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Isolation And Identification Of Active Compound Of Ethylacetate Fraction Of Kasturi (*Mangifera casturi* Konsterm.) Fruit From South Kalimantan Indonesia

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ABSTRACT

Kasturi (*Mangifera casturi* Konsterm.) is a native plant of Kalimantan and has a chemical compound of potential as a drug. A test was conducted for isolation and identification of the active compound from ethyl acetate fraction of *M. casturi* fruit. The ethyl acetate fraction was obtained from methanolic extract of *M. casturi* that was extracted using *n*-hexane; *n*-hexane insoluble extract was re-extracted using ethyl acetate (ethyl acetate soluble extract is called ethyl acetate fraction). Isolation and identification of active compound from ethylacetate fraction of *M. casturi* fruit had been done using liquid liquid extraction method followed by column chromatography. The compound was isolated using column chromatography with *n*-hexane-ethyl acetate as solvents at gradients of (10:1, 8:2, 6:4, 5:5, 4:6, 2:8) v/v and 100% ethyl acetate. The isolate was white crystal with melting point ranging from 189 - 190°C. Identification of the isolate was conducted with spectroscopic analyses of UV-Vis, FT-IR, ¹HNMR, ¹³CNMR, DEPT, and LCMS. The identification found a polyphenol compound, namely, 3,4,5-trihydroxy-benzoic acid methyl ester (methyl gallate).

Keywords : *Mangifera casturi* Konsterm, ethyl acetate fraction, isolate, 3,4,5-trihydroxy benzoic acid methyl ester

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INTRODUCTION

Kasturi (*Mangifera casturi* Kosterm.) is a plant of genus *mangifera*, *anacardiaceae* family. The genus *mangifera* consists of more than 51 species and can thrive in Asia, particularly in Indonesia. In the Southern Kalimantan, there are three varieties, namely, *casturi*, *cuban/castuba*, and *asem pelipisan/palipisan*. Casturi fruit has something in common with mango fruit, but casturi fruit is smaller, round up to ellipsoid, 5-6 cm length, 4-5 width, and \pm 65.6 grams of weight. The ripe fruit is green-purple. It tastes sweet and smells good. It is edible for the community in fresh condition. The casturi can thrive well in the Southern Kalimantan Indonesia, but it is more scarcely found. Casturi is one of the specific plants of Kalimantan. It is also known as Borneo Mango. The plant grows widespread in the Southern Kalimantan, such as Martapura, Kandungan, and Tanjung. In addition, it is also widespread in the Central Kalimantan and Eastern Kalimantan, such as Kutai and Tenggarong Sebrang[1].

The research made use of casturi fruits collected from Banjar District, Southern Kalimantan. The fruits were harvested from December 2015 to January 2016. Some parts of the kasturi fruit had been studied. For instance, the juice of kasturi fruit flesh was reported to have an antioxidant activity[2]. Phytochemical test on the casturi stem bark found polyphenol, terpenoid, and saponin compounds[3]. [4]Reported that ethylacetate fractions of casturi fruit had an antioxidant activity with an $IC_{50} = 6.01 \mu\text{g/mL}$. Phytochemical test on the ethylacetate fractions of casturi fruit found compound of polyphenol group, which had antioxidant activity.

Isolation was conducted for the ethylacetate fractions of *M. casturi* fruit, guided by spot monitoring with thin-layer chromatography. Elucidation and identification of the isolated compound were conducted by analyzing the data of UV-Vis spectroscopy, FT-IR, ^1H NMR, ^{13}C NMR, DEPT, and LCMS spectroscopy. This research is very important to get the active compounds in plants. Therefore, in this study we focused on the awareness for *M. casturi* fruits as biologically active compounds with medicinal.

