

**A PHENOLIC ACID AND ITS ANTIOXIDANT ACTIVITY FROM STEM BARK
OF CHLOROFORM FRACTION OF *SYZYGIUM LITTORALE*(BLUME)
AMSHOFF (MYRTACEAE)**

**SUATU ASAM FENOLIK DAN AKTIVITAS ANTIOXIDAN DARI FRAKSI
KLOROFORM KULIT BATANG TUMBUHAN *SYZYGIUM LITTORALE*(BLUME)
AMSHOFF (MYRTACEAE)**

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ABSTRACT

This study describes the chemical investigations of *Syzygiumlittorale* (*S.littorale*), a plant belonging to the family Myrtaceae, and their antioxidant properties. As far, there is no study due to chemical investigations conducted to the plant either on leaves, fruits, stem bark or the others. The stem bark of *S. littorale* was extracted with organic solvent and the extracts were then fractionated and purified by using standard chromatographic techniques. The molecular structure of pure isolated pure compound was elucidated and established through extensive spectroscopic studies and by comparing with literature data and authentic sample. The dried and powdered stem bark of *S. littorale* was extracted with methanol and then partitioned by chloroform. Chromatographic (vacuum liquid chromatography and gravitational column chromatography) fractionation and purification of the chloroform soluble fraction that is always monitored by TLC yielded a pure isolated pure compound. The purified compound was then identified and justified as a phenolic acid i.e. gallic acid. This compound is found for the first time from this plant, although it has previously been found in other *Syzygium* species such as *S. aromaticum*, *S. cumini*, *S. polyanthum*, *S. cordatum*, etc. The chloroform fraction, isolated compound, and vitamin C showed very strong antioxidant activity against 2,2'-diphenyl-1-picrylhydrazyl (DPPH) with the IC₅₀ value of 23.2; 7.5; and 12.5 µg/mL, respectively.

Keywords: chloroform fraction, DPPH, gallic acid, *Syzygiumlittorale*, vitamin C.

ABSTRAK

Penelitian ini menyajikan hasil penyelidikan kimia terhadap tumbuhan klampok alas/watu, *Syzygiumlittorale* (*S. littorale*), suatu tumbuhan yang termasuk family Myrtaceae dan sifat anti oksidannya. Sejauh ini, belum ada penelitian terkait dengan penyelidikan kimia yang dilakukan terhadap tumbuhan tersebut baik pada daun, buah, kulit batang atau bagian lainnya. Kulit batang tumbuhan *S. littorale* diekstraksi dengan pelarut organik dan selanjutnya ekstrak difraksinasi (diisolasi) dengan menggunakan teknik-teknik kromatografi yang standar. Struktur molekul dari senyawa murni hasil isolasi dielusidasi dan ditetapkan melalui kajian spektroskopi secara mendalam dan juga dilakukan dengan cara membandingkannya dengan data literature dan sampel asli. Serbuk kering kulit batang *S. littorale* diekstraksi dengan methanol dan kemudian dipartisi dengan kloroform. Isolasi terhadap fraksi kloroform melalui kromatografi cair vakum dan kromatografi kolom gravitasi yang selalu dimonitor dengan KLT menghasilkan suatu senyawa murni. Senyawa murni ini selanjutnya diidentifikasi dan dipastikan sebagai senyawa asam fenolik, yaitu asam galat. Diperolehnya asam galat dari tumbuhan *S. littorale* merupakan laporan penemuan untuk pertama kalinya, meskipun sebelumnya senyawa ini telah ditemukan pada tumbuhan *Syzygium* lainnya, seperti *S. aromaticum*, *S. cumini*, *S. polyanthum*, *S. cordatum*, dll. Fraksi

kloroform, senyawa hasil isolasi dan vitamin C telah menunjukkan aktivitas antioksidan sangat kuat (aktif) terhadap DPPH dengan nilai IC₅₀ masing-masing adalah 23,2; 7,5; dan 12,5 µg/mL.

