An Ellagic Acid Derivative and Its Antioxidant Activity of Chloroform Extract of Stem Bark of *Syzygium polycephalum* Miq. (Myrtaceae)

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ABSTRACT

The investigation of the Syzygium polycephalum *Miq. (Myrtaceae) aimed to assess the phytochemical contents and antioxidant activity of the chloroform fraction. In this study, the fraction was obtained from methanol extract of* S. polycephalum *stem bark partitionated by chloroform. An ellagic acid derivative was successively isolated from the chloroform fraction. The molecular structure of isolated compound was elucidated and established as 3,4,3'-tri-*O*-methylellagic acid through extensive spectroscopic studies including UV-Vis, FTIR, NMR and LC-MS analyses and by comparison with literature data. The finding of the isolated compound is the first time from the plant, although the isolated compound previously have been found in the other* Syzygium *species such as* S. cumini *together with ellagic acid and 3,3'-di-O-methylellagic acid. The chloroform fraction, isolated compound, and vitamin C showed antioxidant activity against 2,2'-diphenyl-1-picrylhydrazyl (DPPH) with* IC_{50} *value of 163.6, 72.1, and 11.5 µg/mL, respectively.*

Keywords: *antioxidant; ellagic acid derivative; Myrtaceae;* Syzygium polycephalum

ABSTRAK

Penelitian pada tumbuhan Syzygium polycephalum *(Myrtaceae) ini bertujuan untuk mengetahui kandungan fitokimia dan sifat antioksidannya dari fraksi kloroform. Dalam penelitian ini, fraksi kloroform diperoleh dari ekstrak metanol kulit batang tumbuhan* S. polycephalum *yang dipartisi oleh pelarut kloroform. Suatu senyawa turunan asam elagat telah berhasil diisolasi dari fraksi kloroform. Struktur senyawa hasil isolasi tersebut dielusidasi melalui kajian spektroskopi (UV-Vis, FTIR, NMR, dan LC-MS) dan juga melalui perbandingan dengan data literatur, dan ditetapkan sebagai asam 3,4,3'-tri-*O*-metilelagat. Penemuan senyawa hasil isolasi ini merupakan pertama kalinya dari tumbuhan tersebut, meskipun sebelumnya senyawa ini juga telah ditemukan pada tumbuhan* Syzygium *lainnya, yakni* S. cumini *bersama dengan asam elagat dan asam 3,3'-di-*O*-metilelagat. Fraksi kloroform, senyawa hasil isolasi, dan vitamin C telah menunjukkan aktivitas antioksidan terhadap 2,2'-diphenyl-1-picrylhydrazyl (DPPH) dengan nilai* IC_{50} *masing-masing adalah 163,6; 72,1; dan 11,5 µg/mL.*

Kata Kunci: *antioksidan; Myrtaceae;* Syzygium polycephalum*; turunan asam elagat*

INTRODUCTION

Syzygium is a genus in the Myrtaceae family that includes a number of popular species cultivated for their many purposes such as colorful, edible and fleshy fruit. The genus name *Syzygium* is derived via Latin from the Greek word 'syzygos', meaning yoked together, possibly referring to the paired leaves [1]. *Syzygium*, the genus of flowering plants, is one of the important genera in Myrtaceae family, mostly distributed in the tropical and sub-tropical region of the world, with the greatest diversity of species taking place in South East Asia such as Indonesia, Malaysia and also in East India. It is represented by around 140 genera and 1100 species [2].

Recent studies focusing on the exploitation of natural compounds from *Syzygium* species for medicinal purposes have drawn much attention to the effective extraction of the desired bioactive ingredients from natural products. On basis of literature study, phytochemical studies on *Syzygium* species have led to the identification and isolation of main compounds such as phenolic compounds (e.g. eugenol, eugenol acetate), and β-caryophyllene found in *S. aromaticum* [3]. The compound had been also found in *S.*

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samarangense [4]. Then, eucalyptol and α-cadinol had been isolated from *S. caryophyllatum* [5]. In addition, friedelane, friedelin, 3-α-friedelinol and stigmasterol had been obtained from *S. alternifolium* (Wight) Walp. [6], then flavonoids from *S. polyanthum* (Wight) Walp leaves [7]. Six flavonoid compounds had been found from *S. aqueum* leaf [8], flavonoid eucalytin from *S. alternifolium* Walp. leaves [9] and others.