MATERIAL AND METHOD

Plant materials

Fifteen kilograms of *M. casturi* fruits were collected from Banjar District, Southern Kalimantan, Indonesia. The sample of ripe *M. casturi* fruits was collected from December 2015 – January 2016. Plant Identification was conducted in Pharmaceutical Biology Laboratory, Gadjah Mada University, Yogyakarta. Parts of the fruit used in the research were the fruit flesh and fruit bark (isolated from the seed), desiccated under 50°C for 3 days. The desiccated sample was made into powder using a grinder and extracted with methanol as a solvent.

Extraction and fractionation

Two kilogram of sample powder of casturi flesh was extracted in maceration using methanol as a solvent. The extract was thickened using a rotary vacuum evaporatory under a temperature of 60°C . Fractionation was conducted with liquid-liquid extraction method by weighing 100 grams of thick methanolic extracts and then extracting the powder in 150 mL of *n*-hexane solvent. The extraction was repeated seven times; *n*-hexane non-soluble compound was re-extracted using 100 mL of ethylacetate; re-extraction was repeated 5 times. Ethylacetate soluble compound was evaporated (called ethylacetate fraction).

Isolation procedure

The compound isolation was conducted with bioassay guided method, for extraction, fractionation, and isolation of its activity as an antioxidant. Thin-layer chromatography (TLC) was conducted upon the ethylacetate (EtoAc) fraction, using immobile phase of silica gel 60 F₂₅₄ and mobile phase of *n*-hexane-EtoAc (6:4)v/v. The compound to isolate was monitored through a chromatogram sprayed with a specific reactant. The reactant used was 0,4 nM of DPPH to monitor antioxidant compounds and cerium sulfate for general compounds. Spots indicated as antioxidant were isolated vacuum liquid chromatography a solvent gradients of *n*-hexane-EtoAc [8:2 (Fraction A); 7:3 (fraction B); 6:4 (fraction C); 5:5 (fraction D); 4:6 (fraction E); 3:7 (fraction F); and 2:8 (fraction G)]. The isolation was followed by column chromatography on fraction E, using solvent gradients of *n*-hexane-EtoAc (10:1, 8:2, 6:4, 5:5, 4:6, 2:8)v/v and 100% EtoAc.

Structure analysis with spectroscopy

Structure analysis conducted on the isolated pure compounds using spectroscopic data visible light Ultra Violet (UV-vis, Spectronic® 20 genesys™), Infrared spectroscopy (FT-IR, Perkin Elmer 100), hydrogen nuclear magnetic resonance spectroscopy (¹HNMR, JEOL 500 MHz), carbon nuclear magnetic resonance spectroscopy (¹³CNMR, JEOL 500 MHz), liquid chromatography mass spectroscopy (LCMS, Mariner biospectrometry Hitachi L 6200) from the Indonesian institute of Science (LIPI). Spectra were analyzed to determine the chemical structure of the isolated compounds.

Antioxidant activity test

Antioxidant activity test on the isolated compound was conducted with DPPH (1,1-diphenyl-2-picrylhydrazyl) method. DPPH 0.4 mM solution in methanol pa used for the test must always be fresh. DPPH reactant was prepared by dissolving 15.7 mg in 100 mL of methanol. The isolated compound was dissolved in methanol, prepared at concentrations of (0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0) µg/mL b/v, and then reacted in 1 mL of DPPH solution. Enough volume was obtained by adding up to 5 mL of methanol. The mixture was homogenated with vortex, let under room temperature for 20 minutes, and calculated for absorbance with a spectrophotometer at a wavelength of 517 nm. Quercetin was used as a positive control. Antioxidant activity of the sample was presented in the reduction ratio of DPPH absorbance (%) that is calculated using the formula:

$$\text{Reduction ratio of DPPH absorbance} = \frac{\text{abs. blanco} - \text{abs. sample}}{\text{abs. blanco}} \times 100\%$$

RESULTS AND DISCUSSIONS

Phytochemical test

Phytochemical test on the ethylacetate fraction of casturi fruit showed that it contained a compound of polyphenol group and had an antioxidant activity, as shown in table 1. The compound of polyphenol group is shown using a specific reactant (FeCl₃), while the antioxidant property is shown with 0,04 mM DPPH reactant, sprayed on a TLC plate.