Kata Kunci: asam galat, DPPH, fraksi kloroform, *Syzygium littorale*, vitamin C.

INTRODUCTION

Syzygium, the genus of flowering plants, is one of the important genus in Myrtaceae family, widely presented 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. On the basis of literature study, phytochemical studies on *Syzygium* species have led to the identification and isolation of main compounds such as phenolic compounds (eugenol and eugenol acetate found in *S. aromaticum* (Alma, Murat, Siegfrie, & Hubert, 2007). Also, the other phenolic compounds namely flavonoids had been obtained from *S. polyanthum* (Wight) Walp leaves (Har & Ismail, 2012), *S. aquem* leaf (Manaharan, David, Hwee, & Uma, 2012), and from *S. alternifolium* Walp. Leaf (Komuraiah et al., 2014). On the other hand, several *Syzygium* species reported have antioxidant properties are *S. cumini* leaves (Ruan, Liang, & Yi, 2008), *S. polyanthum* (Wight) Walp leaves (Har & Ismail, 2012), *S. aqueum* (Osman, Afidah, Norhafizah, & Nornaemah, 2009) and *S. lanceolatum* (Muthumperumal, Nadarajan, Arun, & Swamy, 2016).

Syzygium littorale that has many synonyms with *Eugenia littorale* (Blume) Meijer Drees, *Eugenia subglauc* Koord. & Valeston and *Jambosalittorale* Blume is a species in the Myrtaceae, native to Indonesia with common name “klampok”. Local people call it as ‘klampok watu’ or ‘klampok alas’. This species is a wild species, mostly found along the riverbanks, e.g. Resort Seroja. The wood can be used as building materials since it is long lasting and strong (Ariyanti, Rony, Lia, & Deden, 2012). Based on the literature data, no information about the total phenolic contents, antioxidant and other bioactivity potentials of the

medicinal organic compounds in the stem bark of *S. littorale* that have been reported so far. Therefore, we are interested strongly to investigate them especially for phenolic contents and their antioxidant bioactivity from the stem bark extract of this plant. The present study however, reports the finding of gallic acid for the first time from the chloroform fraction of the stem bark of *S. littorale*.

EXPERIMENTAL SECTION

Materials

Chemicals and plant materials

Isolated compound from the stem bark of *S. littorale*, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 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 obtained from local sources and were of analytical grade (Grade AR). The stem bark of *S. littorale* (c.a. 25 kg) was collected from a local area in Bojonegoro, East Java, Indonesia in December 2014. The identification of the plant was performed by the staff of Herbarium-LIPI, Purwodadi, East Java, Indonesia. A voucher sample is kept in the Herbarium of LIPI with Identification No. 0116/IPH.06/HM/I/2015, January 10, 2015.

Equipment and instruments

The equipment used to do extraction and fractionation (isolation) are filter paper, Buchner funnel, Hirsch funnel, Erlenmeyer flask, pipet, spatula, measuring glass, vials, containers, separating funnel, and vacuum rotary evaporator type BUCHI Rotavapor R-215. The equipment used to measure melting point of an isolate is Fisher Scientific. Whereas, chromatographic techniques

used to isolate phenolic compounds from chloroform fraction included Vacuum Liquid Chromatography (VLC) (using silica gel 60, 0.040-0.063 mm), Gravitational Column Chromatography (GCC) (silica gel 60, 0.063–0.200 mm and 0.200–0.500 mm or 70–230 mesh ASTM).

TLC analyses 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 (E.Merck) and the spots were detected by exposure to UV-lamp at 254 nm or 366 nm.

Some instruments needed to identify and characterize an isolate included spectrophotometer FTIR-8400S and spectrophotometer UV-1800 (SHIMADZU). The ^1H 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 (δ_{H} 3.30; δ_{C} 49.0)] on the tetramethylsilane (Sigma) scale.

