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ANTIOXIDANT PROPERTIES OF THE ETHYL ACETATE EXTRACT OF ENDOPHYTIC FUNGUS *Penicillium Citrinum* FROM *Kalanchoe Millotii* STEM THROUGH SECONDARY METABOLITES

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Penicillium citrinum.

Abstract

Kalanchoe, as Cocor Bebek, is a genus of plants that is often used in traditional medicine because it has various biological activities, one of which is as an antioxidant. The development of research was carried out on the secondary metabolites compounds of endophytic fungi from the stem of *K. millotii* as the Cocor Bebek species. Endophytic fungi are microorganisms that live in plant tissues, generally producing secondary metabolites that have bioactivities such as anticancer, antioxidant, and antibacterial. One of the endophytic fungi isolated from the stem of *K. millotii* was identified as *Penicillium citrinum*. The potential antioxidant activity of the secondary metabolites contained in the ethyl acetate extract of the endophytic fungus *P. citrinum* is interesting to study. The sample extract was extracted using non-polar, semi-polar, and polar solvents. The ethyl acetate extract was separated and purified by various chromatographic methods to obtain 8 mg compound **1**, as a yellow solid. The chemical structure was determined by spectroscopic methods. Based on spectrum analysis of 1D-NMR, 2D-NMR, FTIR, and UV-Vis, compound **1** was identified as 4-(2-ethylbutoxy)-3,5-dimethoxybenzaldehyde. Antioxidant activity testing of ethyl acetate extract and compound **1** was carried out using the DPPH (2,2-diphenyl-1-pikrilhidrazyl) method. The test results on the extract showed weak activity with an IC_{50} value of 401.01 ppm while compound **1** showed moderate activity with an IC_{50} value of 221.41 ppm.

INTRODUCTION

In today's modern age, degenerative diseases affect many people, even at productive ages. According to the National Health and Nutrition Examination Survey (NHNES III), around 50 million people worldwide suffer from degenerative diseases [1]. These diseases are caused by oxidative stress and an unhealthy lifestyle [2]. To prevent damage to body tissue cells due the exposure to free radicals, it is recommended to use substances that contain antioxidants [3]. However, some artificial antioxidants like Butyl Hydroxy Anisole (BHA) and Butyl Hydroxy Toluene (BHT) can cause side effects such as liver damage and even tumors in the long term [4]. Due to the discovery of these carcinogenic side effects, people tend to choose natural antioxidants as a safer alternative. Natural antioxidants can be obtained from plants or fruits [5].

Extract from plant parts shows antioxidant activity driven by the presence of secondary metabolites such as alkaloids, saponins, tannins, terpenoids, flavonoids, and phenolics [6][7]. This active phytochemical compound is the result of plant biosynthesis and is a significant source of medical compounds.

Kalanchoe millotii is one of the succulent plants from the Crassulaceae family. *K. millotii* belongs to the genus Kalanchoe which has 145 species, and only 33 species have been studied [8]. According to previous studies, this family contains flavonoids, steroids, alkaloids, and bufadienolides, which have potential as antimalarial, insecticidal, antiviral, antimicrobial, antioxidant, and cytotoxic agents [9]. Several types of Kalanchoe plants have been found to possess antioxidant properties. According to Sylvia, the ethanol extract of *K. pinnata* leaves has a stronger antioxidant activity compared to the *n*-hexane and ethyl acetate extracts. There have been limited studies reporting the secondary metabolites in *K. millotii* plants, hence, research and development on this species are being carried out [10].

Kalanchoe in its natural habitat has endophytic fungi that form, a symbiotic relationship with the plant through mutualism. These fungi can produce bioactive compounds and

secondary metabolites [11]. The Penicillium family of fungi is a widely distributed group that can be found in various environments. Studies have shown that *P. citrinum* fungi from different sources can produce bioactive secondary metabolites with a range of chemical structures [12]. Research conducted by Chen has successfully isolated the secondary metabolite compound 2,3,4-trimethyl-5,7-dihydroxy-2,3-dihydrobenzofuran from the endophytic fungus *P. citrinum* which was identified as a new antioxidant with an IC_{20} value of 10.39 μM [12]. In addition, the endophytic fungus P. citrinum was also successfully isolated from the flowers of *Ocimum tenuiflorum* L. which produced the compound 5-methyl alternariol ether with IC_{50} values of 1.0 μg/mL, 3.7 μM [14].

One of the endophytic fungi successfully isolated from the stems of the *K. millotii* plant is *P. citrinum*, which has been shown to have moderate antioxidant activity in its ethanol extract [15]. Ethyl acetate, which is a semi-polar solvent, can attract both polar and nonpolar compounds, has low levels of toxicity, and tends to evaporate easily [16].