Syzygium polycephalum, locally known as gowok or kupa or kepa, is an indigenous tree growth in Indonesia. It has synonyms: *Eugenia polycephala* Miq. and *Jambosa cauliflora* DC. It was reported that the plant can be able to lower high blood pressure and high cholesterol level and it exhibits antioxidant activity [10]. A recent study reported that a decoction of the bark of the plant is used for the treatment dysentery [11]. Additional information, it has been reported the presence of several compounds found in the plant such as ursolic acid, oleanolic acid, squalene, and β-sitosterol from *S. polycephalum* leaves [12]. It has been known that all of these compounds are non phenolic compounds. On the other hand, it was reported that the wood extracts (ethyl acetate extract) of *S. polychephalum* potentially contained anti-fungal compound (i.e. 3-*O*-glucosyl-3',4',5-trihydroxyflavonol) to inhibit the growth of *S. commune* Fr. and *Pleurotus* sp. fungi [13]. However, there is no literature on the total phenolic contents (except the compound as mentioned above), antioxidant and other potential bioactivity of the medicinal organic compounds in the stem bark of *S. polycephalum*. Therefore, it is a great interest to carry out a proper scientific investigation of the stem bark extract of this plant. The present study however, reports for the first time the isolation and structural elucidation of 3,4,3'-tri-*O*-methylellagic acid from the chloroform fraction of the stem bark of *S. polycephalum*.

EXPERIMENTAL SECTION

Materials

Isolated compound was found from the stem bark of *S. polycephalum*. DPPH (2,2'-diphenyl-1-picrylhydrazyl) and vitamin C (Vit. C, ascorbic acid, as antioxidant standard) were purchased from Merck (Germany) and sigma Aldrich (USA). Hexane, chloroform, ethyl acetate, methanol and silica gel used were analytical grade (Grade AR) and purchased from Merck (Germany) and sigma Aldrich (USA). The stem bark of *S. polycephalum* (c.a. 27 kg) was collected from a local area in Ngawi, East Java, Indonesia in December 2014. The identification of the plant was performed by staff of Herbarium-LIPI, Purwodadi, East Java, Indonesia. A voucher sample is kept in the Herbarium of LIPI with Identification No. 0117/IPH.06/HM/I/2015, January 5, 2015.

Instrumentation

The equipment used to do extraction and fractionation (isolation) were filter paper, Buchner funnel, Hirsch funnel, Erlenmeyer flask, pippet, spatula, measuring glass, vials, containers, separating funnel, and vacuum rotary evaporator type BUCHI Rotavapor R-215. The equipment used to measure melting point of isolate was Fisher Scientific. Whereas, chromatographic techniques used to isolate phenolic compounds from chloroform fraction included Gravitational Column Chromatography (GCC) (silica gel 60, 0.063–0.200 mm and 0.200–0.500 mm or 70–230 mesh ASTM), TLC analysis were carried out on silica gel 60 F254 chromaplates with the developing solvent systems. Checking the homogeneity of the compounds were made by TLC on Kieselgel gel 60 F254 pre-coated sheets (Merck) and the spots were detected by exposure to UV-lamp at 254 or 366 nm.

A number of instruments were needed to identify and characterize the isolates including spectrophotometer FTIR-8400S Shimadzu, spectrophotometer UV-1800 Shimadzu. The ^{1}H-NMR spectra were recorded with a Bruker DRX-600 NMR Spectrometer (600 MHz, CD_3OD) instrument and the ^{13}C-NMR spectra were obtained with the same instrument at 150 MHz in CD_3OD. Chemical shifts are given in δ (ppm) values relative to those of the solvent signal [CD_3OD (δH 3.30; δC 49.0)] on the tetramethylsilane (Sigma) scale.

Procedure

Extraction and isolation

The fresh stem bark of *S. polycephalum* (c.a. 27 kg) was washed under tap water and dried under sunlight for one week. Then, it was dried in an oven at reduced temperature (not more than 50 °C) to make it suitable for grinding purpose. It was then ground to fine powder using electric grinder to obtain 8.3 kg and transferred to air tight container. The dried and powdered stem bark (8.3 kg) of *S. polycephalum* was macerated with methanol (c.a. 25 L) at room temperature. The container with its content was sealed by foil and kept for a period of 24 h accompanying occasional shaking and stirring and carried out for three times. The whole mixture was then filtered using Buchner funnel and the filtrate was concentrated at 50 °C with a vacuum rotary evaporator. The concentrated extract obtained is termed as crude extract (349 g) of a yellowish brown thick syrupy. After removal of the solvent by evaporation, the residue was suspended in

