 13 fractions (E.1 – E.13). Fraction E.10 (324.9 mg) was then washed with dichloromethane and the precipitant (217.6 mg) was purified by crystallization with dichloromethane: methanol: water: acetic acid (90:10:1:0.1 v/v/v/v). The isolate compound was found in E10.11 (12.2 mg).

The purified compound (E10.11) was analyzed using BRUKER proton nuclear magnetic resonance spectroscopy (^{1}H NMR) 400 MHz in acetone ($(CD_3)_2CO$).

2.7 Statistical analysis

One-way analysis of variance (ANOVA) with significant differences ($P < 0.05$) was used for statistical analysis in Microsoft Excel Office 365 and IBM SPSS statistics 22 with Duncan's post hoc test. Results are displayed as mean values with standard deviations (mean±SD) from three replications of each sample.

3. Results and Discussion

3.1 TPC, TAC, DPPH, and ABTS of extracts

The leaf ethanol extract had a high phenolic content and high antioxidant activities as listed in Table 1. The ethanol extract and ascorbic acid had similar inhibitions of DPPH and ABTS at the same concentration (100 µg/mL) in the range from 94.98% to 100%. The ethanol extract of this plant had an excellent ability to scavenge free radicals, with IC_{50} values of 3.81 µg/mL DPPH and 13.51 µg/mL ABTS, compared with 12.72 µg/mL and 3.07 µg/mL for ascorbic acid (a well-known antioxidant compound), respectively.

Ethanol is a solvent that is commonly used to extract phenolic compounds. Phenolics have several distinctive characteristics, including their color, flavor, odor, bitterness, and biological activities, which have led to their use in many food and medicinal applications (Naczk & Shahidi, 2006).

The present study determined that ethyl acetate extract was not significantly different from the methanol extract. Both extracts had the highest phenolic content with no significant difference (504.92 and 542.81 mg GAE/g DW). They also had excellent activity to scavenge DPPH and ABTS free radicals with an IC_{50} range from 7.67 to 14.00 µg/mL, while the least activity was for the hexane extract. Based on

Table 1. TPC, TAC, DPPH and ABTS inhibitions of *Elaeocarpus submonoceras* Miq. extracts. The percentage inhibition of DPPH and ABTS is given for a concentration of 100 µg/mL. All results in this table are shown as mean ± SD (n = 3). Significant differences ($P < 0.05$) are shown by different superscript letters.

Extraction process/sample		TPC (mg GAE/g DW)	TAC (mg AAE/g DW)	DPPH		ABTS	
				Inhibiton (%)	IC_{50} (µg/mL)	Inhibiton (%)	IC_{50} (µg/mL)
Positive control	Ascorbic acid	-	-	96.60 ± 1.05^{a}	12.72 ± 0.32^{c}	100.00 ± 0.10^{a}	3.07 ± 0.04^{a}
Maceration	Ethanol	578.45 ± 31.44^{a}	419.73 ± 3.95^{a}	94.98 ± 0.29^{a}	3.81 ± 0.06^{a}	96.85 ± 0.11^{b}	13.51 ± 0.67^{b}
Successive	Hexane	7.65 ± 0.55^{c}	53.22 ± 3.08^{e}	35.37 ± 2.31^{c}	92.10 ± 4.69^{d}	11.04 ± 0.34^{d}	>1000
Soxhlet	Dichloromethane	57.23 ± 4.08^{c}	90.11 ± 4.59^{d}	64.99 ± 2.69^{b}	307.33 ± 0.06^{e}	13.98 ± 0.76^{c}	827.82 ± 5.20^{c}
extraction	Ethyl acetate	504.92 ± 43.23^{b}	274.21 ± 6.43^{c}	94.71 ± 0.66^{a}	7.81 ± 0.19^{b}	95.94 ± 1.10^{b}	14.00 ± 0.57^{b}
	Methanol	542.81 ± 50.11^{ab}	343.45 ± 1.51^{b}	94.07 ± 0.14^{a}	7.67 ± 0.09^{b}	96.75 ± 0.18^{b}	13.89 ± 0.34^{b}

these results, it was determined that the antioxidant activities originated from polar compounds were related to the abundance of phenolics in the extract. Similar to another report, the successive extraction of three species of *Mentha* (*M. spicata*, *M. pulegium*, and *M. piperita*) also had DPPH inhibition only from polar extracts (methanol and water) rather than from non-polar ones (hexane and dichloromethane) (Barchan, Bakkali, Arakrak, Pagán, & Laglaoui, 2014). Polar extracts were related to the phenolic content. Li *et al.* (2017) identified 15 phenolic compounds and concluded that DPPH and ABTS scavenging activities had a positive correlation with the phenolic constituents.

