Antioxidant Activities and Identification of an Active Compound from Rambutan (*Nephelium lappaceum* L.) Peel

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Abstract: *The consumption of rambutan fruit resulted in a vast amount of peels and seeds waste. Therefore, the exploration of active compounds having beneficial effects on human health, such as antioxidants, is very lucrative. This research was aimed to isolate and to identify the active compound as an antioxidant from rambutan peel. The powdered rambutan peel was extracted with a maceration technique using methanol then fractionated using petroleum ether, chloroform, and ethyl acetate to get the corresponding fractions. The extract and fractions were determined for its antioxidant activities in vitro using 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and metal-chelating assay. The ethyl acetate fraction exhibited the highest antiradical activity with an IC_{50} value of 26.22 μg/mL and metal-chelating activity, accounting for 12.32%. The antioxidant activities of extract and fractions correlated with its phenolics and flavonoid contents. Identification of active compounds using FTIR, GC-MS, and NMR resulted in the chemical formula of $C_7H_6O_4$, identified as 3,4-dihydroxybenzoic acid.*

Keywords: *rambutan peel; DPPH; metal-chelating; 3,4-dihydroxybenzoic acid*

■ INTRODUCTION

Rambutan (*Nephelium lappaceum* L.) belonging to *Sapindaceae* family is widely distributed in South East Asian regions such as Indonesia, Malaysia, Thailand, and Vietnam. Rambutan is consumed as the fresh fruit results a vast amount of waste from seeds and peels. Therefore, it is very challenging to take benefits of rambutan waste as natural antioxidant sources. The antioxidant obtained from natural materials usually derived from secondary metabolites of plants like alkaloids, phenols, and flavonoids [1]. An antioxidant is a compound capable of protecting the human body from degenerative diseases caused by oxidative stress such as arteriosclerosis, arthritis, cataract, and premature aging. Free radicals have unpaired electrons that are very highly unstable and very reactive to react with other molecules. They include reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS) which able to initiate the reactions that damage organic molecules in the biological systems [2]. These reactions are taken into account to be the cause of some degenerative diseases such as diabetes and aging [3-4].

Rambutan peel has been reported to have antiradical, antioxidant, and antibacterial activities caused by the content of phenolic compounds [5-6]. The ethanol extract of rambutan peel is reported to contain ellagic acid, corilagin, and geraniin [7]. Palanisamy et al. [8] also isolated geraniin in rambutan peel. These compounds were reported to be responsible for antiradical and antioxidant activities. Ethyl acetate fraction of rambutan peel was reported to have radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical with IC_{50} of 2.73 μg/mL, while quercetin used as a positive control has IC_{50} of 1.99 μg/mL [9].

However, to obtain more active activities, it is necessary to further fractionation of the ethyl acetate fraction to obtain sub-fraction. Mistriyani et al. [10] also reported that ethyl acetate fraction revealed the highest antioxidant activities as determined by Ferric reducing activity power (FRAP) method. However, Permatasari and Rohman [9] and Mistriyani et al. [10] did not take further fractionation and did not elucidate the active compound responsible for this activity in the fraction of ethyl acetate. Therefore, in this research, the isolation and structure elucidation of the active component contained in the most active fraction of the sub-fraction of ethyl acetate was elucidated. The activity of natural antioxidant was generally attributed to the content of total phenolic and flavonoid contents; therefore, the phenolic and flavonoid contents in rambutan peel extract and fractions were correlated with antioxidant activities [11-13].

■ EXPERIMENTAL SECTION

Materials

Rambutan fruit was obtained from Bantul, Yogyakarta, Indonesia, and the authentication process was determined in the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Procedure

Preparation of methanol extract

The preparation of extract was done according to Permatasari and Rohman [9]. Rambutan peel was cleaned from impurities by using water, then chopped up and dried in an oven at a temperature of 60 °C for 2 × 24 h. Then, the simplicia was powdered and weighed. The powder of rambutan peel was extracted using the maceration method using methanol. The maceration was carried out for 3 days, occasionally stirred, and subjected to re-maceration 2 times. The filtrate obtained was then evaporated using a vacuum rotary evaporator at a temperature of 60 °C to obtain a methanolic extract.