Procedure

Extraction and isolation

The fresh stem bark of *S. littorale* (c.a. 25 kg) was washed with under tap water and dried under sunlight for one week. It was then ground to be smooth powder as much as 9.5 kg. The dried powder of plant was macerated with methanol at room temperature for 24 hours. The mixture was then filtered and the filtrate obtained was concentrated to be crude extract (967.6 g).

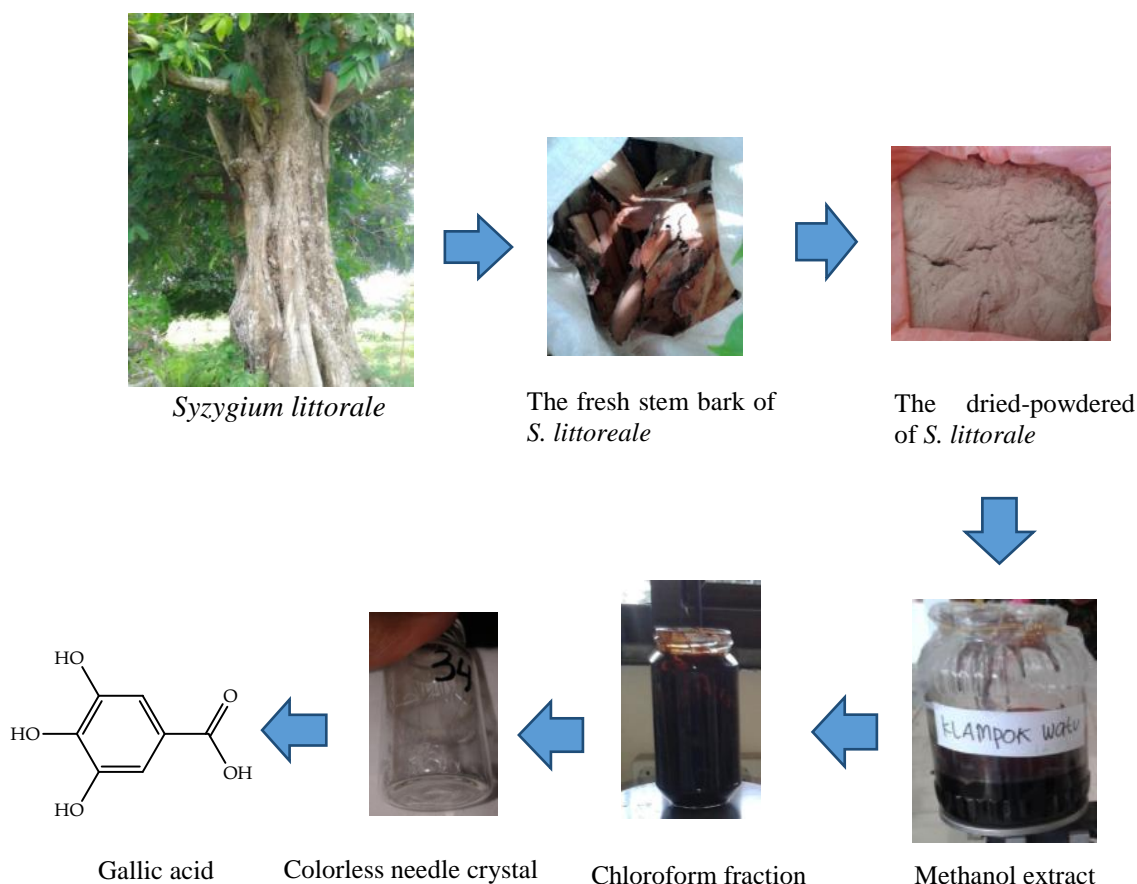


Figure 1. Isolation process to find gallic acid from chloroform fraction of *Syzygium littorale* stem bark

The extract was then suspended in methanol and defatted with hexane to yield two layers. The upper part (hexane soluble parts) was evaporated to gain hexane extracts (15.044 g) and the lower part (methanol soluble parts) is called residue. By using the same manner, the residue was suspended in methanol and defatted with chloroform to yield two layers as well. The lower layer (chloroform soluble parts) was also evaporated to obtain chloroform fractions (107.787 g).