Antioxidant activity is a biological activity highly related to phenolics due to the donation of a hydrogen cation from the phenolic hydroxyl group to free radicals, generating relatively stable free radicals (Prihantini, Tachibana, & Itoh, 2015). Studies of the antioxidant activity are often performed by phosphomolybdenum, DPPH, and ABTS scavenging methods, which are widely used in plant and food research (Basumatary, Das, Nanjian, & Sharma, 2015; Pientaweeratch *et al.*, 2016).

Phenolic compounds that are also active as antioxidants can be found in various *Elaeocarpus* species. For example, an ethanol extract of leaves of *E. lanceofolius* contained 4'-methylmyricetin and myricetin-3-O-rhamnoside (Ray, Dutta, & Dasgupta, 1976). Mearnsetin was found in *E. serratus*, *E. oblongus*, and *E. floribundus*. Quercetin, kaempferol, and ethyl gallate were found in *E. tuberculatus*. Ellagic acid was found in *E. serratus* and *E. ganitrus* Roxb. (Chand *et al.*, 1977). Ethanol extract from leaves of *E. sphaericus* also showed the presence of gallic acid (Garg, Goswami, & Khurana, 2012). Gallic acid, a simple phenolic compound, possesses strong antioxidant activity and is easily absorbed by the human metabolic system (Badhani, Sharma, & Kakkar, 2015). Its mechanism in the human body involves counteracting with reactive oxygen species to produce antioxidants. However, an imbalance in the amount of antioxidants in the body can cause many diseases (Kurutas, 2016). Therefore, natural antioxidant agents that may be in leaf extracts could have an important role to play in safeguarding human health. Ethanol extract from *E. submonoceras* Miq. is promising as an antioxidant agent and could inhibit ABTS better than *E. ganitrus* leaf ethanol extract. *E. ganitrus* showed 55.77% ABTS inhibition at a concentration of 500 µg/mL and had a higher IC_{50} value (297.12 µg/mL). This species also had a lower TPC at 56.79 mg GAE/g DW (Kumar, Shanmugam, Palvannan, & Kumar, 2007). *E. ganitrus* bark ethanol extract was also found to have a higher IC_{50} value (81.85 µg/mL) against DPPH free radicals and phenol compounds were detected in a phytochemicals test (Talukdar, Dutta, Chakraborty, & Das, 2017).

3.2 Cytotoxic effects of *E. submonoceras* Miq. extracts

For commercial applications, comprehensive information about the content, including cytotoxicity against normal cells, is needed (Fonseca-Santos *et al.*, 2015). The extracts of *E. submonoceras* Miq. in concentrations ranging from 10–1000 µg/mL reduced viability of HaCaT, human skin non-carcinoma cells, in a dose-dependent manner (data not shown). Interestingly, the calculated IC_{50} values of ethanol, ethyl acetate, and methanol were 258.98, 446.25, and 230.27 µg/mL, respectively (Figure 2) and did not indicate a toxic effect compared to the IC_{50} values of antioxidant assays (Table 1). These results were similar to those from a previous study, which involved treating HaCaT and fibroblast cells with a leaf ethanol extract of *Helianthus tuberosus* L. The proliferation of normal cells after extract treatment may be related to the high concentration of phenolic compounds (Nizioł-Łukaszewska, Furman-Toczek, & Zagórska-Dziok, 2018). Methanol extracts of *E. tuberculatus* and *E. serratus* also showed low cytotoxicity against Vero cells (normal African green monkey kidney epithelial cells), in a concentration dependent manner (Shailasree, Sampathkumara, Niranjana, & Prakash, 2014).

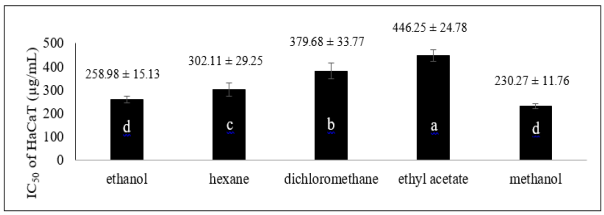

Figure 2. IC_{50} of *Elaeocarpus submonoceras* Miq. leaf extracts when treating HACAT cells for 48 h. All results are represented as mean + SD (n = 3).