There hasn't been a lot of research done on the secondary metabolite compounds in the endophytic fungus *P. citrinum* found in the stems of *K. millotii* plants. Therefore, it is interesting to conduct research and study the group of secondary metabolite content to determine its potential as a natural antioxidant agent.

EXPERIMENT

The method used in this research is the true experimental method which includes work stages, including liquid-liquid extraction of ethanol extract with *n*-hexane and ethyl acetate solvents. Subsequently, isolation and purification of secondary metabolite compounds by Thin Layer Chromatography (TLC), Gravity Column Chromatography (GCC), Chromatotron, and Preparative Thin. The next stage was the elucidation of compound structure with 1D-NMR, 2D-NMR, UV-Vis, and FTIR spectroscopy. Finally, antioxidant activity testing of extract isolates using the DPPH method.

Material

The materials used in this study include concentrated ethanol extract of endophytic fungus *P. citrinum* from the stem of *K. millotii*, ethyl acetate ($CH_3COOC_2H_5$), *n*-hexane (C_6H_{14}), chloroform ($CHCl_3$), methylene chloride (CH_3C_{l2}), acetone (C_3H_6O), methanol (CH_3OH), sulfuric acid (H_2SO_4), silica gel 60 (0.0063-0.0200) mm, TLC plate (Silica gel 60 GF254), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, distilled water.

Instrument

The equipment used are laboratory glassware, separatory funnel, rotary evaporator brand Heidolph Laborata 4000 type Heizbad W, desiccator, Ohaus cp214 analytical balance, dropper pipette, spatula, distillation apparatus, vial bottles, gravity chromatography column, chamber, capillary tube, mortar and pestle, UV lamp λ 365 nm and 254 nm, glass plate chromatotron, JEOL JNM ECA-500 NMR Spectroscopy, Hewlett Packard 8453 Ultraviolet Spectroscopy, Shimadzu Type Prestige-21 Infrared Spectroscopy.

Procedure

Secondary Metabolites Extraction

Ethyl acetate extract propagation was done by adding an ethyl acetate solvent to the previously obtained ethanol extract. This extract propagation is done by liquid-liquid extraction technique until the filtrate is colorless (near clear) and detected by a TLC as a control. The next step was filtering ethyl acetate extract, the filtrate was collected and concentrated using a rotary evaporator resulting in 17.52 g of ethyl acetate concentrated extract [17].

Secondary Metabolites Isolation

The concentrated ethyl acetate extract obtained was further purified by various chromatographic techniques such as Gravity Column Chromatography, Chromatotron, and Preparative TLC. The purity of the separated compounds was identified based using TLC on various eluents that showed a one-stain chromatogram [18].

Thin-layer chromatography

The ethyl acetate extract was analyzed using Thin-Layer Chromatography (TLC). Samples were spotted on the TLC plate using a capillary pipe at a distance of 0.5 cm from the bottom of the plate. The plate with the sample is added to a chamber filled with eluent, previously saturated to a certain ratio.The plate was kept aside until the elution was complete. After that, the TLS plate was lifted and dried. It was then observed under a UV lamp with wavelengths of 254 nm and 365 nm. Finally, it was sprayed with an H_2SO_4 reagent in ethanol to determine the compounds present in each spot on the TLC plate.

Gravity column chromatography

A set of gravity column chromatography (GCC) equipment was cleaned and assembled. Silica gel 60 with a size range of 0.0063-0.0200, which had been activated by n-hexane, was used as the stationary phase. The ratio of sample to silica was 1:20. Then, 7.5 grams of ethyl acetate extract that had been impregnated using silica gel 60 with a size range of 0.0063-0.0200, and a ratio of sample to silica of 1:2 were added to the column, just above the stationary phase. The elution process began with 100% n-hexane eluent. The polarity of the eluent was then gradually increased using a variation of n-hexane: ethyl acetate eluent until it reached 100% ethyl acetate. Finally, 100% acetone and 100% methanol were used as eluents [17]. Each fraction was then concentrated, weighed, and analyzed using the thin-layer chromatography (TLC) method with n-hexane: ethyl acetate (3:7) eluent. The fractions with the same separation pattern were combined, resulting in 10 fractions.

The isolation process continued on fraction 15-5 with the GCC method, using a smaller column than before, namely 60 cm high and 1.5 cm in diameter. From the fractionation process, 18 fractions were obtained, and then TLC tests were carried out to determine the eluate that had the same chromatogram pattern.