Fractionation of methanolic extract of rambutan peel

The methanolic extract (50 g) was added with warm distilled water (1000 mL), fractionated using petroleum ether (PE) at a ratio (1:1 v/v). The residue of methanol extract (water fraction) was then fractionated again using chloroform and ethyl acetate to get the fractions of PE, chloroform, ethyl acetate, and water. The initial methanolic extract and the fractions were further used for evaluation of the antioxidant activity

Determination of antioxidant activity using DPPH method

The antiradical activity of extract and fractions toward 2,2-diphenyl-1-picrylhydrazyl (DPPH) was evaluated using a visible spectrophotometer at 517 nm according to Blois method [6]. The stable DPPH radical solution (0.1 mM) in methanol was prepared, and then 1 mL of this solution was mixed with 3 mL of the sample at different concentrations. A control containing 1 mL of DPPH radical solution and 3 mL of methanol was prepared. The mixture was left to stand at ambient temperature for 20 min, and the absorbance was subsequently measured at 517 nm against the blank of methanol.

Determination of metal-chelating activity

The determination of metal-chelating activity was carried out as in Gülçin et al. [14]. A test sample was added with 0.05 mL of $FeCl_2$ 2 mM. The mixture was added with 0.2 mL of ferrozine 5 mM and vortexed for 10 sec. The absorbance was measured at a wavelength of 562 nm. The inhibition percentage of complexes of Fe^{2+}-ferrozine was calculated as follows:

$$\text{Percent (\%) inhibition chelating} = \frac{A^0 - A^1}{A^0} \times 100$$

where, A^0 and A^1 are the absorbances in control and with the presence of the tested sample, respectively. EDTA is used as a positive control.

Determination of total phenolic content

The levels of total phenolic in samples were analyzed using the Colorimetric Method (Folin-Ciocalteau method), according to Chun et al. [15]. Total phenolic contents of samples were expressed as gram of Gallic acid equivalent/100 g of dry samples. Gallic acid for the preparation of the calibration curve was made in the range of 1.0–10.0 mg/100 mL.

Determination of total flavonoid

Flavonoid contents of samples were determined

using the aluminum chloride colorimetric method, according to Zou et al. [16]. Total flavonoid contents of extracts and fractions were expressed as gram rutin equivalent/100 g dry material. Rutin for preparation of calibration curve was made in the range of 1.0–10.0 mg/100 mL.

Fractionation of the most active fraction and identification of the isolate

The fraction of rambutan peel which showed the most active antioxidant (ethyl acetate (EA) fraction) was further fractionated using vacuum liquid using silica gel G 60 GF_{254} (200 g) as in Rohman et al. [12]. The EA fraction was added with anhydrous sodium sulfate previously heated for 2 h at 110 °C, allowed to stand for one night and then filtered using a filter paper. The solvent was evaporated using a vacuum rotary evaporator. Samples were added to the column that has been let stand overnight. The EA fraction was eluted using 250 mL of chloroform, then the mixture of chloroform:EA with the volume ratio of 225:25, 200:50, 175:75, 150:100, 125:125, 100:150, 75:175, 50:200, 25:225, and 0:250 to give total volume of 250 mL. The eluates were collected for each 50 mL and were evaporated using a vacuum rotary evaporator. The isolated compound was identified by FTIR, NMR, and gas chromatography-mass spectrometry (GC-MS) methods [17].

The purity test

The purity of isolate was checked using two methods, namely melting point and thin-layer chromatography (TLC). The melting point of the crystal was checked using Buchi Melting Point B-450. The test was repeated by measuring the temperature of ± 10 °C below the melting point obtained, ramped at 1 °C/min. For purity test using TLC, the isolate was eluted using three eluent systems with a mobile phase having different polarity index, namely acetone: ethyl acetate: chloroform with a ratio of 4:3:3 v/v. The compound is considered to be pure when the TLC spot presents a single spot. The spot detection was performed visually using UV_{254} and UV_{366}.