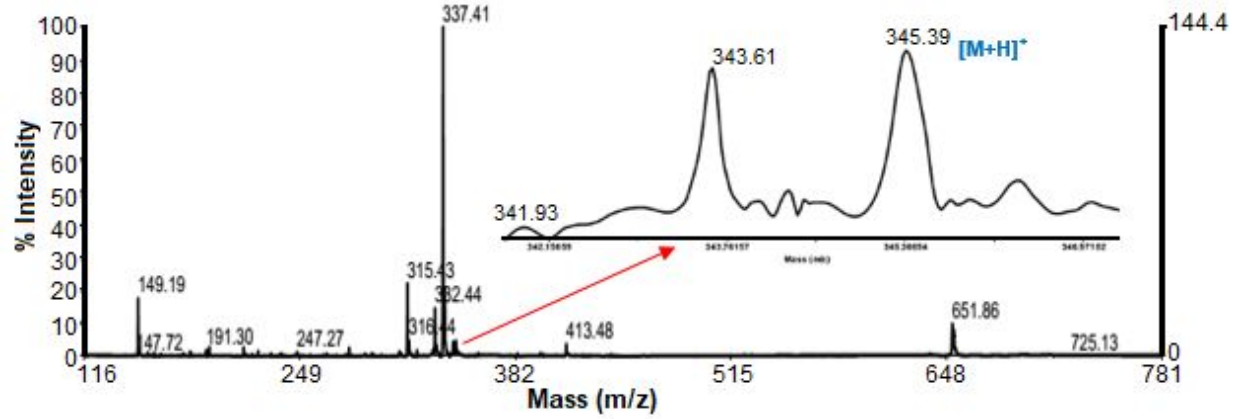


Fig 1. LC-ESI-MS spectral data of isolated compound

methanol and defatted with hexane yielded two layers in which the upper layer (hexane soluble parts) was then separated to gain hexane fraction. This process was repeatedly conducted for three times and hexane fraction obtained is evaporated to gain hexane extracts (22.38 g). By using the same manner, the residue was then suspended in methanol and defatted with chloroform yielded two layers. The under layer (chloroform soluble parts) was separated to obtain chloroform fractions. This process was also repeatedly carried out for three times and yielded chloroform fraction (5.66 g).

The chloroform fraction was then divided by two portions in which the first portion of chloroform fractions (3 g) and the second portion (2.66) was subjected to silica gel GCC using hexane-chloroform-methanol system (5:4:1) to yield 55 fractions. On the basis of TLC, from 55 fractions of each portion that give the same value of Rf can be grouped into 5 fractions A (1-4), B (5-6), C (7-37), D (38-51), and E (52-55). Based on TLC analysis, the fraction B (5-6) seemed that the fraction gave simple chromatogram profile and allowed to evaporate at room temperature yielded a pure enough isolate (termed as isolate GW-1) as off-white amorphous powder (10.3 mg) with mp. 267-269 °C. The isolate was then characterized by UV-Vis, FTIR, LCMS and NMR spectroscopies and by comparing with literature data. The structure might be 3,4,3'-tri-*O*-methylellagic acid.

Antiradical assay

The antioxidant assay using 2,2'-diphenyl-1-picrylhydrazyl (DPPH) was performed on extracts, fractions, and purified compounds and was described in detail as follows. Each test samples (the chloroform fraction [0.3 mL: 10, 15, 25, 50 and 100 µg/mL] and isolated compound as well as antioxidant standard (Vit. C) (0.3 mL: 1, 5, 10, 15 and 20 µg/mL) in methanol were mixed with methanol solution (3 mL) containing DPPH radicals (0.004%, w/w). The mixture was vigorously shaken and was left to stand for 30 min in the dark before measuring the absorbance at 515 nm against a blank [14] with slight modifications. Then, the scavenging ability (the percentage inhibition, I%) was calculated using eq. 1.

$$\%\text{Inhibition} = \frac{\text{A blank - A sample}}{\text{A blank}} \times 100 \quad (1)$$

where A blank is the absorbance value of the control and A sample is the absorbance value of the test samples [15]. Ascorbic acid can be used as positive controls. Percentage radical scavenging ability was plotted against the corresponding antioxidant substance concentration. The results were expressed as IC_{50} values and were calculated by linear regression analysis of tests conducted in triplicates. The equation for the line is used to obtain the IC_{50} value, which is defined as the amount of antioxidant substance required to scavenge 50% of free-radicals (DPPH) present in the assay system. On the other words, IC_{50} (50% inhibitory concentration) values were obtained through extrapolation from concentration of test samples necessary to scavange 50% of free-radicals (DPPH). A lower IC_{50} value indicates greater activity. IC_{50} < 50 µg/mL is very active; 50 µg/mL < IC_{50} < 100 µg/mL is active; 100 µg/mL < IC_{50} < 200 µg/mL is moderately active; and IC_{50} > 200 µg/mL is not active [16].