Table 1. Chromatogram of ethylacetate fraction of casturi fruit

Spot	Eluent	Spot Color			HR _f	Result
		UV ₂₅₄	DPPH	FeCl ₃		
1	N-hexane-ethylacetate (4:6) v/v	-	Yellow	-	80	Antioxidant
2		Purple	Yellow	Blue	35	Polyphenol-antioxidant
3		Brown	Yellow ^a	Blue	4	Polyphenol-antioxidant

Antioxidant activity test

Antioxidant activity test for the isolated compound used quercetin as a positive control. Both the isolated compound and quercetin showed an activity as a free radical scavenger in DPPH. At concentration of 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32,0 µg/mL, the isolate showed a very strong antioxidant effect, with an IC₅₀ value of 2.35 µg/mL (stronger than that of quercetin, IC₅₀ 2.96 µg/mL). The results of antioxidant activity test for the isolated compound using DPPH method are presented in Table 2.

DPPH is the most commonly used method in antioxidant activity test. Advantages of the method include simplicity, quickness, and sensitivity to test the antioxidant activity of certain compounds or plant extracts[1],[5]. The mechanism is based upon the reaction that involves DPPH as an electron scavenger or as a hydrogen radical scavenger by producing a stable diamagnetic compound to neutralize free radicals in the DPPH. Antioxidant activity test for the isolated compound using DPPH method showed an IC₅₀ value of 2.35 µg/mL, compared to the positive control (quercetin), IC₅₀ = 2.96 µg/mL. ANOVA analysis at a Confidence Interval (CI) of 95% (P<0.05) showed that within the same range of concentration, no significant difference was

observed in the activity as a DPPH free radical scavenger between the quercetin (positive control) and the isolated compound.

Table 2. Antioxidan activities of isolate compound and quercetin (positif control) free radical scavenging.

Sample	Concentrations ($\mu\text{g/mL}$)							IC ₅₀
	0,5	1,0	2,0	4,0	8,0	16,0	32,0	
Isolat	21,51 ^a	22,89 ^a	50,10 ^a	64,37 ^a	75,74 ^a	87,33 ^a	92,14 ^a	2.35 ^b
Quercetin	20,43 ^a	25,84 ^a	38,41 ^a	54,42 ^a	66,48 ^a	88,41 ^a	90,52 ^a	2,96 ^b

^a percentage inhibition of DPPH radical

^b inhibitory activity was expressed as the mean of 50% inhibitory concentration

Structure identification

Active compound resulted from the isolation was white crystals with a melting point (m.p) of 189-190°C. Chemical structure characterization with UV-Vis, FT-IR, ¹HNMR, ¹³CNMR, DEPT, and LCMS spectroscopy. UV-Vis spectroscopy with a λ_{max} absorbance spectrum of 226 indicated a methoxy molecule (-OCH₃) and a λ_{max} absorbance of 278 indicated an aromatic compound. In FT-IR (KBr) spectroscopy, a ν_{max} absorbance of 3367.85 cm⁻¹ characterized a hydroxyl group (OH); an absorbance of 2950.15 cm⁻¹ indicated C - H aromatic; an absorbance of 2370.75 cm⁻¹ indicated C - H alifatic; an absorbance of 1692.63 cm⁻¹ indicated a characteristic group of C = O, and an absorbance of 1619.21 cm⁻¹ indicated C = C group of aromatic compound (Figure 1).