Data analysis

All data of antioxidants and their related data were analyzed in triplicate and expressed as mean ± standard deviation using Excel (Microsoft Inc., USA). The statistical analysis, including normality and homogeneity tests, were performed using SPSS software (SPSS Inc., USA).

■ RESULTS AND DISCUSSION

The evaluation of antioxidant activities in this study was done using several antioxidant assays. Among antioxidant mechanisms, radical scavenging and chelating agent activity were the most reported ones for the evaluation of antioxidant capacity derived from plants. The scavenging activity of DPPH radicals can be employed to measure the antioxidant activities in a relatively short time [18]. The DPPH radical is stable and soluble in polar solvents such as methanol and ethanol. Sharma and Bhat [19] have revisited the use of solvents during DPPH radical scavenging assay, and the results showed that methanol and buffered methanol were selected as solvents due to the capability to provide the highest absorbance value. Therefore, methanol was used in this study.

The results of the antioxidant activity test using DPPH radical assay, as expressed with IC_{50} were compiled in Fig. 1. It is found that ethyl acetate fraction was the most active fraction with an IC_{50} value of 26.22 μg/mL, followed by MeOH (IC_{50} 31.56 μg/mL), PE and $CHCl_3$ (IC_{50} 31.91 μg/mL) and then water fraction (IC_{50} 40.36 μg/mL). This can be explained that ethyl acetate fraction contains more active compounds as radical scavenging such as phenolic and flavonoids. The similar results were also reported by Rohman et al. [12], Permatasari and Rohman [9] and Rohman et al. [20] in which ethyl acetate fractions in the methanol extract of Mengkudu, rambutan dan red fruit also revealed the highest antiradical activities among extracts and fractions evaluated. Mistriyani et al. [10] have also previously reported that ethyl acetate also revealed the highest reducing power and antiradical activity toward ABTS radical.

In this study, the IC_{50} value of ethyl acetate fraction was higher than that of vitamin C (as a positive control) in which vitamin C had an IC_{50} value of 3.34 μg/mL, but the IC_{50} values of ethyl acetate fraction and other fractions were still categorized as a very strong antioxidant (less

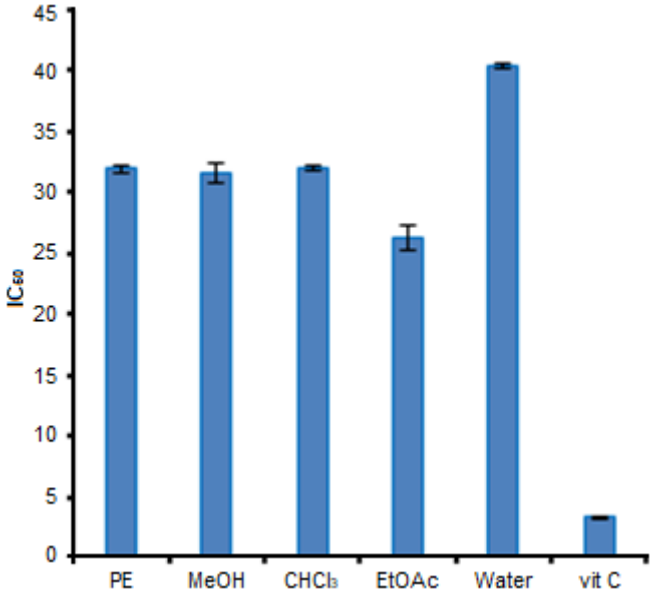

Fig 1. The IC_{50} values of methanol extract of rambutan peel and its fraction using DPPH radical scavenging assay. PE = petroleum ether fraction; MeOH = methanol extract; $CHCl_3$ = chloroform fraction; EtOAc = ethyl acetate fraction; water = water fraction; Vit. C = vitamin C

than 50 µg/mL) [21]. Therefore, the ethyl acetate fraction was further fractionated.