Secondary Metabolites Purification

Chromatotron plates that have been coated with silica gel GF245 are mounted on an electric motor and are rotated at a speed of 800 rpm. Elusion is performed using n-hexane until the first drop appears, after which, the eluen crane is closed for 15 minutes before the 25 mg sample is dissolved in the acetone and inserted carefully. The eluent opening is followed by the elusion with *n-hexane*, then continued with a mixture of eluent chloroform and ethyl acetate. (8,5: 1,5). The fluid resulting from the elusion is collected according to the pattern of stains seen using UV lights λ 254 nm and λ 365 nm. After collection, the fluid is applied and identified using the KLT technique. Chromatotron fractions with similar patterns are combined into one [18].

Thin-layer chromatography

The preparatory KLT method was chosen because the fraction to be separated has a small mass, only 20.9 mg, and shows a tight pattern of stains, so GCC or chromatotron is not recommended for use. For this process, a 8 x 5 cm KLT plate with 60 GF254 silica gel is used as a silent phase, while the eluen used is a mixture of chlorophorus and ethyl acetate. (8,5:1,5). Photographed samples on plates using capillary tubes and subsequently diluted with the same mixture of eluent chlorophorus and ethyl acetate. Tape stains of preparatory KLT observed under UV light are marked and cut, then inserted into a vial containing a solution of chlorophorus and di-vortex to dissolve the compound. The mixture is then filtered to separate the solute filters of the compound from the silica, and the sample is dried to form a solid insulation [20].

Purity Test

Fraction 28-1 from preparative TLC was then tested for purity using TLC on various eluents. If the results show a single spot stain, then the fraction can be said to be a pure isolate by TLC.

NMR, UV-Vis, FTIR Identification

Core *Magnetic Resonance Spectroscopy* (NMR) analysis is one of the spectroscopic methods often used in structure determination. UV-Vis analysis is performed to determine the conjugated system that has or is bound to a functional group. FTIR analysis is performed to determine the functional group.

Antioxidant Activity Test

Antioxidant activity tests on isolates were carried out using the DPPH method known as 1,1-diphenyl-2-picrylhydrazyl free radical silencing [20]. This is because the DPPH method has the advantage that the analysis is easy, efficient, and requires a fairly fast test time.

Preparation of DPPH solution

A Total of 15.7 mg of DPPH was dissolved in 100 mL of methanol p.a and stored in a dark bottle, resulting in a DPPH solution concentration of 0.4 mM.

Preparation of ascorbic acid master standard solution

A 10 mg amount of ascorbic acid was put into a 100 mL volumetric flask and dissolved with ethanol p.a until the limit mark.

Preparation of extract and isolate solution

A total of 50 mg of extract and 5 mg of pure isolate were dissolved with ethanol p.a and the volume was sufficient in a 50 mL volumetric flask.

Determination of λmax DPPH

A 0.4 mM DPPH solution was added with 3 mL of ethanol p.a and incubated for 30 minutes. Then the absorbance was measured at a wavelength of 300-800 nm.

Measurement of DPPH free radical binding activity with ascorbic acid

The test was carried out by pipetting 0.05 mL; 0.1 mL; 0.015 mL; 0.2 mL; 0.025 mL; 0.3 mL; 0.035 mL; and 0.4 mL of 100 ppm ascorbic acid mother liquor, the mixture was added with 1 ml of DPPH and then the volume was sufficient to 10 ml with ethanol p.a to obtain a solution with a concentration of 0.5; 1; 1.5; 2; 2.5; 3; 3.5; and 4 ppm, then homogenized and left for 30 minutes. Then the absorbance was measured by UV-Vis spectrophotometry at the maximum wavelength (750 nm).

Measurement of DPPH free radical binding activity with samples

Ethyl acetate extract solution was done by pipetting each 0.5 mL; 1 mL; 1.5 mL; 2 mL; and 2.5 mL of 1000 ppm test solution then adding 1 mL of DPPH and sufficient volume to 10 ml with ethanol p.a to obtain a solution with a concentration of 50; 100; 150; 200; and 250 ppm. Then homogenized and incubated for 30 minutes, then measured the absorption with UV-Vis spectroscopy at λmax.

RESULT AND DISCUSSION

Sampling

The material utilized in this study is the ethanol extract of the endophytic fungus *P. citrinum,* which was previously obtained from the stem of the K. millotii plant. The extract has undergone phytochemical screening, resulting in a positive test for secondary metabolites of alkaloid, steroid, terpenoid, flavonoid, quinone, and tannin groups [14].