A portion of chloroform fractions (10 g) was then chromatographed through VLC using hexane-ethyl acetate system (100: 0 → 0: 100) to yield 43 fractions. The same way, it was also re-chromatographed to the fractions for four times and called to be VLC-1, VLC-2, VLC-3, and VLC-4. Of these, fractions 17-20 of VLC-1, fractions 21-34 of VLC-2, fractions 18-30 of VLC-3 and fractions 21-31 of VLC-4 were combined by TLC to yield 3 g (as fraction B). Fraction B was then separated through GCC using eluent system (hexane: ethyl acetate = 4: 6) to get 22 fractions. On the basis of TLC, the fractions can be grouped into three fractions (B1, B2 and B3). When observed on TLC plate, the fraction B2 was seemed to give simple chromatogram profile (0.5 g).

Finally, the fraction was then purified through GCC using eluent system (hexane: chloroform: methanol = 1: 8: 1) to yield 59 fractions that can be combined into four fractions [B21 (1-13), B22 (14-29), B23 (30-42), and B24 (43-59)]. The fraction B23 was allowed to evaporate at room temperature and yielded a colorless needle crystal (50 mg) with mp. 256-257 °C. The crystal was then characterized by UV-Vis, FTIR, and NMR spectroscopies and by comparison with literature data and authentic sample (gallic acid) and determined its structure to be gallic acid. Extraction and isolation that had been

carried out to find the isolate can be seen in **Figure 1**.

Free radical scavenging ability on 2,2-diphenyl-1-picrylhydrazyl (DPPH)

In order to assess the scavenging ability of 2,2'-Diphenyl-1-picrylhydrazyl (DPPH), each test samples (the chloroform fraction or isolated compound or antioxidant standard (Vit. C) (0.3 mL: 10, 25, 50, 75 and 100 µg/mL) in methanol was mixed with methanol solution (3 mL) containing DPPH radicals (0.004%, w/w). The mixture was shaken vigorously and left to stand for 30 minutes in the dark before measuring the absorbance at 515 nm against a blank (Braca et al., 2001) with slight modifications. Then, the scavenging ability (the percentage inhibition, I%) was calculated using the following equation:

$$I\% = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100$$

Where A_{blank} is the absorbance value of the control and A_{sample} is the absorbance value of the test samples (Khan, Khan, Sahreen, & Ahmed, 2012). Percentage radical scavenging ability was plotted against the corresponding antioxidant substance concentration. The results were analyzed 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 scavenge 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 (Reynertson, 2007).