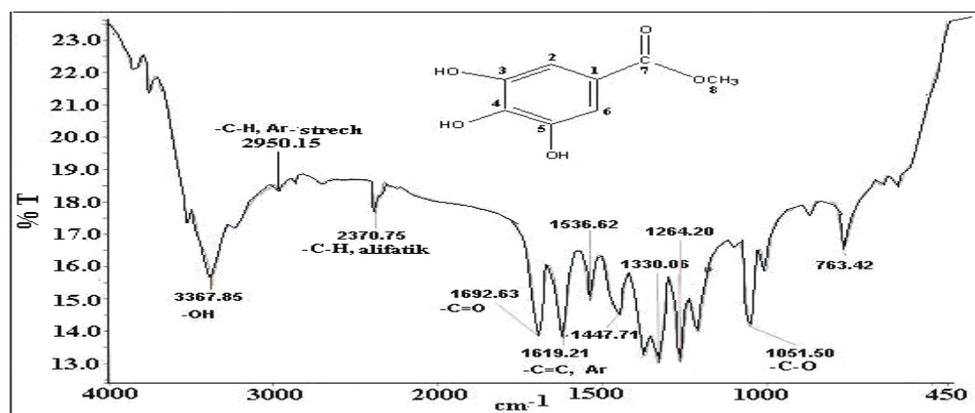


Figure 1. FTIR spectrum of isolate compound (pellet KBr)

¹HNMR spectroscopy analysis indicated two characteristic signals on the 3,4,5-trihydroxy-benzoic acid methyl ester compound, namely, a shift at δ 7.0 characteristic of aromatic proton type; the signal of two integrated protons from H-2 and H-6, which were symmetric at the shift of δ 3.8, indicated a specific signal of methoxy proton type (Figure 2).

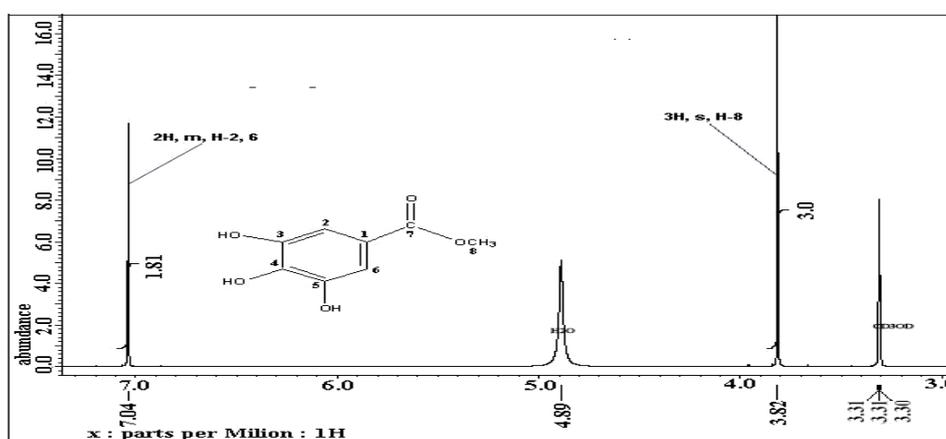


Figure 2. ¹HNMR spectrum of isolate compound (Jeol 500 MHz, CD₃OD)

^{13}C NMR spectrum (Figure 3) indicated 8 carbon atoms and DEPT analysis showed 5 quaternary carbon atoms (C), 2 metin atoms (CH), and 1 methyl (CH_2). There are seven types of carbon, consisting of C = O signals at δ c 169.10 (indicating ester carbonyl). The signal at δ c 146.59 indicated 2 aromatic oxygenated carbons, at C-3 and C-5. The signal at δ c 139.86 indicated one of aromatic carbons, C-4. The signal at δ c 121.49 indicated quaternary carbon at the aromatic ring, C-1. The signal at δ c 110.08 indicated two symmetric metin carbons at the aromatic ring, C-2 and C-6. The signal at δ c 52.36 indicated carbon atoms of methylene, namely, aliphatic C Ester.