Extraction

The concentrated ethanol extract of endophytic fungus *P. citrinum* from the stem of *K. millotii* after the fermentation process weighs 313.57 g, then carried out the liquid-liquid extraction process using the *n*-hexane solvent to draw non-polar compouds such as fats, terpenes, xanthophyll in the extract. This action was followed by extraction using an ethyl acetate solvent, which was selected because of its evaporative, non-hygroscopic, low toxicity, and semi-polar nature, and is expected to attract both polar and non-polar compounds. The results of analysis using the TLC technique with UV rays at λ 254 nm and λ 365 nm showed the potential of ethyl acetate extracts containing secondary metabolite compounds.

The extraction process is carried out continuously until a filtrate is obtained that has faded in color (close to clear). Furthermore, all the filtrates obtained were combined into one to be concentrated using a rotary evaporator at 40°C and 240 mBar pressure until the solvent no longer drips, and a concentrated extract of 17.52 g was obtained.

Purification by Column Chromatography

The fractionation process of the concentrated ethyl acetate extract was carried out using the GCC method. This process aims to separate the active compound components from the extract that has been obtained. The results of the GCC obtained 22 fractions and identified by TLC to be combined based on the same stain pattern so that 10 fractions were obtained (**Figure 1**).

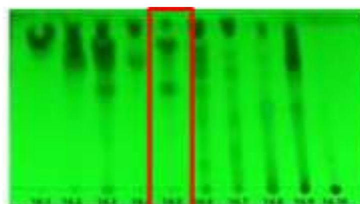


Figure 1. TLC of the combined fraction of the results of the GCC.

The purification process was continued on fraction 15-5 because it had sufficient weight with the expected TLC stain. Isolation was carried out by the GCC method, but using a smaller column than before. 18 fractions were obtained, then merged based on the same stain pattern on the TLC results so that 9 fractions were obtained (**Figure 2**). Inhibition under UV light 254 nm showed that the compound had at least two conjugated double bonds.

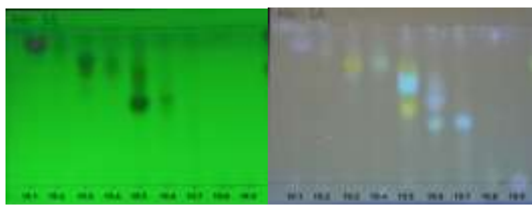


Figure 2. TLC of the combined fractions from the 15-5 GCC.

Purification by Chromatotron

Fraction 19-5 was analyzed by TLC to select the appropriate eluent for the chromatotron process. The separation technique in this chromatotron uses centrifugal force and gravity, so the process can take place faster, where the plate is mounted on the electric motor and rotated at a speed of 800 rpm.

The mobile phase system used is chloroform: ethyl acetate eluent with a ratio of 8,5: 1,5 so that 14 fractions were obtained which after merging resulted in 6 fractions (**Figure 3**).

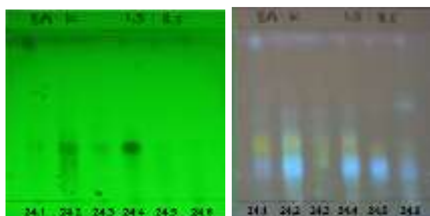


Figure 3. KLT of combined fractions from chromatotron 1 and 2.

Separation by Preparative Chromatography

The preparative TLC method was chosen for the separation process because the fraction to be separated has a small mass of only 20.9 mg and has a closely matched stain pattern. It is not recommended to use the GCC or chromatotron method for this purpose due to these reasons. **Figure 4** illustrates the preparative TLC process.

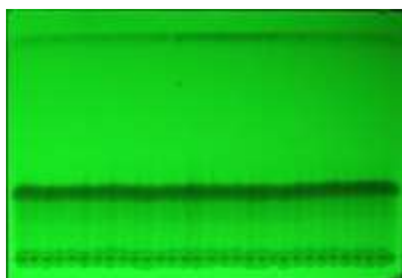


Figure 4. Preparative KLT results.

The plate that has been cut according to the target stain pattern is put into a vial containing chloroform solvent. Furthermore, the vial is kept for 24 hours so that it can dissolve completely. Then the mixture was filtered to separate the sample in the form of filtrate with silica gel from preparative TLC. As a result, fraction 28-1 with a mass of 8 mg was obtained.

Purity Test

To determine the purity of fraction 28-1, purity tests were carried out by means of TLC until one stain appeared on at least three eluent systems.

