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Secondary Metabolites from The Active Antioxidant Fraction of The Stem Bark of *Syzygium zeylanicum*. L. DC.

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ABSTRACT

Syzygium zeylanicum (L.) DC, known by the local community in South Sumatra, Indonesia, as *jambu nasi-nasi*. This plant has been used to treat various diseases including hypertension, diabetes, joint pain, headaches, and fever. The use of plants as medicine is closely related to their secondary metabolite content. This research is an experimental study aimed at determining the antioxidant activity of fractions from the stem bark of *S.zeylanicum* and isolating and determining the chemical structure of its active compounds. The fractionation of the dry powder of *S. zeylanicum* stem bark was carried out using a gradient extraction method with solvents n-hexane, ethyl acetate, and methanol. Each fraction was tested for antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The active antioxidant fractions were further isolated into pure compounds using chromatography techniques, and their chemical structures were identified through spectroscopy, including 1D and 2D Nuclear Magnetic Resonance (NMR). The antioxidant activity test results (IC_{50}) for each fraction, namely n-hexane, ethyl acetate, and methanol, were 338.1: 114.1: and 88.71 µg/mL. The results of the separation of the ethyl acetate and methanol fractions using a chromatography column showed that the SE7 subfraction from the ethyl acetate fraction had the highest IC_{50}, which is 83.25 µg/mL. The SE7 fraction was further separated by column chromatography until compound 1 was obtained as a white solid with an IC_{50} value of 62.22 µg/mL. Based on spectroscopic analysis including ^{1}H-NMR, ^{13}C-NMR, HSQC, HMBC and UV, compound 1 is proposed as 1-(4-isopropoxyphenyl)propane-1,2,3-triol

Keywords: *Syzygium zeylanicum, Medicinal plants, Secondary metabolites, Antioxidants*

Introduction

Antioxidants are substances that have the ability to neutralize free radicals, thereby inhibiting oxidative damage to target molecules.[1] These antioxidant compounds can inhibit damage caused by reactive oxygen species (ROS), which are responsible for various degenerative diseases such as cancer, arteriosclerosis, inflammation, diabetes mellitus, arthritis, aging, skin damage, liver injury, cardiovascular diseases, and Parkinson's disease.[2] Natural sources of antioxidants, such as vegetables, fruits, spices, and medicinal plants, are widely known for containing vitamin C, vitamin E, and phenolic compounds. [3,4,5,6]
Syzygium zeylanicum (L.) DC, one of Indonesia's tropical plants, is known to the local community as "jambu nasi-nasi." This plant belongs to the Myrtaceae family, has a soft woody stem, and spreading branches. This plant can reach a height of 5 meters and grows in the Indo-Malaya region. [7,8,9] People in various regions have utilized this plant as a traditional medicine to prevent and treat various diseases such as diabetes, joint pain, headaches, arthritis, fever, and it has antimicrobial and antifungal properties.[10,11,12] Based on the research reported by Mai, et al (2007) the methanol extract of *S. zeylanicum* leaves contains polyphenols and exhibits antioxidant activity, marked by a relatively strong inhibitor concentration (IC_{50}) value.[13] The same thing was reported by Nomi, et al., (2012) who successfully isolated the macrocyclic ellagitannin compound zeylaniin A and showed antioxidant activity that could serve as a source of natural antioxidants based on the DPPH test (*2,2-diphenyl-1-picrylhydrazyl*).[14] Other parts of the plant, such as the bark of *Syzygium zeylanicum*, have also been reported to have strong antioxidant bioactivity, especially in the ethyl acetate fraction using the DPPH test. [15] Phytochemical screening of *S. zeylanicum* successfully reported the presence of alkaloids, glycosides, phenolics, flavonoids, tannins, saponins, and steroids.[7,11,12,14,16] In addition, the leaves of the jambu nasi-nasi also contain essential oils, namely: (Z)-β-ocimene, linalool, α-copaene, viridiflorol, humulene epoxide II, epi-α-muurolol, α-cadinol, δ-cadinol, α-humulene.[17,18,19,20] The phytochemical content found in plants depends on where they grow. Differences in growing locations will affect the phytochemical content, even with the same species.[17,21] Light, environmental pH, and soil nutrient levels are components that affect the content in *S.zeylanicum*.[22] For that reason, further research is needed on the secondary metabolite content of *S. zeylanicum* and its antioxidant activity.