RESULT AND DISCUSSION

Structural Determination of the Isolated Compound from Chloroform Extract

Isolated compound was obtained as an off-white amorphous powder (10.3 mg), m.p. 267-269 °C and its molecular formula $C_{17}H_{12}O_8$ was determined by the LC-ESI-MS (m/z 345.39 [M^+H^+] as seen in Fig. 1). The UV-Vis (MeOH, λ_{max}) spectrum of isolated compound

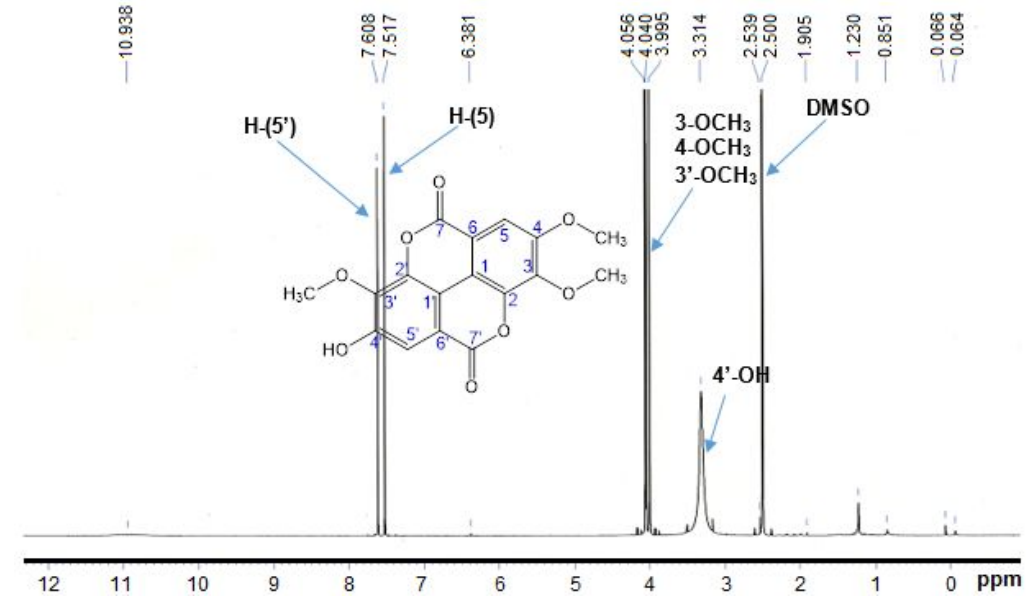


Fig 2. The H-NMR spectral data of isolated compound

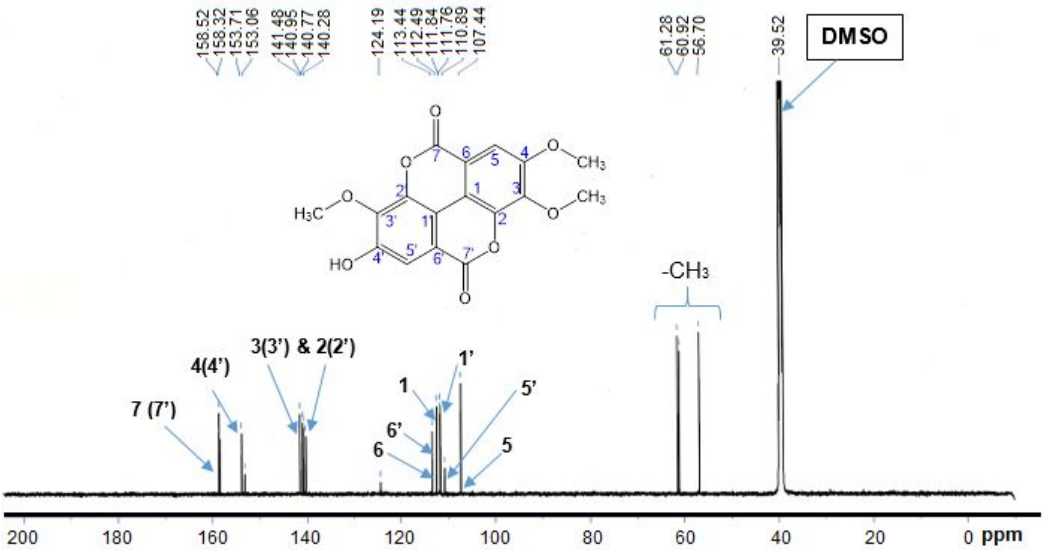


Fig 3. The C-NMR spectral data of isolated compound

showed maximum absorption at 247 and 371 nm indicating phenolic compound with conjugated benzene ring of carbonyl group. The IR (KBr, v_{max}) spectrum exhibited the following absorption frequencies: 3441, 2957, 2918, 2851, 1753, 1728, 1611, 1578, 1493, 1361, 1298, 1115, 1092, 988, and 914 cm^{-1}. The IR spectrum of the isolate showed sharp absorption bands at 3441 cm^{-1} indicating hydroxyl group, at 2957, 2918, and 2851 cm^{-1} representing C-H stretching, and at 1753 and 1728 cm^{-1} revealing the presence of two carbonyl groups. The characteristic absorption bands at 1611, 1578, and 1493 cm^{-1} indicated benzene ring system. The presence of methyl group is shown specifically at 1361 cm^{-1}. For a while, absorption bands at 1298, 1115, and 1092 cm^{-1} indicated –O-aryl and –O-CH_3, respectively. The last absorption band at 988 and 914 cm^{-1} showed substituted benzene.

The ^{1}H-NMR spectrum (600 MHz, DMSO-d6) of isolated compound revealed the presence of six significant proton signals that could be explained as follows (in Fig. 2). Two signals located at δ_H 7.61 ppm (1H, s) and 7.52 ppm (1H, s) indicated two aromatic protons due to the ellagic acid skeleton. The spectrum of the compound also displayed one signal at δ_H 3.31 ppm (1H, br) suggesting the presence of an aromatic hydroxyl group (aryl–OH). In addition, three signals located at δ_H 4.06, 4.04, and 3.99 ppm (3H, s) showed three methoxyl groups.