The metal-chelating activity test was performed using EDTA as a positive control because EDTA has a strong metal-chelating characteristic [22-23]. The activity of metal-chelating of the methanol extract of rambutan peel and its fraction as well as EDTA was shown in Fig. 2. The water and ethyl acetate fractions have higher metal-chelating activities, accounting for 12.53 and 12.32%, respectively, but lower than that of EDTA, accounting for 35.50%. Based on the results of one-way ANOVA test, it can be shown that water and EA fractions did not differ significantly in a metal-chelating activity.

The antioxidant activity of methanol extract of rambutan peel and its fraction were correlated with phenolics and flavonoid contents. The linear regression equation obtained for the correlation between total phenolic content (TPC) (y-axis) with IC_{50} values of DPPH radical scavenging (x-axis) has R^2 of 0.279. This result showed that 27.9% of the antioxidant activities against DPPH were from the contribution of phenolic compounds while 72.1% of the antioxidant activities came from other classes of compounds. It indicated a very low correlation between IC_{50} values and TPC. The correlation between TPC with metal-chelating activity had R^2 of 0.4322, which showed that 43.22% of metal-chelating activities was coming from the contribution of phenolic compounds. This indicated that phenolics compounds present in rambutan peel more active as metal chelator than a radical scavenger. However, the individual phenolics responsible for these activities have not reported yet.

The correlation between the total flavonoid content (TFC) (y-axis) expressed as % w/w RE and IC_{50} values of methanol extract of rambutan peel and its fraction using DPPH radical scavenging activity having R^2 of 0.445. This R^2 value indicated that 44.5% of DPPH radical scavenging activity came from the contribution of flavonoid content contained in rambutan peel, while the correlation between TFC with metal-chelating activities had R^2 of 0.283, which showed that 28.3% of metal-chelating activities was from the contribution of flavonoid compounds. Based on the results, it can be stated that flavonoid compounds contained in rambutan peel were more active as a radical scavenger than metal chelators.

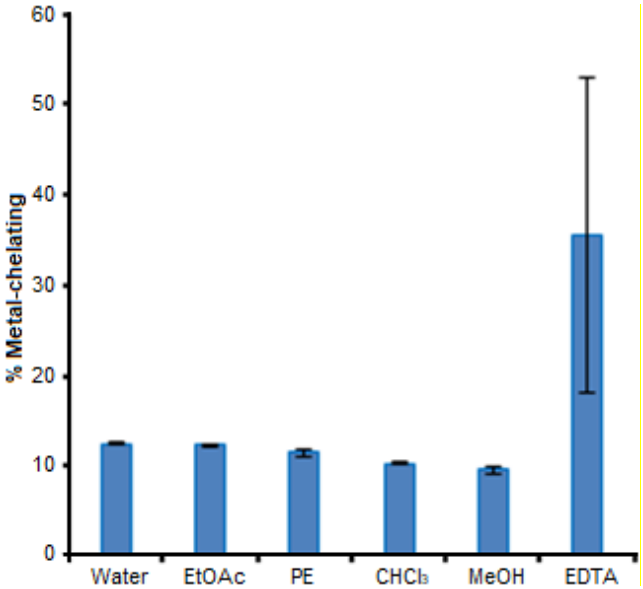

Fig 2. The results of metal chelating activity of methanol extract of rambutan peel and its fraction. The chelating activity data were represented as mean values ± SD, n replication = 3. Water = water fraction; EtOAc = ethyl acetate fraction; PE = petroleum ether fraction; $CHCl_3$ = chloroform fraction; MeOH = methanol fraction

Isolation and Identification of Active Compounds in Ethyl Acetate Fraction

Isolation of ethyl acetate fraction resulted in 104 sub-fractions. Among these, there was a precipitate of crystalline yellow in sub-fraction 61–65. The combined sub-fraction was subjected to gravity column chromatography (GCC) to obtain more pure compounds. Isolate obtained was yellowish-colored with a crystalline shape resembling small needles. Based on TLC result using three systems of eluents with different polarities, there was one spot with Rf value of 0.7 indicating that the compound has been purified. The melting point of the isolate is 196.3–197.7 °C. The compound was considered pure if it has a melting point with a range of 2 °C [24]. Therefore, the target compound could be further isolated and identified using the spectroscopic method.