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Materials and Methods

Collection and Identification of Plant Material

The sample was taken in Manggar, East Belitung, Bangka Belitung Islands, Indonesia (-2.8797964, 108.2705523) and identified at the Herbarium Laboratory of the Indonesian Biology Generation Foundation, Gersik, Indonesia, with herbarium number: BT-072478. As much as 3 kg of *S. zeylanicum* stem bark was cleaned, cut into small pieces, and dried at room temperature in an open space, assisted with a

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fan for two weeks until the weight became constant. The dried material was then finely ground.

Extraction of the powdered stem bark of S. zeylanicum

A total of 1 kg of fine powder was placed into a 5 L tube, then 4 L of hexane was added, left to stand for 24 hours, and filtered. The filtrate was collected, and the residue was re-macerated in the same manner three times. The combined filtrate obtained was concentrated using a vacuum evaporator until a concentrated extract was obtained. This concentrated extract was weighed, transferred into a bottle, tightly sealed, and stored in the refrigerator at 4° celsius. The residue was dried at room temperature until solvent-free. In the same way, the residue was successively macerated again with ethyl acetate and methanol for 24 hours with three repetitions. Each obtained concentrated extract was subjected to an antioxidant test. The extract showing antioxidant activity was subjected to the separation and purification stage.[23,24,25]

Antioxidant Test

Antioxidant activity was determined using the DPPH method (*2,2-diphenyl-1-picrylhydrazyl).* Extracts/fraction were made into different concentrations (1000: 500: 250: 125: 62.5: 31.25: 15.625 µg/mL). 0.2 mL of each concentration was added to 3.8 mL of 0.05 mM DPPH.[23] The mixture was homogenized and left in the dark tube for 30 minutes. Absorption was measured using a UV-Vis spectrophotometer at λ 517 nm. In this test, ascorbic acid was used as the standard positive control and DMSO as the negative control.[26] Antioxidant activity can be represented by the DPPH absorption inhibition value, which is calculated using the percentage of DPPH absorption inhibition and the IC_{50} value. The percentage of inhibition and the IC_{50} value are calculated to determine antioxidant activity.[27,28]

$$\%\text{Inhibition} = \frac{A_k - A_s}{A_s} \times 100\%$$

A_k : control absorbance
A_s : sample absorbance

The IC_{50} value is determined by creating a concentration curve plot and obtaining the linear regression equation y = b + ax. Thus, the IC_{50} value of the equation can be expressed as 50 = b + ax. Based on this equation, the IC_{50} value can be calculated as follows:

$$X = \frac{50 - b}{a}$$

b : intercept, a : slope

Isolation and identification of pure compound

The selected fraction (ethyl acetate fraction) was separated using the gravity column chromatography technique (Ø 2 cm) with various eluents of increasing polarity. The column was prepared using G60 silica gel (70–230 mesh). The column was compacted by flowing n-hexane solvent into it. The sample was prepared by pre-adsorption using silica gel G60 (70–230 mesh) in a 1:1 mass ratio, then placed into the column and eluted with an increasing polarity eluent. The separation results were collected in vials sized ±10 mL. The separation results in the vials were evaporated to a concentrated solution, then analyzed using thin-layer chromatography (TLC) to determine the stain pattern. Vials with the same stain pattern were combined into one subfraction (SE1–SE8). The column subfractions were then tested for antioxidant activity to determine the potential subfraction groups for purification. The selected fraction (SE7) was further purified using column chromatography until a pure compound was obtained (Compound 1). The purity of the isolated compound was determined using a TLC test with various eluents. The compound was considered pure if it showed a single spot on the TLC plate with different eluents. Identification of compound structures using NMR spectroscopy methods including ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC and ultra violet (UV) spectroscopy