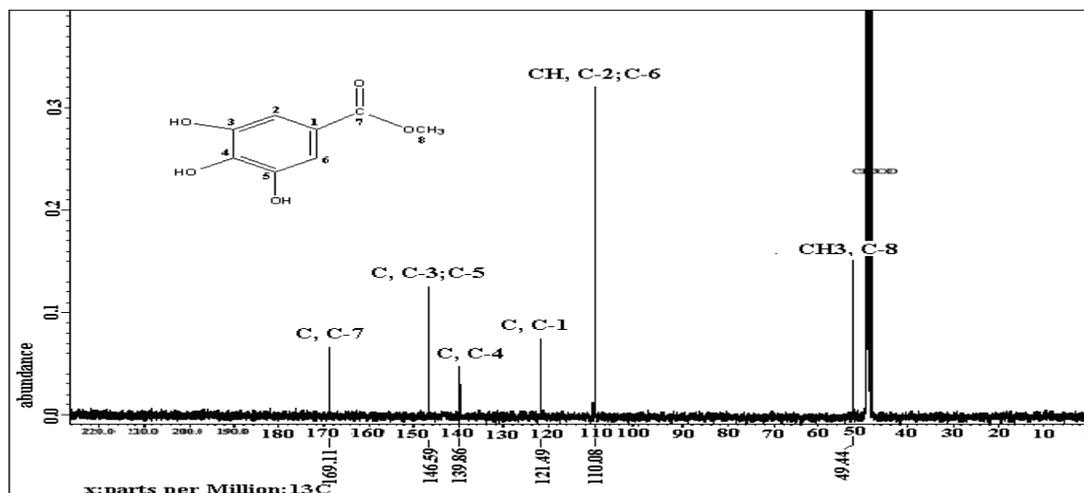


Figure 3. ^{13}C NMR spectrum isolate compound (Jeol 500 MHz, CD_3OD)

Post-ion LC MS-ESI spectroscopy data showed an ion peak at m/z 185.153 ($M + 1$) which indicated a point of molecular weight of 3,4,5-Trihydroxy-benzoic acid methyl ester. The ion peak at m/z 153 indicated the release of methoxy molecule group ($-\text{OCH}_3$) from the compound. Spectroscopic analysis concludes that the compound isolated from the ethylacetate fraction of casturi is 3,4,5-Trihydroxy-benzoic acid methyl ester (methyl gallate), as shown in Figure 4.

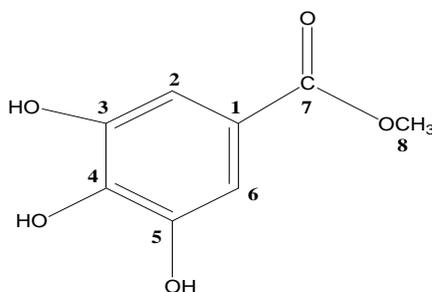


Figure 4. Structure of compound isolated (3,4,5-Trihydroxy-benzoic acid methyl ester)

Methyl gallate compound had been isolated from *Toona sureni* (Blume) Merr. Leaves[6], *Labisia pumila* Benth leaves[7], and *Galla Rhois* leaves[8], [9]. This is the first research to isolate the methyl gallate from *M. casturi* fruit. Methyl gallate compound had been reported to have antioxidant activity[6], [10], antibacterial activity[9],[11], and anti-herpes simplex[12] and to stimulate the production of inflammatory mediator interleukin-6 and interleukin-8[11] and cyclooxygenase-2 inhibitor[13]. Its derivatives such as methyl, propyl, butyl, pentyl, isopentyl, phenyl gallate, 3, 4, 5 triacetoxylgallic acid, 3, 4, 5-trimethoxy gallic acid, and methyl 3, 4, 5-trimethoxy gallic acid had an antioxidant and anti-Parkinson activity[10].

CONCLUSIONS

Isolation of ethylacetate fraction of *Mangifera casturi* Kosterm fruit successfully isolated 3,4,5-Trihydroxy-benzoic acid methyl ester (methyl gallate), isolated for the first time from *M. casturi* plant. The isolated compound has an antioxidant activity with value IC_{50} 2.35 $\mu\text{g}/\text{mL}$.

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