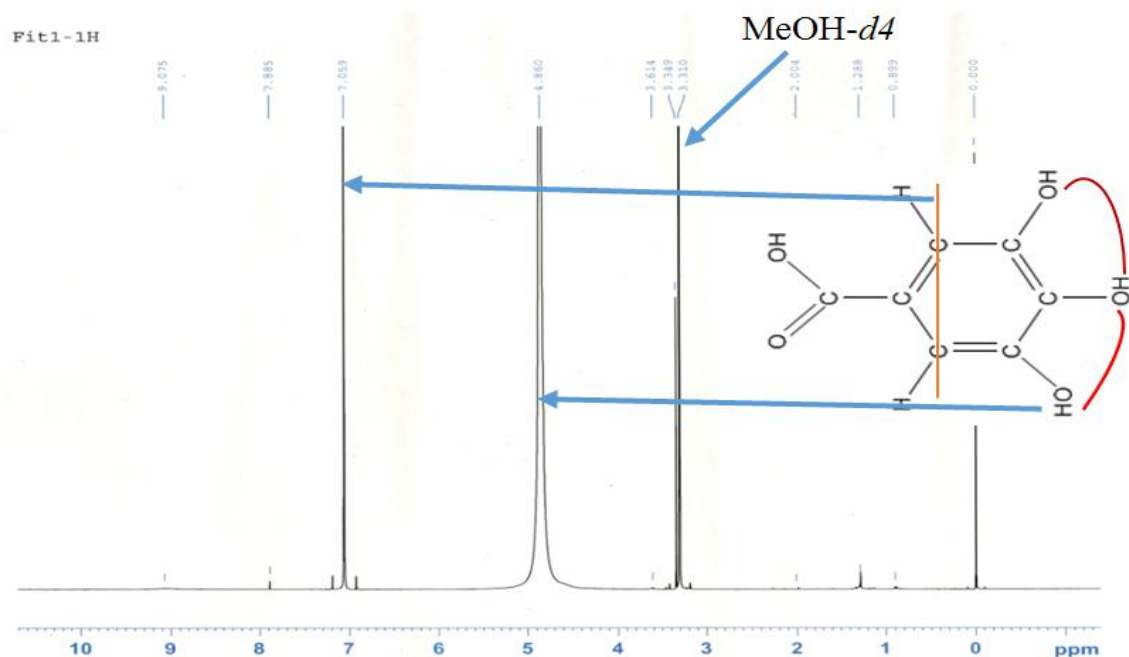


Figure 2. The H-NMR spectral data of isolated compound (gallic acid)

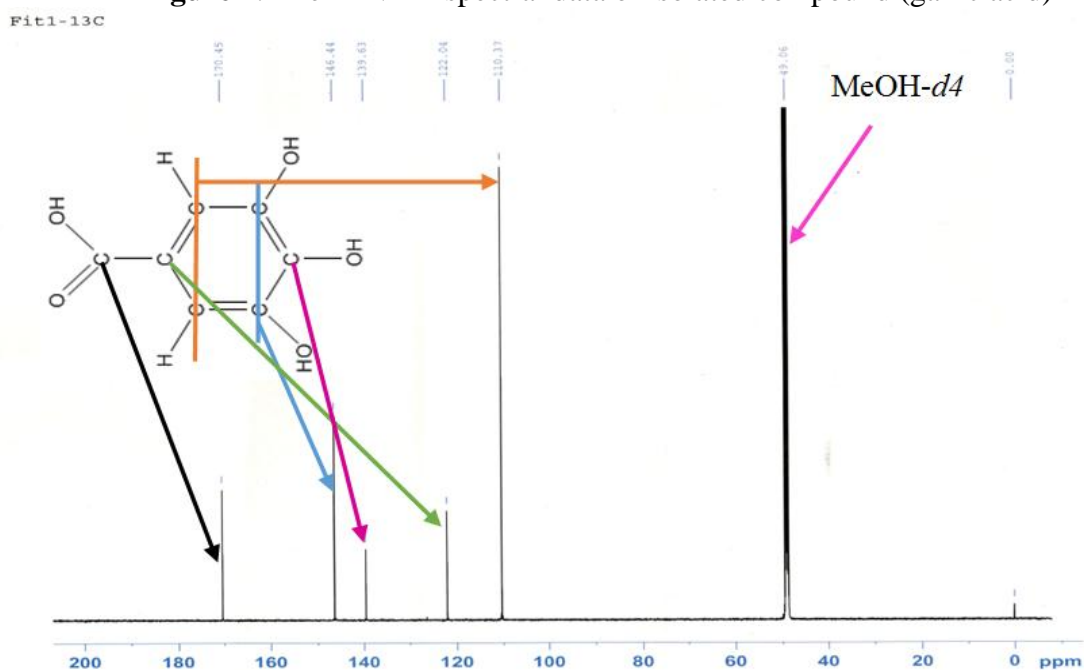


Figure 3. The C-NMR spectral data of isolated compound (gallic acid)