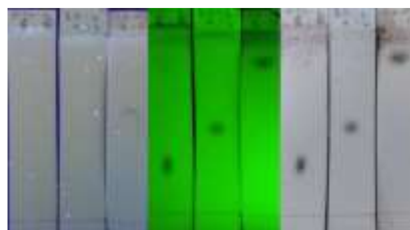
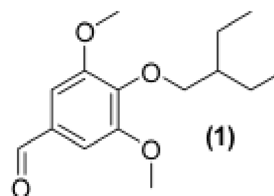


Figure 5. TLC results of isolates with various eluents.

The three eluent systems chosen for fraction 28-1 are MTC: chloroform (6:4); ethyl acetate: chloroform (4:6); ethyl acetate: methanol (8:2) as shown in **Figure 5**. The TLC results obtained one stain in three different eluents, so it can be said that the fraction 28-1 obtained is a pure isolate by TLC. The next fraction 28-1 is called compound **1**.

Structure Elucidation of Compound 1



4-(-2-ethylbutoxy)-3,5-dimethoxybenzaldehyde (1), obtained in the form of yellow solids; UV-Vis (MeOH) λ_{max} nm 275,00, 260,01 nm; IR ν_{max} cm^{-1} : 1906,76 (C=O), 1588,19 (C=C aromatic), 997,34 (C-C), 760,77 (C-H aromatic); ^{1}H-NMR (500 MHz, CD_3OD, TMS) δ ppm: 0,9 (3H, t, J=7,5 Hz), 1,17 (2H, m), 1,24 (3H, t, J=7 Hz), 1,32 (2H, m), 2,01 (1H, s), 3,60 (3H, q), 4,10 (2H, s), 4,61 (3H, s), 6,68 (1H, d, J=3. 5 Hz), 7,40 (1H, d, J=3. 5 Hz), 9,54 (1H, s), ^{13}C-NMR (125 MHz, CD3OD, TMS) δ ppm: 12,65 (C-methyl), 18,43 (C-methylene), 14,55 (C-methyl), 21,00 (methylene), 29,14 (C-methine), 57,65, 61,79, 57,38 (C-O), 110,4, 124,9 (C-aromatic), 129,6, 131,2, 151,9, 153,9 (C-O aromatic), 179,9 (C-carbonyl).

According on UV-Vis spectrum analysis, λmax 260 nm shows the presence of $\pi \rightarrow \pi^*$

transition indicating the presence of chromophore groups or C=C conjugated double bonds in the structure, while at λmax 275.00 nm shows the presence of n→π* electron transition indicating the presence of C=O double bonds [22].

Based on the results of FTIR analysis of compound **1**, it shows the presence of an aromatic system in the compound, supported by the absorption in the 1588 cm^{-1} region indicating the presence of a conjugated C=C bond. This is also reinforced by the presence of aromatic C-H bending vibrations in the 600-900 cm^{-1} region. The sharp absorption in this region indicates the presence of substituents in the aromatic system. Another typical absorption in the 1906 cm-1 wave number range is the presence of a C=O carbonyl group, also at wave number 997 cm^{-1} indicating the presence of alkane C-C bonds.

The ^{1}H-NMR data for compound **1** shows the presence of 11 proton signals including one signal for RCH=O at a chemical shift (δ_H) 9.54 (s, H-7), other protons are in the shear range of 6-8 ppm namely (δ_H) 7.39 (d, J = 3.5 Hz, H-6) and 6.68 (d, J = 3.5 Hz, H-2) which indicates the shear range of aromatic protons. Where the two protons are interconnected as indicated by the presence of couplings that have the same value, namely coupling in the meta position (J = 3.5 Hz). Furthermore, the chemical shifts (δ_H) 3.60 (q, H-12); 4.61 (d, H-13); 4.10 (q, H-14); indicate the presence of protons in the $-OCH_2$ (methylene oxy) and -OCH3 (methyl oxy) groups. Other protons that have not been mentioned are 6 protons for methyl and methylene which appear at chemical shift regions (δ_H) 2.01 (s, H-15); 1.32 (q, H-16); 1.24 (t; J=7.5 Hz, H-17); 1.17 (t, H-18); and 0.90 (m; J=7 Hz, H-19). The coupling constants for protons H-17 and H-19 are 7.5 and 7 which means that the two protons are in the cis position. The spectrum of ^{1}H-NMR compounds is shown in **Figure 6**. And the results of ^{1}H-NMR analysis are shown in **Figure 7**.