Results and Discussion

The results of the stepwise maceration of the dry powder of *S. zeylanicum* stem bark with n-hexane, ethyl acetate, and methanol yielded a thick n-hexane fraction of 6.962 g: ethyl acetate 11.637 g: and methanol 145.45 g. The difference in mass was caused by the conditions when the extract was weighed. The methanol extract had a paste-like texture that made it even heavier. Another condition was that the particle size of methanol was smaller compared to the solvents ethyl acetate and n-hexane, making it easier to penetrate cell walls. Methanol entered the cell tissue due to the large concentration difference between the solvent and the cell tissue: the compounds present in the cell tissue diffused into the solvent. In this case, the cell tissue had a high concentration, so the compounds diffused into the methanol solvent, which had a lower concentration

Antioxidant activity test

Antioxidant activity is observed based on the IC_{50} values of the three extracts with the positive control being ascorbic acid. Antioxidant activity was measured by preparing extracts at different concentrations. Based on the research results, the antioxidant activity of the methanol extract has strong antioxidant activity with an IC_{50} value of 88.12. The ethyl acetate extract shows fairly good antioxidant activity, while n-hexane shows weak antioxidant activity with IC_{50} values of 114.13 and 338.11, respectively.

Separation and purification of secondary metabolite compounds

The active antioxidant fractions, namely the ethyl acetate and methanol fractions, were separated using the gravity column chromatography method with G60 silica gel as the stationary phase. (70-230 mesh). The pre-absorbed fractions are placed into the chromatography column and eluted with solvents of increasing polarity, namely n-hexane:EtOAc (10:0 → 0:10) and EtOAc:MeOH (10:0 → 0:10). A total of 5 grams of ethyl acetate fraction and 5 grams of methanol fraction have been separated. The separation of 5 grams of ethyl acetate fraction resulted in 166 vials and was grouped into 8 column subfractions (SE1-SE8). The separation of 5 grams of methanol fraction resulted in 60 vials and was grouped into 5 column subfractions. (SM1-SM5). Column subfractions are grouped based on the stain pattern in thin-layer chromatography (TLC) tests. A total of 13 column fractions from the separation of ethyl acetate and methanol extracts were tested for antioxidant activity, and the results are presented in Table 1.

The SE7 column subfraction (vials 138-151) of the ethyl acetate fraction showed the strongest antioxidant activity among all subfractions from the ethyl acetate and methanol fractionation, with an IC_{50} value of 83.25 µg/ml. This SE7 subfraction comes from the ethyl acetate fraction, which has moderate antioxidant activity with an IC_{50} value of 114.13 µg/ml. Among the eight subfractions obtained from column chromatography (SE1-SE8), it is observed that the other seven subfractions exhibit weak to inactive antioxidant activity. This indicates that secondary metabolites with antioxidant activity are concentrated in the SE7 subfraction, thus providing strong antioxidant activity. Therefore, the SE7 subfraction is continued to the purification stage.

The selection of the appropriate eluent using the thin-layer chromatography (TLC) method is necessary to optimize the separation process. Chloroform: acetone is used in the separation process based on the eluent test. Purification by column chromatography using that eluent in the ratio (100:0: 95:5: 90:10) . The purification results yielded a pure compound characterized by only one spot in the purity test using TLC with various different eluents and the mass of compound 1 was obtained as 8.2 mg. The pure compound was then subjected to antioxidant activity testing and showed strong antioxidant activity with an IC_{50} value of 62.22 µg/ml (Table 1).

Identification of Compounds 1

The ^{1}H-NMR spectrum of compound 1 (700 MHz, $CDCl_3$) of the isolated compound was observed at a chemical shift of δH 0.80 - 8.30 ppm, which indicated that the isolated compound was an aromatic compound. The ^{1}H-NMR spectrum showed two aromatic proton signals

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appearing at δH 7.58 and 8.22 ppm, each with an integration of 2 and a coupling constant of 8.4 Hz, indicating that compound 1 had a para-substituted benzene ring. Furthermore, there was an oxygenated methylene proton signal at δH 4.00 ppm and three oxygenated methine signals at δH 4.18, 5.34, and 5.80 ppm. In addition, there was a signal at δH 1.25 ppm with an integration of 6, which was suspected to be a proton signal corresponding to the gem-dimethyl group. Thus, the ^{1}H-NMR spectrum revealed that compound 1 had a disubstituted benzene ring, where the substituents contained an oxygenated methylene group