RESULTS AND DISCUSSION

Structural Determination of The Isolated Compound

Isolated compound was obtained as a colorless needle crystals (50 mg) with mp. 256-257 °C. The UV-Vis (MeOH, λ_{max}) spectrum of isolated compound showed maximum absorption at 216 and 271 nm indicating a phenolic compound. The IR spectrum of the isolated compound

displayed absorption bands broadly at 3462 - 3013 cm^{-1} and at 1705 cm^{-1} indicating hydroxyl and carbonyl groups, respectively. The absorption bands at 1620, 1541, and 1450 cm^{-1} and 1265, 1246, and 1208 cm^{-1} indicated the presence of benzene ring system and three -O-aryls directly attached to the benzene ring, respectively. The last absorption band at 702 cm^{-1} showed substituted benzene.

The ^1H -NMR spectrum (600 MHz, $\text{MeOH-}d_4$, ppm) of the isolated compound displayed the presence of an aromatic singlet at δ_{H} 7.06 (2H, s). The ^1H -NMR spectrum of it also showed a broad singlet at δ_{H} 4.86 showed the presence of three aromatic hydroxyl groups (aryl-OH) as can be seen in **Figure 2**. The ^{13}C -NMR spectrum (150 MHz, $\text{MeOH-}d_4$, ppm) of isolated compound displayed five carbon signals, one carbon signal at δ_{C} 170.45 confirming significantly for a carbonyl group, two carbon signals at δ_{C} 110.37 and 146.44 confirming as benzene ring attached at *meta*-hydrogen and *meta*-hydroxyl group, respectively. Finally, the rest carbon signals at δ_{C} 139.63 and 122.04 confirming as substituted benzene as shown in **Figure 3**.

When ^1H - and ^{13}C -NMR spectra of the isolated compound were compared with those of gallic acid as reported in literature data (Hiranrat, 2010), it could be identical as shown in **Table 1**. Also, by comparing the IR data of the isolated compound with that of an authentic sample (gallic acid) as shown in **Figure 4**, it was confirmed as gallic acid. Needed to note that this is the first report of its occurrence in *S. littorale*, although it has previously been found in other *Syzygium* species such as *S. cumini* (Sikder et al., 2012) and *S. Polyanthum* (Har & Ismail, 2012), etc.

Table 1: H- and C-NMR spectral data for the isolated compound and gallic acid

Position	Isolated Compound ($\text{MeOH-}d_4$)		Gallic acid ($\text{DMSO-}d_6$) ^a	
	δ_{C} (150 MHz)	δ_{H} (600 MHz)	δ_{C} (75 MHz)	δ_{H} (300 MHz)
1	122.04	-	120.89	-
2(6)	110.37	7.06 (s)	109.16	6.90 (s)
3(5)	146.44	-	145.84	-
4	139.63	-	138.43	-
7	170.45	-	169.96	-

^aGallic acid compared was isolated *Rhodomyrtus tomentosa* (Hiranrat, 2010)