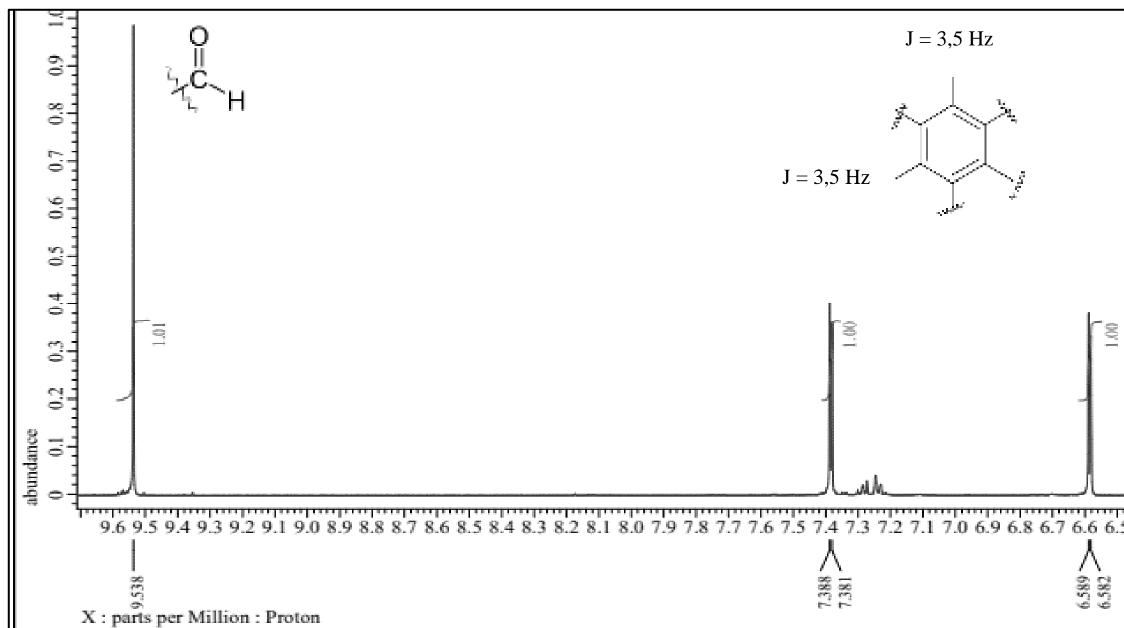


Figure 6. Fragment of ^{1}H-NMR spectrum of isolated compound at δ_H 9.54 -6.68 ppm.

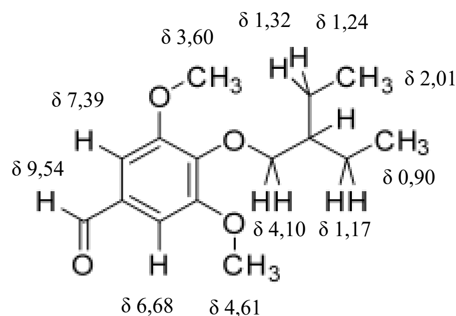


Figure 7. Chemical shift value (^{1}H).

The ^{13}C-NMR spectrum shows a typical signal at a certain chemical shift region. Based on the spectrum analysis of compound **1** shows the presence of carbon in 4 methyl groups, 3 methylene groups, 4 methine groups, and 4 quaternary carbons. Where 2 methyl carbons are shown in carbon peaks at chemical shifts (δ_C) 61.79; 57.38; 14.55 and 12.65 ppm. Three methylene groups are shown at chemical shifts (δ_C) 57.65; 21.00; 18.43 ppm. Furthermore, four methine groups are shown at chemical shifts (δ_C) 179.77; 124.94; 110.38, and

29.14 ppm. Finally, four quaternary carbons at chemical shifts (δ_C) 151.94; 151.9; 131.2 and 129.6 ppm indicate the presence of a symmetrical aromatic C=C signal. the results of ^{13}C-NMR analysis are shown in **Figure 8**.

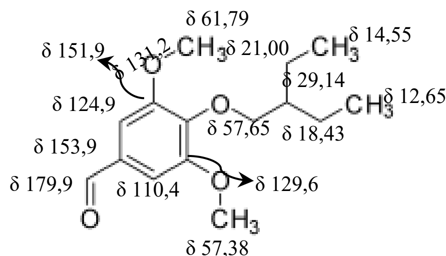


Figure 8. Chemical shift value (^{13}C).

The ^{1}H-^{1}H COSY (*Correlated Spectroscopy*) spectrum is used to determine the correlation of neighboring protons and strengthens the correlation on the ring, which can be seen from the cross-peaks between the protons. In **Figure 9**. The ^{1}H-^{1}H spectrum of COSY can be observed that there is a correlation between proton (H-6) (1H, d) δ_H 7.39 ppm correlated with (H-2) (1H, d) δ_H 6.68 ppm; (H-14) (2H, s) δ_H 4.10 ppm correlated with (H-15) (1H, s) δ_H 2.01 ppm; (H-15) (1H,s) δ_H 2.01 ppm correlates with (H-16) (2H,m) δ_H 1.32 ppm; and (H-16) (2H,m) δ_H 1.34 ppm correlates with (H-17) (3H,t) δ_H 1.24 ppm.