3.2 Isolation of the active compound

The isolation process started from the separation of the compounds based on their polarity, followed by conducting TPC and antioxidant assays. DPPH and ABTS inhibition assays were used to evaluate all the extracts and

fractions until the pure compound was obtained. To date, no information on the phenolic content and antioxidant activities related to successive extracts of *Elaeocarpus* genus have been reported. The ethyl acetate extract was selected for further fractionation, purification, and isolation of the pure compound because its phenolic content, antioxidant activities, TLC; and this extract was the most non-toxic when tested against HaCaT cells (section 3.2).

This compound displayed as blue color when reacted with Folin–Ciocalteu's reagent (data not shown) and only one spot when confirmed by a TLC plate with variation of the solvent system (data not shown). The compound (E10.11) was obtained at a weight of about 12.2 mg as a white powder. ^{1}H NMR was used to identify the compound ($(CD_3)_2CO$, 400 MHz), δ (ppm): 7.15 (d, J= 1.2 Hz, 2 H (H-2 and H-6)) (Figure 3), and the results revealed that it was gallic acid. The ^{1}H NMR result is similar to previous reports related to the *Elaeocarpus* genus. Leaf extracts of *E. sylvestris* and *E. tonkinensis* also contained gallic acid (Dao *et al.*, 2018; Prihantini *et al.*, 2015). Gallic acid is also one of the major compounds in *E. tuberculatus* and *E. ganitrus* (Chand *et al.*, 1977). Gallic acid or 3,4,5-trihydroxybenzoic acid is a member of the phenolic group, with one aromatic ring, one carboxylic acid, and three hydroxyl substituents. It has a low molecular weight with a simple structure, but possesses high antioxidant activities (Badhani *et al.*, 2015).

4. Conclusions

Information from the Dayak tribe regarding *E. submonoceras* Miq. used for skin and wound treatment was validated in this study. Leaf extracts from *E. submonoceras* Miq. showed abundant phenolic content, indicating it may be used as a safe antioxidant agent. Gallic acid was identified as one of the active compounds. Information of antioxidant activities from this study could help expand the utilization of this plant as a supplement for skin care or in cosmetics production, and could lead to a further study of the antioxidant activities of this plant extract *in vivo*.

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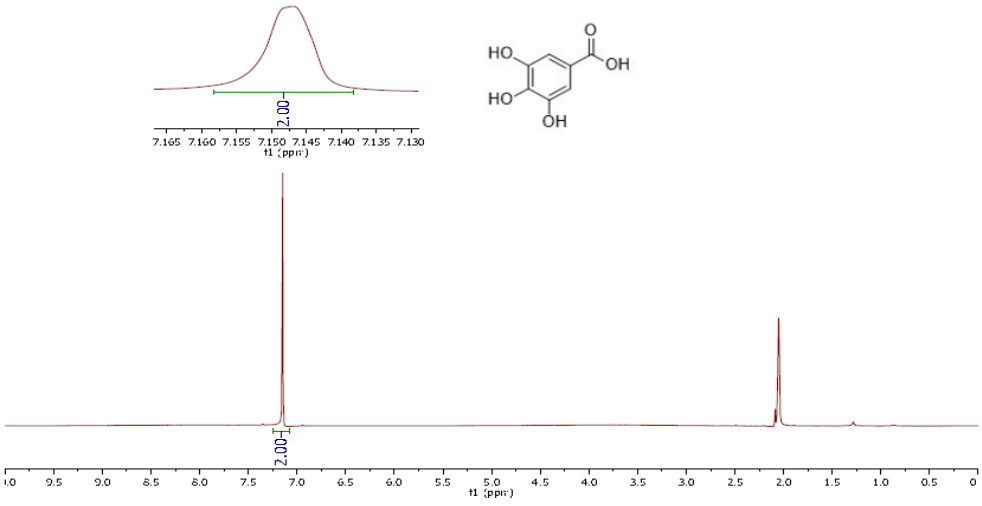

Figure 3. ^{1}H NMR of the purified compound (E10.11)

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