Table 1: Antioxidant activity (IC_{50}) of ethyl acetate subfractions (SE1-SE8) and methanol (SM1-SM5) from the bark of *S. zeylanicum* and the standard antioxidant ascorbic acid using the DPPH Method

No.	Test Sample	IC_{50} (µg/mL)
1	SE1	> 500[ia]
2	SE2	> 500 [ia]
3	SE3	> 500 [ia]
4	SE4	> 500 [ia]
5	SE5	> 500 [ia]
6	SE6	307.77*
7	SE7	83.25***
8	SE8	217.94**
9	SM1	> 500 [ia]
10	SM2	> 500 [ia]
11	SM3	93.56***
12	SM4	324.67*
13	SM5	> 500 [ia]
14	Compound 1	62.22***
15	Ascorbic acid	9.81****

Note : antioxidant activity IC_{50} (µg/mL): **** very strong < 20µg/mL ***strong < 100 µg/mL: **moderate 100-250 µg/mL:*weak 250-500 µg/mL: [ia] in active > 500 µg/mL

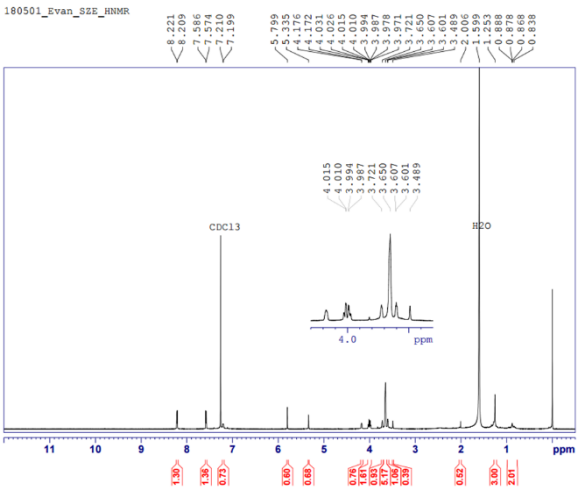

Figure 1: The ^{1}H-NMR spectra of compound 1

and three oxygenated methine groups. The ^{13}C-NMR spectrum of compound 1, shown in Figure 2, revealed the presence of 10 carbon signals. Four sp^2 carbon signals were observed at δ_C> 100 ppm, including a characteristic signal for an aryl ether carbon at δ_C164.6 ppm and an aromatic quaternary carbon at δ_C 147.7 ppm. Two aromatic carbon signals, which exhibited high intensity, indicated the presence of two pairs of equivalent carbons in a substituted benzene ring. These carbons resonated at δ_C 123.9 and 126.9 ppm. Additionally, five carbon signals were detected at δ_C 50–80 ppm, suggesting the presence of five oxygenated carbon atoms in compound 1. Lastly, a carbon signal at δ_C 29.9 ppm corresponded to a methyl carbon.The HSQC (Heteronuclear Single Quantum Correlation) spectrum of compound **1** (Figure 3) shows the presence of eight proton correlations with sp^2 and sp^3 carbons. There are two equivalent aromatic proton correlations, one methylene proton correlation to an oxygenated sp^3 carbon, four methine proton correlations to oxygenated sp^3 carbons, and one methyl proton

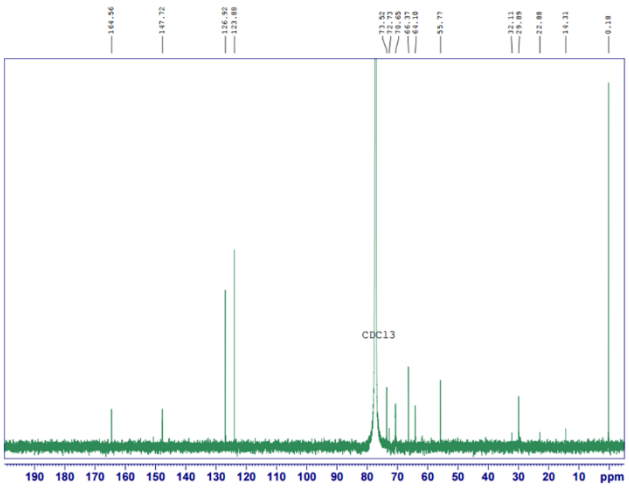

Figure 2: The ^{13}C-NMR spectra of compound 1

correlation. Thus, it is known that compound **1** has a para-substituted benzene ring, a gem-dimethyl group attached to an oxygenated methine carbon, a methylene group attached to an oxygenated carbon, three methine groups attached to oxygenated carbons, and a methoxyl group.