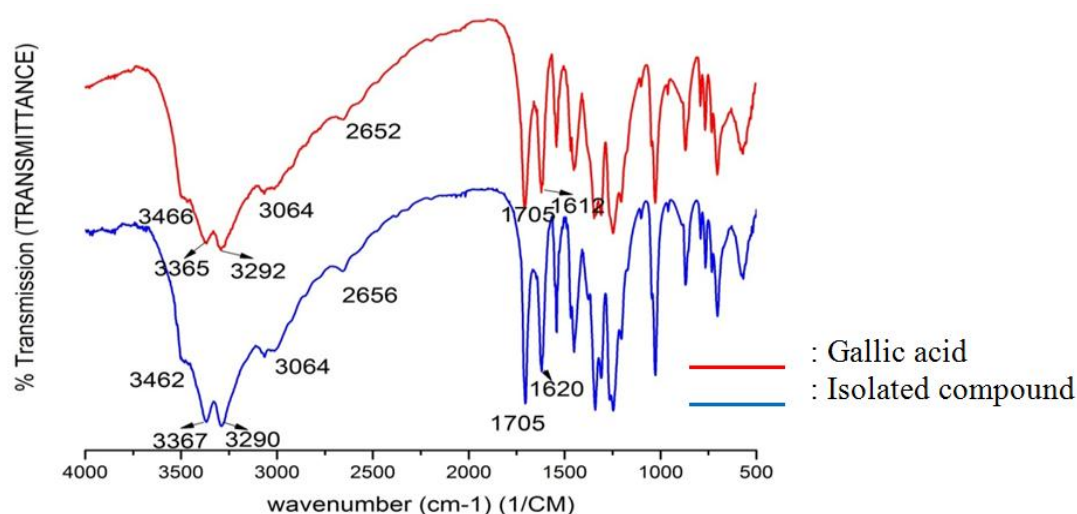


Figure 4. The comparison of IR spectral data of isolated compound and that of authentic sample (gallic acid)

DPPH free radical-scavenging assay

In this paper, the free radical-scavenging activities of the tested samples including chloroform fraction, isolated compound from *S. littorale* and also vitamin C toward DPPH radical could be explained as follows. DPPH radical has been used frequently as a reactive hydrogen acceptor for the determination of radical scavenging activity of various natural and synthetic compounds. In this study, tested samples used (chloroform fraction, isolated compound and vitamin C) can be natural and synthetic compounds. The IC_{50} value of an antioxidant can be defined as the concentration of antioxidant required for 50% scavenging of DPPH radical in the period of 30 minutes. After doing the free radical scavenging assay of tested samples, the IC_{50} values of them was determined to be 23.2; 7.5; and 12.5 $\mu\text{g/mL}$, respectively. It can be declared that isolated compound showed a more effective hydrogen donating capacity (IC_{50} 7.5 $\mu\text{g/mL}$) than the chloroform fraction (IC_{50} 23.2 $\mu\text{g/mL}$) and vitamin C (IC_{50} 12.5 $\mu\text{g/mL}$). But, vitamin C displayed a more effective hydrogen donating capacity than the chloroform fraction. The graphs showed percentage inhibition of chloroform fraction, isolated compound

and vitamin C could be plotted and compared as shown in **Figure 5**.

It was observed that the regression equations of tested samples (chloroform fraction (CF), isolated compound (IC) and vitamin C (Vit. C)) and the good correlations of them are $y = 0.1545x + 46.421$ ($R^2 = 0.992$), $y = 0.1528x + 48.850$ ($R^2 = 0.993$), and $y = 0.1570x + 48.036$ ($R^2 = 0.991$) for CF, IC, and Vit. C, respectively (as shown in **Table 2**). The free radical scavenging activity of the chloroform fraction was significantly related to its phenolic content ($R_{\text{DPPH}} = 0.992$) (**Figure 5**). The chloroform fraction exhibited the high radical scavenging activity because of the presence of phenolic compounds in the fraction such as gallic acid as isolated from the fraction. As reported that gallic acid (3,4,5-trihydroxybenzoic acid) represents a secondary metabolites found in large family of plant and possess natural antioxidant properties (Patrick-Iwuanyanwu, Onyeike, & Adhikari, 2014). The high free radical scavenging ability of the fraction of *S. littorale* is also similar to the other *Syzygium* genera like *S. cumini* that might be responsible for this high antioxidant activity (Ruan, Liang, & Yi, 2008).