The chemical structure of the pure isolate (as analyzed using TLC and melting point) having active antioxidant was identified using spectroscopic methods (UV-Vis, FTIR, GC-MS, and NMR). Identification of functional groups using the IR spectrum (Fig. 3) was as follows: a broad peak at 3400–2400 cm^{-1} indicated the presence of O–H (hydrogen bonding of a carboxylic acid). The peaks at 2924 cm^{-1} indicated asymmetrical stretching vibration CH_2 group whereas the peak at 2863 cm^{-1} corresponded to the symmetric vibration of CH_3. Peak at 1667 cm^{-1} originated from C=O stretching vibration, peak at 1597 cm^{-1} revealed C=C benzene, and the peaks at around 1095 cm^{-1} indicated C–O stretching. While peaks at 790 and 760 cm^{-1} indicated aromatic out of plane bending system [25-26].

In order to confirm the chemical formula of isolated compounds, gas chromatography-mass spectrometry (GC-MS) was used. The mass spectrum (Fig. 4) exhibits the base peak at m/z 154 consistent with a molecular formula $C_7H_6O_4$, while the peak at m/z of 137 was formed due to the loss of OH group. The NMR measurement of isolated compounds is presented in Table 1.

The ^{1}H-NMR spectrum exhibited 2 proton groups that appear at the chemical shift, δ 7.4 and 6.8 ppm with integration 2 and 1, respectively (Fig. 5). The expanded spectra inform clearly that one proton showed peaks at δ 6.8 ppm as a doublet with J = 8 Hz was due to signal of H-5. While peaks at δ 7.4 ppm were the signal of 2 protons H-2 and H-6. H-2 rise as a doublet at δ 7.41 ppm J = 2 Hz, while H-6 appeared at δ 7.43 ppm as a doublet of doublet $J_{5\text{-}6}$ = 8 Hz and, $J_{6\text{-}2}$ = 2 Hz.

The ^{13}C-NMR spectrum of the isolated compound showed signals representing for 7 carbons present in the

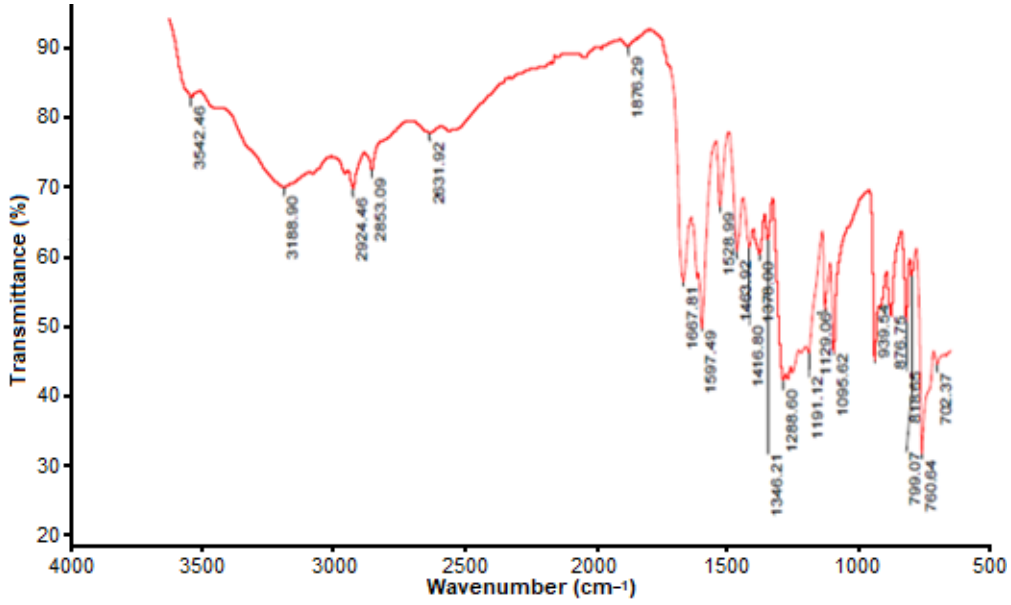

Fig 3. Attenuated total reflectance (ATR)-FTIR spectrum of the target compound, scanned at wavenumbers of 4000–650 cm^{-1}