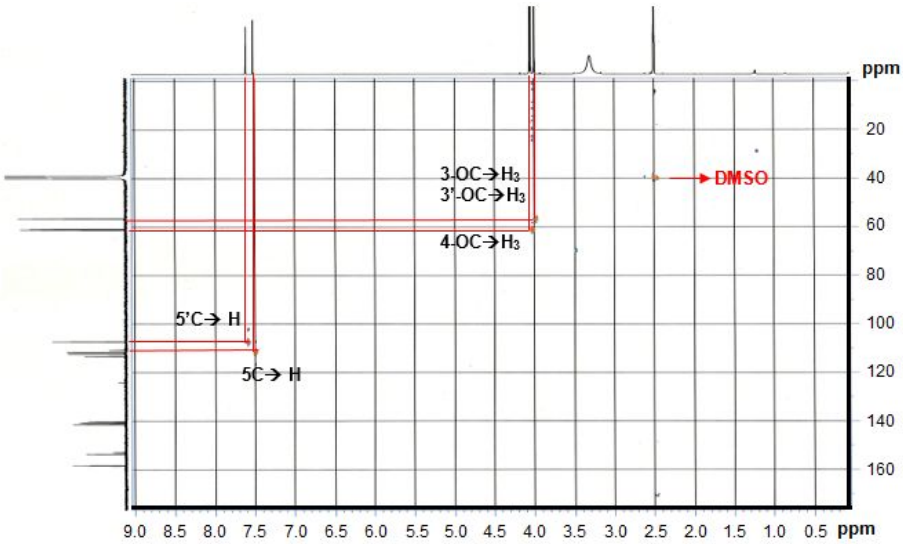


Fig 4. The HSQC spectral data of isolated compound

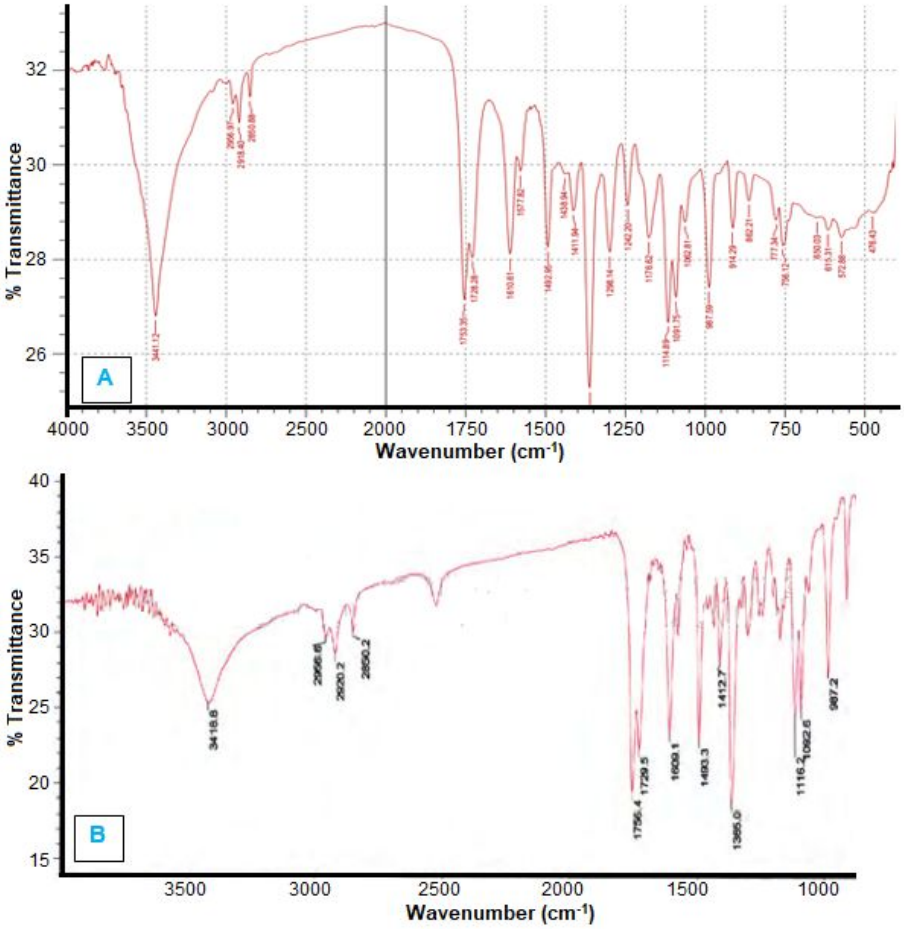


Fig 5. The comparison of IR spectral data of isolated compound (**A**) and that of literature data (**B**) [17]

The ^{13}C-NMR spectrum (150 MHz, DMSO-d6) of isolated compound displayed seventeen carbon signals that could be described as follows (in Fig. 3). The spectrum showed 17 signals, of which 14 were assigned to the ellagic acid portion and the rest signals were three methoxyl groups. Two carbon signals

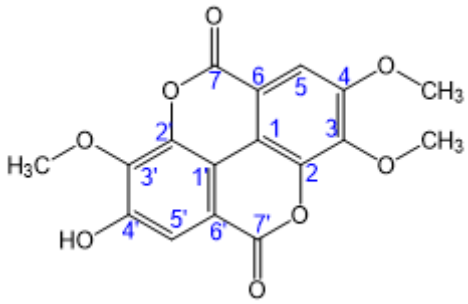
located at δ_C 158.52 and 158.32 ppm confirming clearly for two carbonyl groups [C-7(7')] were attributed to ellagic acid lactone carbonyl signals, two carbon signals at δ_C 153.71 and 153.06 ppm were confirmed as benzene ring attached by methoxyl and hydroxyl groups [C-4(4')], and two carbon signals at δ_C 112.49 and 107.44 ppm indicated benzene ring attached by hydrogen [C-5(5')]. Then, two carbon signals located at δ_C 141.48 and 140.28 ppm with high intensity indicated as benzene ring attached by methoxyl groups [C-3(3')]. For a while, two carbon signals on