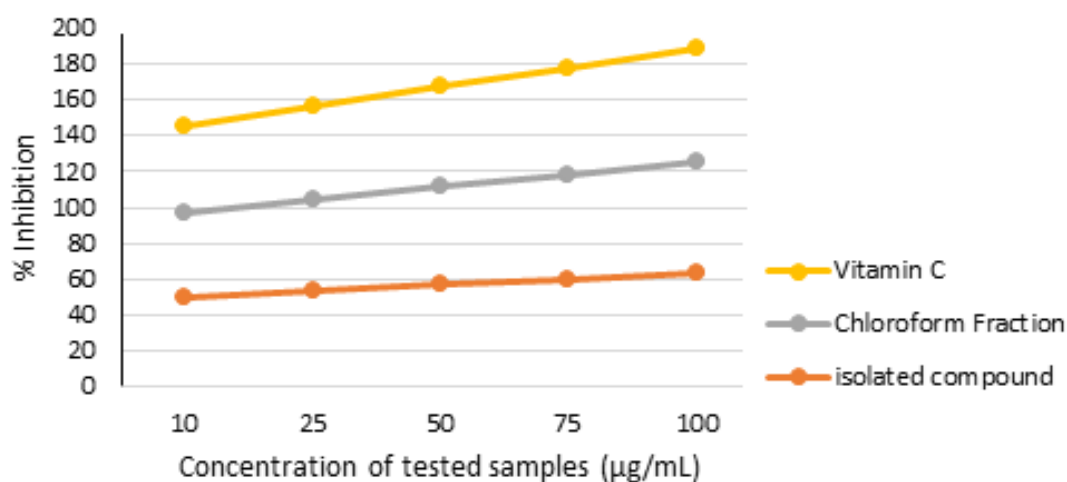
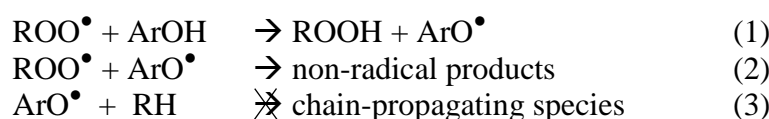


Figure 5. Comparison of percentage inhibition of isolated compound (gallic acid) from stem bark of *S. littorale*, chloroform fraction and vitamin C

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

Tested Samples	Linear Regression	Antioxidant activity (IC ₅₀ µg/mL) ^a
CF	$y = 0.1545x + 46.421$ ($R^2 = 0.992$)	23.2
IC	$y = 0.1528x + 48.850$ ($R^2 = 0.993$)	7.5
Vit. C	$y = 0.1570x + 48.036$ ($R^2 = 0.991$)	12.5

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

**Figure 6.** The role of radical-scavenging antioxidants (e.g. ArOH) in an autoxidation inhibition process

Phenolic compounds are almost universally used for industrial/commercial applications as well as in nature. The compounds (called be ArOH) possessing high reactivity properties may chain peroxy radicals (ROO[•]) to form hydrogen peroxide derivative (ROOH). The mechanism of the reaction displaying the formal transfer of an H-atom from the ArOH to the peroxy radical (ROO[•]) can be shown at reaction 1 (**Figure 6**).

Peroxy radical (ROO[•]), then can react with phenoxyl radical (ArO[•]) to produce a non-radical products (reaction 2 in **Figure 6**). According to Valgimigli et al., (2013), under some circumstances such as when diffusion of the antioxidant is impeded, and it has limited opportunity to encounter other radical species, it is possible for the antioxidant-derived phenoxyl radical to propagate the chain reaction reaction 3 (**Figure 6**).

However, data obtained from the present study suggest that chloroform fraction of stem bark of the plant might depend on the antioxidant properties. These results showed 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 gallic acid (isolated compound) from chloroform fraction of this plant. Nowadays, we still continue investigating the other phenolic compounds and their antioxidant activity of the fraction and the other fractions of the plant, beside to *S. polycephalum*, *S. polyanthum*, and *S. malaccensis*.

CONCLUSION

A phenolic compound isolated from the chloroform fraction of stem bark of *S. littorale* have been determined as gallic acid. The compound is found for the first time from the chloroform fraction of the stem bark of *S. littorale*., although it has previously been found in other *Syzygium* species such as *S. aromaticum*, *S. cumini*, *S. polyanthum*, *S. cordatum*, etc. Antioxidant activity of isolated compound (IC₅₀ value of 7.5 µg/mL) is more active than that of vitamin C as standard (IC₅₀ value of 12.5 µg/mL).

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