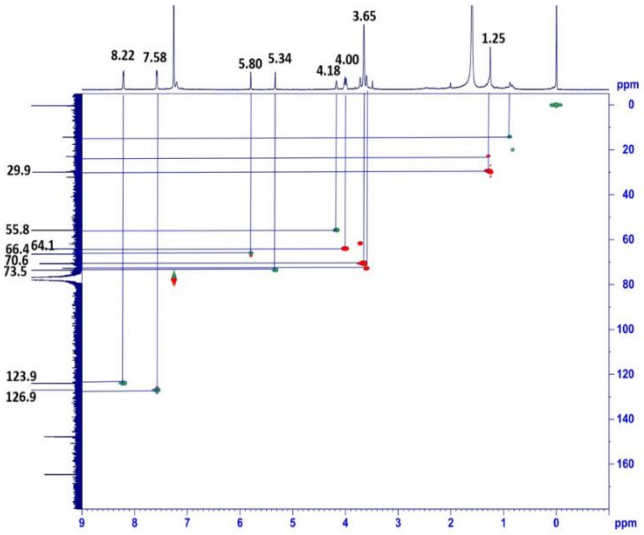

Figure 3: The HSQC Spectral of Compound 1

The correlation between protons and neighboring carbons that are two to three bonds apart is determined through the HMBC (Heteronuclear Multiple-Bond Correlation) spectrum. The HMBC spectrum of compound 1 (Figure 4) shows the correlation of equivalent aromatic protons at δ_H 8.22 (1H, dd, J= 8.4 Hz) with two aromatic carbon atoms at δ_C 123.9 and 147.7 ppm, including their equivalent carbon atoms. Aromatic equivalent protons at δ_H 7.58 (1H, dd, J= 8.4 Hz) correlate with three aromatic carbons at δ_C 126.9: 123.9: and 147.7 and an sp^3 methine carbon oxygenated at δ_C 73.5 ppm. The correlation of these two equivalent aromatic protons indicates that compound **1** has a para-substituted benzene ring. There is an oxidized methylene carbon (δ_C

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73.5 ppm) directly bound to a quaternary aromatic carbon (δ_C 147.7 ppm). The methine proton at δ_H 5.80 ppm correlated over three bonds to the oxiaryl carbon at δ_C 164.6 ppm. Furthermore, there was a methyl proton at δ_H 1.25 ppm that was bound to the carbon at δ_C 29.9 ppm, correlating to the same methyl carbon atom at δ_C 29.9 ppm. This indicated the presence of a dimethyl group bound to the oxygenated methine carbon. Thus, it was determined that another substituent of the substituted benzene ring was an isopropoxy group bound to a quaternary carbon at δ_C 164.6 ppm.