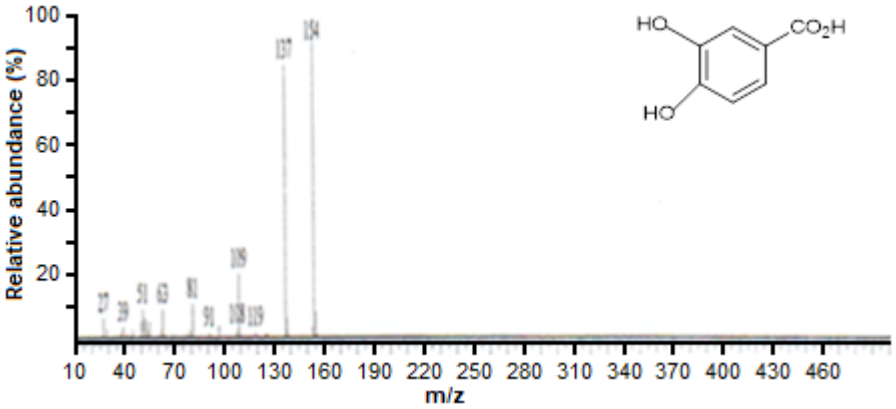

Fig 4. The mass spectrum of the isolated compound

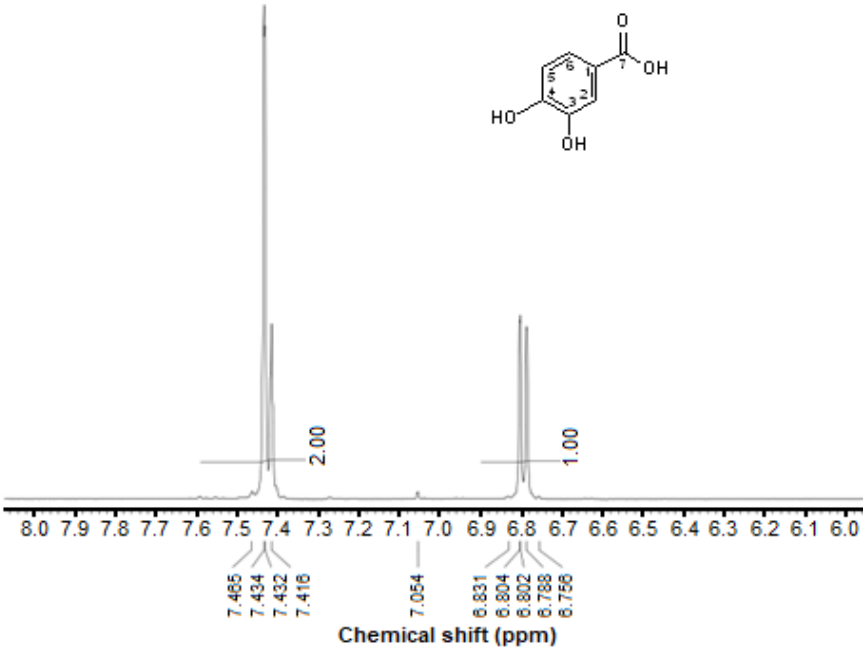

Fig 5. Partial ^{1}H-NMR spectrum

Table 1. NMR data of isolated compound

Carbon number	^{13}C (ppm, type of carbon)	Proton H (ppm)	HSQC correlation
1	124.03 Cq	-	
2	117.81 Cq	7.43 *d.J* = 2 Hz	H-2
3	146.22 Cq	-	
4	151.16 Cq	-	
5	121.27 Ct	6.8 *d.J* = 8 Hz	H-5
6	115.88 Ct	7.41 *dd.J* = 8 Hz, 2 Hz	H-6
7	170.31 (C=O)	-	