the position of δ_C 140.95 and 140.77 ppm represented benzene ring attached by the respect lactone groups [C-2(2')] and δ_C 113.44 and 111.84 ppm revealed benzene ring attached by carboxyl groups [C-6(6')]. Finally, two carbon signals located at δ_C 111.76 and 110.89 ppm revealed benzene ring attached by other phenyl and vice versa [C-1(1')] and assigned as ellagic acid skeleton.

The assignments of all protonated carbons were accomplished by interpretation of the HSQC NMR spectrum indicated five connections between: δ 7.52 (H-5) and 107.44 (C-5), δ 7.61 (H-5') and 112.49 (C-5'), δ 4.06 and 60.92 (3'-OCH_3), δ 4.04 and 61.28 (3-OCH_3), and δ 3.99 and 56.70 (4-OCH_3) as shown in Fig. 4. The comparison of IR spectral data of isolated compound and that of literature data [17] as shown in Fig. 5 might be justified that the compound is 3,4,3'-tri-*O*-methylellagic acid. This is supported by looking the comparison ^{1}H- and ^{13}C-NMR spectral data of isolated compound that were identical with those reported in literature data [18-19] as shown at Table 1. On this basis, the isolated compound was characterized as 3,4,3'-tri-*O*-methylellagic acid.