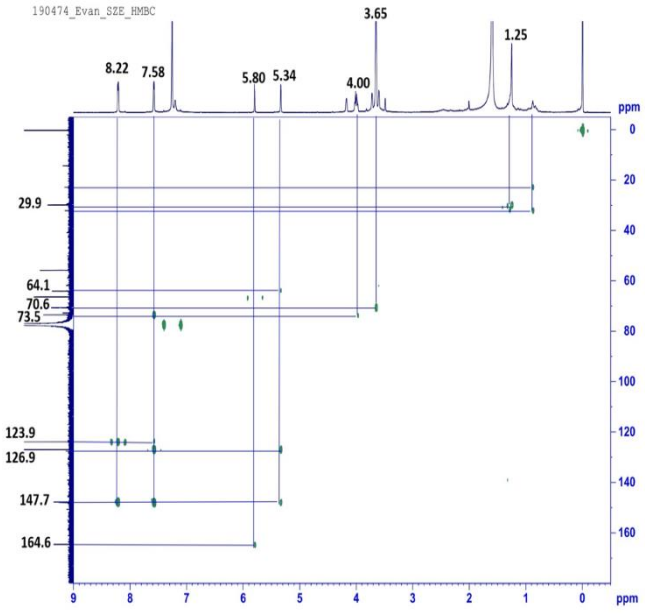

Figure 4: The HMBC Spectral of Compound 1

The next correlation is from other substituent protons. The correlation of the methine proton at δ_H 5.34 ppm correlates with two aromatic carbons at δ_C 126.9: 147.7 ppm and also correlates with a methylene carbon at δC 64.1 ppm. Similarly, the methylene proton at δ_H 5.34 ppm correlates with three bonds to the methine carbon at δ_C 73.5 ppm. This indicates that the substituent attached to the para position of the isopropoxy group is a carbon chain consisting of oxygenated carbon atoms. The 1D and 2D NMR spectrum data for compound **1** are listed in Table 2. The results of ultraviolet spectroscopy (Figure 5) showed no wavelength shift when shift reagents ($AlCl_3$ and NaOH) were used, indicating that the structure of compound 1 did not have a substituent directly binding to the hydroxyl group. This further supported the presence of an isopropoxy group on the benzene ring. Based on the analysis of the ^{1}H-NMR, ^{13}C-NMR, HSQC, and HMBC spectra and UV spectroscopy, it can be explained that compound 1 has a para-substituted benzene ring. An isopropoxy group acts as one of the substituents. Meanwhile, the other substituent in the para position is a carbon chain composed of oxygenated carbon atoms, consisting of two methylene carbons oxygenated with a hydroxyl group and a methylene carbon oxygenated with a methoxy group. The compound 1-(4-isopropoxyphenyl)propane-1,2,3-triol (**1**) has good antioxidant activity with an IC_{50} value of 62.22 µg/mL. This compound is derived from the ethyl acetate fraction of the bark of *S. zeylanicum*, subfraction SE7. Based on the research conducted by Nguyen, et al (2023), the ethyl acetate fraction of the stem bark of S. zeylanicum has a high concentration of phenolic compounds with the highest antioxidant and antihyperglycemic activity in a postprandial zebrafish model.[29] The main contributors to the antioxidant activity in the ethyl acetate fraction are gallic acid, catechin, epicatechin, ellagic acid, quercetin, caffeine, and apigenin, rutin, and ethyl gallate. These compounds reduce α-amylase inhibition activity and affect α-glucosidase inhibition activity. The compound 1-(4-isopropoxyphenyl)propane-1,2,3-triol (**1**) found in this study has not been previously reported from the stem bark of S. zeylanicum, but this compound belongs to the group of phenolic compounds commonly found in the genus Syzygium.[30,31,32,33] The compound (**1**) had a structure similar to the compound 1-(4-hydroxyphenyl)propane-1,2,3-triol, which had been successfully isolated from pinus sylvestris. Lundgren [34] successfully isolated the compound 1-(4-hydroxyphenyl)propane-1,2,3-triol. The compound 1-(4-hydroxyphenyl)propane-1,2,3-triol was isolated using the preparative electrophoresis method and was measured using ^{1}H-NMR spectroscopy (90 MHz) with CD_3OD as the solvent.[34] The comparative data of the isolated compound 1 with the compound from the literature can be seen in Table 2. The compound 1-(4-isopropoxyphenyl)propane-1,2,3-triol (**1**) is an active antioxidant, apparently due to the presence of a 1,2,3-trihydroxyl group. When it undergoes proton abstraction by free radicals, it can form a new, more stable free radical. This stability is due to the free radical produced being able to form a new carbonyl group that is conjugated with the benzene ring, specifically as the compound 4-isopropoxybenzaldehyde.[35]

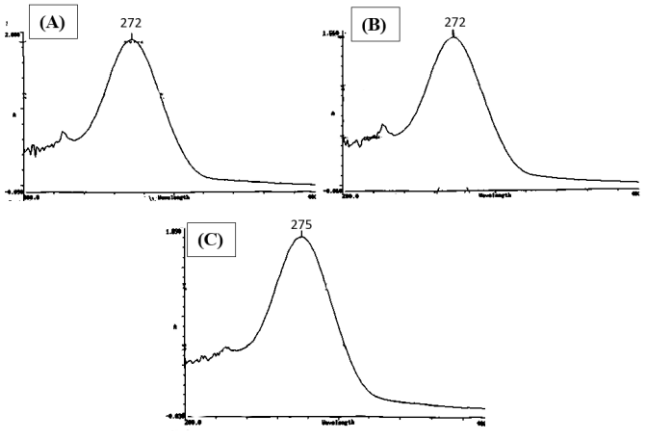

Figure 5: Ultraviolet (UV) spectrum: (A) sample: (B) Sample + NaOH: (C) Sample + $AlCl_3$

Thus, compound 1 is identified as 1-(4-isopropoxyphenyl)propane-1,2,3-triol. The chemical structure of compound 1, including carbon atom numbering and its HMBC correlations, was shown in Figure 6.