For further correlation between heteronuclear (^{1}H^{13}C) using HSQC (*Heteronuclear Single Quantum Coherence*) spectroscopy. In this HSQC data can be known proton-carbon with a distance of one bond, characterized by the same spot in the spectrum. For example, a horizontal line drawn from C-6 at δ_C 124.9 will meet the spot, and then drawn vertically from the spot, a proton signal will be obtained at δ_H 7.40 ppm (H-6). The spectrum of HSQC compounds is shown in **Figure 11**.

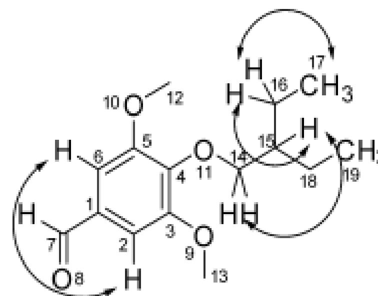


Figure 9. ^{1}H-^{1}H COSY spectra analysis results for the chemical structure of compound **1**.

The results of the last spectrum analysis, namely HMBC (*Heteronuclear Multiple Bond Connectivity*) in **Figure 10**. show the correlation of protons with carbon at a distance of more than one bond. And the spectrum of HMBC compounds is shown in **Figure 12**.

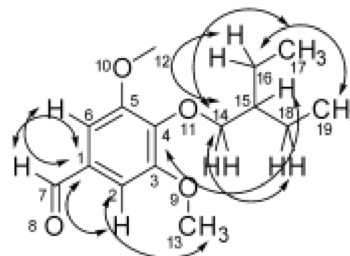


Figure 10. HMBC spectrum analysis results for the chemical structure of compound **1**.

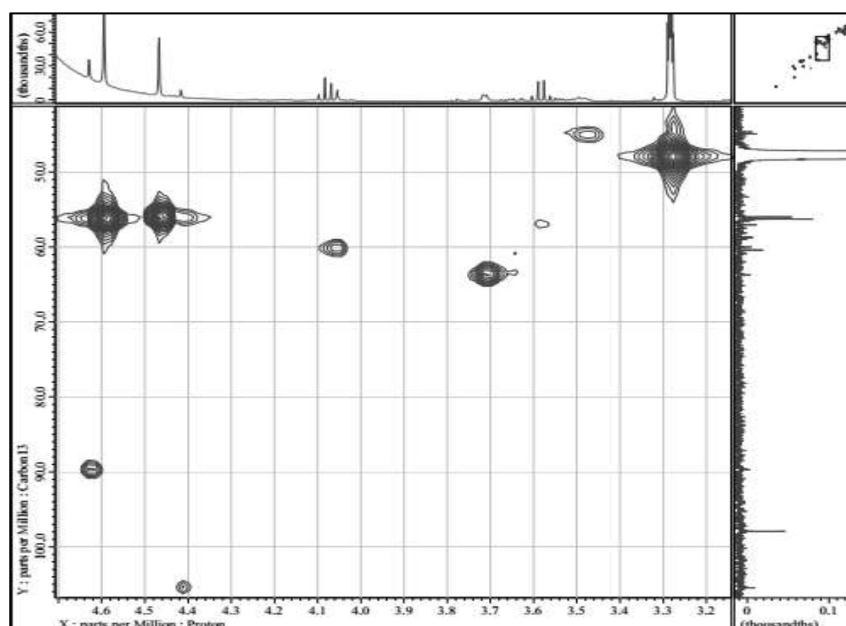


Figure 11. HSQC spectrum of compound **1** at δ_H 4.6 - 3.6 ppm.