molecule (Fig. 6), namely at δ 170.31 ppm which indicated C (C=O) signal. Signals at δ 151.16 and 146.22 ppm were two C quaternary which attached OH groups those were C4 and C3, respectively. The other quaternary carbons were C1 and C2; those signals appeared at δ 124.0 and 117.81 ppm, respectively. While tertiary carbon, those were C5 and C6 which resonated at δ 121 and 115.8 ppm. The assignment of the carbon chemical shift was confirmed

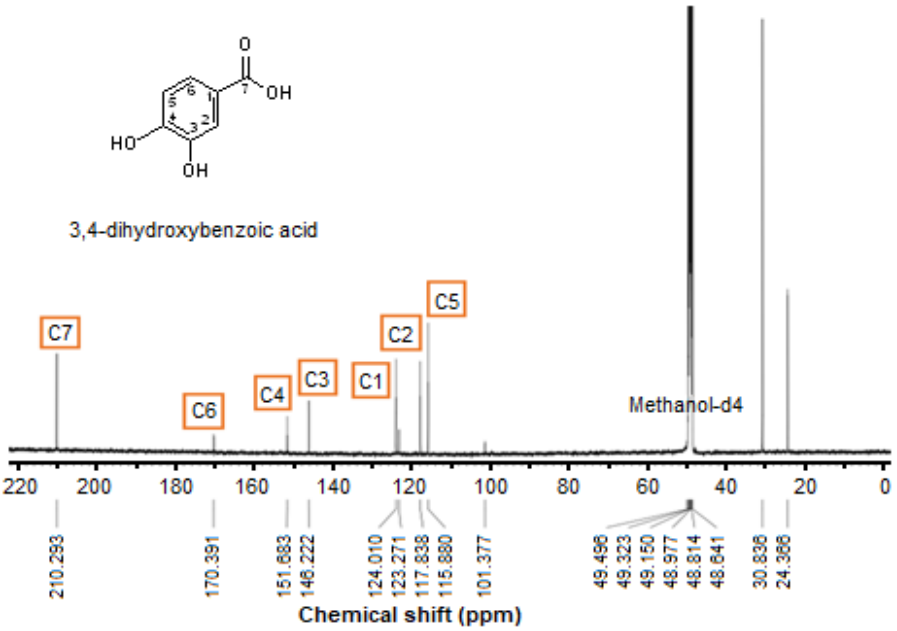

Fig 6. ^{13}C-NMR spectrum of the isolated compound

by DEPT (Suppl. 1). The HSQC spectrum (Suppl. 2) demonstrated that signal H at δ 6.8 ppm attached to C, which resonated at δ 121.27 ppm, while signal H at δ 7.41 ppm correlated to signal C at δ 115.88 ppm. Proton at δ 7.43 ppm attached to C, which resonated at δ 117.81 ppm.

Based on the IR, NMR, and mass spectra of the isolate, it was deduced that the studied compound in isolate had the molecular formula of $C_7H_6O_4$ identified as 3,4-dihydroxybenzoic acid, or protocatechuic acid with chemical structure shown in Fig. 5 and 6. This compound is a phenolic compound. This compound was further used for the evaluation of DPPH radical activity using vitamin C as a positive control. The result showed that the compound had IC_{50} of 5.17 µg/mL, higher than that of vitamin C (IC_{50} of 3.34 µg/mL) [18] indicating that the compound revealed lower antioxidant activity than vitamin C.

■ CONCLUSION

Among the methanolic extract and its fraction, ethyl acetate fraction revealed the highest antiradical activity using DPPH radical with the IC_{50} of 26.22 µg/mL and the metal chelating activity, accounting of 12.32%. The identification of active compounds using spectroscopic (FTIR, GC-MS, and NMR) analyses resulted in an active compound identified as 3,4-dihydroxybenzoic acid or protocatechuic acid.

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