This is the first report of the occurrence of *S. polycephalum*, although it has previously been found in *S. cumini* seed [20] together with ellagic acid and 3,3'-di-*O*-methylellagic acid. The other ellagic acid derivatives found in Myrtaceae family are 3-*O*-ellagic acid-4'-*O*-α-rhampyranonosides, ellagic acid rhamnopyranosides, 3-*O*-methylellagic acid-4'-*O*-α-2"-*O*-acetylrhamnopyranoside and 3-*O*-methylellagic acid-4'-*O*-α-3"-*O*-acetylrhamnopyranoside from *S. guineense* stem bark [21].

DPPH Free Radical-Scavenging Assay

The 2,2'-diphenyl-1-picrylehydrazyl (DPPH) assay is a rapid and effective colorimetric method for estimating antiradical activity. This chemical assay is widely used in natural products research to isolate phytochemical antioxidants and to test general radical absorbing capacity of extracts and pure compounds. The DPPH radical is a stable nitrogen-containing organic compound with a strong absorbance at λ_{max} 517 nm and a dark purple color. After reacting with



3,4,3'-tri-*O*-methylellagic acid

Table 1. H- and C-NMR spectral data for the isolated compound (isolate GW-1) and 3,3',4-tri-*O*-methylellagic acid taken from the literature

Position	Isolate GW-1		3,3',4-Tri-*O*-methylellagic acid [18]		3,3',4-Tri-*O*-methylellagic acid [19]	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	111.76 (C)		112.01 (C)		111.62 (C)	
2	140.95 (C)		141.38 (C)		141.23 (C)	
3	141.48 (C)		141.81 (C)		140.26 (C)	
4	153.71 (C)		154.04 (C)		152.23 (C)	
5	107.44 (CH)	7.52 (s)	107.72 (CH)	7.64 (s)	111.42 (CH)	7.73 (s)
6	113.44 (C)		114.03 (C)		112.11 (C)	
7	158.52 (C=O)		159.06 (C=O)		158.52 (C=O)	
1'	110.89 (C)		111.68 (C)		111.65 (C)	
2'	140.77 (C)		141.03 (C)		141.25 (C)	
3'	140.28 (C)		140.74 (C)		140.23 (C)	
4'	153.06 (C)		152.77 (C)		152.22 (C)	
5'	112.49 (CH)	7.61 (s)	112.77 (CH)	7.68 (s)	111.45 (CH)	7.61 (s)
6'	111.84 (C)		112.70 (C)		112.12 (C)	
7'	158.32 (C=O)		158.74 (C=O)		158.63 (C=O)	
3-OCH_3	61.28 (CH_3)	4.04 (s)	61.76 (CH_3)	4.17 (s)	61.53 (CH_3)	4.19 (s)
4-OCH_3	56.70 (CH_3)	4.00 (s)	56.77 (CH_3)	4.04 (s)	56.51 (CH_3)	4.04 (s)
3'-OCH_3	60.92 (CH_3)	4.06 (s)	61.54 (CH_3)	4.19 (s)	61.26 (CH_3)	4.14 (s)

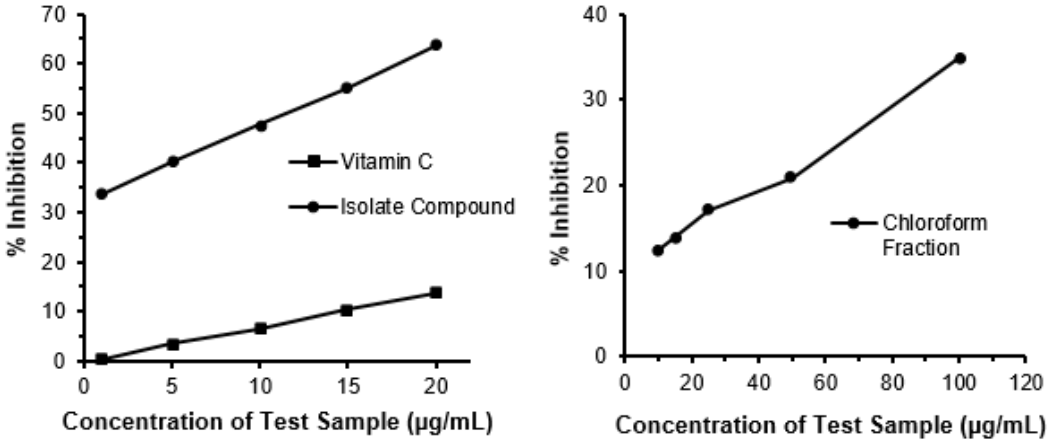


Fig 6. Free radical scavenging activity toward DPPH from test samples (isolated compound from stem bark of *S. polycephalum* and vitamin C (left) and Chloroform Fraction (right)