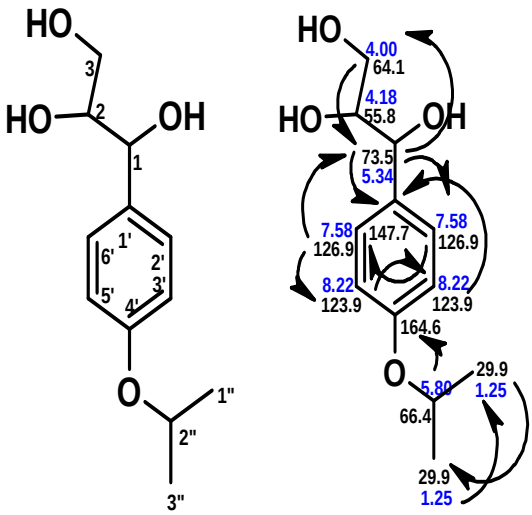

Figure 6: Chemical structure of the compound 1-(4-isopropoxyphenyl)propane-1,2,3-triol with carbon atom numbering and NMR correlation

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Table 2: The NMR data of compound 1, recorded at ^{1}H-500 MHz: ^{13}C-125 MHz in $CDCl_3$

No. C	δ_C ppm 1	δ_H ppm (ΣH. Multiplicity, Hz) 1	δ_H ppm (ΣH. Multiplicity, Hz) 1*	HMBC 1
1	73.5	5.34 (1H: s)	4.5 (1H: d: J=6,6 Hz)	64.1: 126.9: 147.7
2	55.8	4.18 (1H: s)	3.65 (1H: m)	
3	64.1	4.00 (2H: m)	3.45 (1H: dd: J=11,2 & 3.4 Hz)	73.5
1’	147.7			
2’	126.9	7.58 (1H: d: J=8.4 Hz)	6.74 (2H: d: J=8,5 Hz)	73.5: 126.9: 123.9: 147.7
3’	123.9	8.22 (1H: d: J=8.4 Hz)	7.19 (2H: d: J=8,5 Hz)	123.9: 147.7
4’	164.6			
5’	123.9	8.22 (1H: d: J=8.4 Hz)	7.19 (2H: d: J=8,5 Hz)	123.9: 147.7
6’	126.9	7.58 (1H: d: J=8.4 Hz)	6.74 (2H: d: J=8,5 Hz)	73.5: 126.9: 123.9: 147.7
1”	29.9	1.25		29.9
2”	66.4	5.80 (1H: s)		164.6
3”	29.9	1.25		29.9

* data from the literature. [34]

Conclusion

The ethyl acetate fraction of the stem bark of S. *zeylanicum* exhibited moderate antioxidant activity, and the separation results from column chromatography revealed strong antioxidant activity concentrated in the subfraction SE7. The compound *1-(4-isopropoxyphenyl)propane-1,2,3-triol* (1) has been isolated from subfraction SE7, which has not been previously reported from the stem bark of *S. zeylanicum*, and it possesses good antioxidant activity with an IC_{50} value of 62.22 µg/mL. This compound is a group of phenolic compounds commonly found in the genus *Syzygium*. The methanol fraction has strong antioxidant activity, but the subfraction obtained from column chromatography has antioxidant activity lower than that of the ethyl acetate subfraction. Further research is needed to isolate the compounds contributing to the main antioxidant activity from this ethyl acetate fraction to complete the chemical profile of the concentrated antioxidant fraction in the ethyl acetate fraction of the stem bark of *S. zeylanicum*.

Conflict of interest

The author reports no conflicts of interest in this work.

Authors’ Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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