Table 2. Antioxidant activities of test samples (chloroform fraction (CF), isolated compound (IC) from stem bark of *S. polycephalum* and vitamin C (Vit. C))

Test Samples	Linear Regression	Antioxidant activity (IC_{50} µg/mL)[a]
CF	y = 0.2437x + 10.139 (R^2 = 0.9902)	163.6
IC	y = 0.6961x - 0.2079 (R^2 = 0.9981)	72.1
Vit.C	y = 1.5721x + 31.962 (R^2 = 0.999)	11.5

[a] The antioxidant activity was evaluated as the concentration of the test samples (CF, IC and Vit. C) required to decrease the absorbance at 515 nm by 50% in comparison to the control

antioxidant compounds, it is reduced and the color changes to yellow. The change can be measured by a spectrophotometer, and plotted against concentration.

In this paper, the free radical scavenging activities of the chloroform fraction (CF) and isolated compound (IC) from *S. polycephalum* and also vitamin C (Vit. C) toward the DPPH radical were determined as shown in Fig. 6. The good correlation was observed between the DPPH assay (as shown in Table 2), with the regression equations were y = 0.2347x + 10.139 (R^2 =0.9902), y = 0.6961x - 0.2079 (R^2 = 0.9981), and y = 1.5721x + 31.962 (R^2 = 0.999) for CF, IC, and Vit. C, respectively (in Fig. 6). The free radical scavenging activity of the chloroform fraction was significantly related to its phenolic content. Isolated compound showed a more effective hydrogen donating capacity (IC_{50} 72.1 µg/mL) than the chloroform fraction (IC_{50} 163.6 µg/mL). However, vitamin C displayed a more effective hydrogen donating capacity than isolated compound and the chloroform fraction. In addition, the chloroform fraction also exhibited the high radical scavenging activity because the presence of phenolic compounds in the fraction such as ellagic acid derivative as isolated from the fraction. The high free radical scavenging ability of the fraction of *S. polycephalum* is also similar to the other *Syzygium* genera as *S. cumini* that might be responsible for this high antioxidant activity [22].

The previous studies on plants of Myrtaceae family found that phenolic compounds (such as gallic acid and eugenol) and also flavonoids (kaempferol and quercetin) are contributed to be antioxidant activity [23]. Antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. Antioxidant compounds that scavenge free radicals help protect against degenerative diseases [24].

Phenolic compounds belonging to isolated compound which generically denominated as ArOH contain at least one hydroxyl group (OH) attached to the benzene ring and plays as chain breaking antioxidant [25]. Generally, there are two suggested mechanisms by which antioxidants can play their defensive role [26]. In the first mechanism known as hydrogen atom donator (HAT), the free radical removes a hydrogen atom from the antioxidant (ArOH) then becoming itself a radical and terminated the oxidation process by converting free radicals to more stable products (1). In the second mechanism SET (single-electron transfer), the antioxidant (ArOH) donates an electron to the free radical becoming itself a radical cation and terminate the oxidation chain reaction by reducing the oxidized intermediates into the stable form

(2). Several methods have been established based on these two mechanisms [27].

$$R^{\bullet} + ArOH \rightarrow RH + ArO^{\bullet} \quad (2)$$

$$R^{\bullet} + ArOH \rightarrow R^{-} + ArO^{\bullet +} \quad (3)$$

However, data obtained from present study suggest that chloroform fraction of stem bark of *S. polycephalum* may have significant antioxidant. These results show that the possible therapeutic potential of the stem bark of the plant may depend on the antioxidant properties exerted by their phenolic contents. These antioxidant properties could be attributed to the ellagic acid derivative (included isolated compound) from chloroform fraction of this plant. We still continue investigating the other phenolic compounds and their antioxidant activity of the fraction and the other fractions of the plant.

CONCLUSION

An ellagic acid derivative isolated from the chloroform fraction of stem bark of *S. polycephalum* have been determined as 3,4,3'-tri-*O*-methylellagic acid. This is the first report of its occurrence in the plant, although it has previously been found in Myrtaceae family such as *S. cumini* together with ellagic acid and 3,3'-di-*O*-methylellagic acid. Antioxidant activity of isolated compound (IC_{50} value of 72.1 µg/mL) is less active than that of vitamin C (IC_{50} value of 11.5 µg/mL).

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