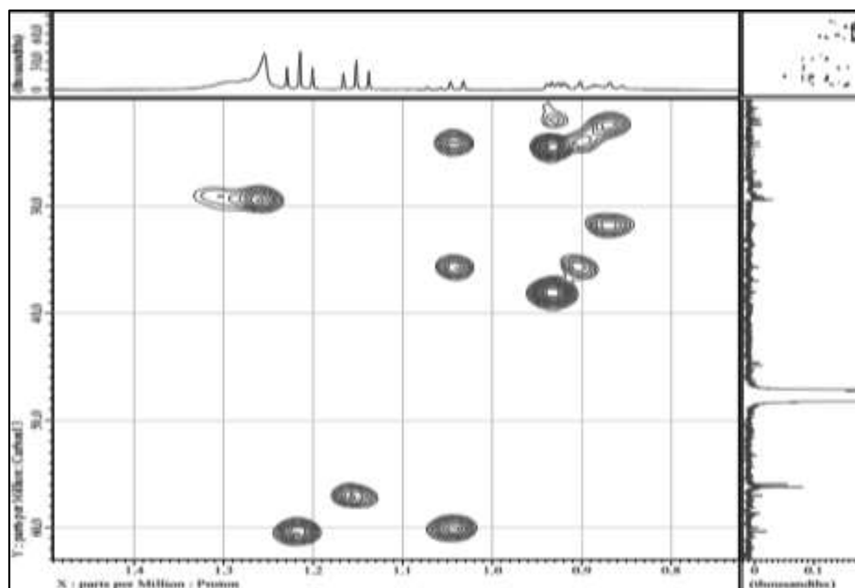


Figure 12. HMBC spectrum of compound **1** at δ_H 0.8 - 1.4 ppm.

Chemical shift data of ^{1}H and ^{13}C-NMR based on 2D-NMR analysis of isolates shown in **Table 1**.

Table 1. Proton and carbon chemical shift data from ^{1}H and ^{13}C-NMR spectra of isolated compounds at 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR, in CD_3OD.

Position C	δ_H (ppm) (ΣH, multisiplitas; J (Hz))	δ_C (ppm) Compound 1
1	-	153,9 C
2	6,68 (d, 1H, J = 3,5 Hz, ArH)	110,4 CH
3	-	129,6 C
4	-	131,2 C
5	-	151,9 C
6	7,40 (d, 1H, J = 3,5 Hz, ArH)	124,9 CH
7	9,54 (s, 1H, CHO)	179,9 CH
8	-	-
9	-	-
10	-	-
11	-	-
12-OMe	3,60 (q, 3H, OMe)	61,79 CH_3
13-OMe	4,61 (q, 3H, OMe)	57,38 CH_3
14	4,10 (s, 2H, OCH_2)	57,65 CH_2
15	2,01 (s, 1H, CH)	29,14 CH
16	1,32 (m, 2H, CH_2)	21,00 CH_2
17	1,24 (t, 3H, CH_3, J = 7 Hz)	14,55 CH_3
18	1,17 (m, 2H, CH_2)	18,43 CH_2
19	0,90 (m, 3H, CH_3, J = 7,5 Hz)	12,65 CH_3

Antioxidant Activity Test

To determine the antioxidant activity of ethyl acetate extract of endophytic fungus *P. citrinum* from *K. millotii* stems and isolates that have been obtained, the DPPH method is used. This is because the DPPH method has the advantage that the analysis is easy, efficient, and requires a fairly fast test time [23]

The IC_{50} value is a parameter used for the concentration of antioxidants needed to inhibit DPPH radicals by 50%. Where the smaller the IC_{50} value, the higher the antioxidant activity [24].

The positive control used is ascorbic acid which has a free hydroxyl group that acts as a free radical catcher and is stable. The results of the antioxidant activity test are shown in **Table 2** below.

Table 2. Antioxidant test results.

Test Solution	Regression Equation	IC_{50} (ppm)
Ascorbic Acid	y = 44,887x - 4,8826 R^2 = 0,997	3,42
Extract ethyl acetate	y = 0,1205x + 2,8115 R^2 = 0,9828	401,01
Pure isolate	y = 22,075x - 69,205 R^2 = 0,9942	221,41

Based on the analysis of the antioxidant activity test of ethyl acetate extract of endophytic fungus *P. citrinum* from the stem of *K. millotii* showed weak antioxidant activity with IC_{50} value of 401.01 ppm compared to antioxidant activity in ascorbic acid which has IC_{50} of 3.42 ppm which is included in the very active category, while the isolate has moderate antioxidant activity with IC_{50} value of 221.41 ppm. This is due to phenol groups substituted by methoxy and carbonyl groups. According to [25], antioxidant activity will decrease with the addition of more carbon groups and high molecular weight.

CONCLUSION

The results of research on the secondary metabolite of the ethyl acetate extract obtained from the *P. citrinum* endophytic fungus in the *K. millotii* strain as an antioxidant show some important findings. The antioxidant activity of the endophytic fungus *P. citrinum* ethyl acetate extract indicated a weak activity category with an IC_{50} of 401.01 ppm. Chemical structural analysis using spectroscopic UV-Vis Spectrophotometer, FT-IR, and 1D-NMR (1H-NMR and 13C-NMR), 2D-NMR (1H-1H COSY, HSQC, and HMBC) Spectrometer, obtained that compound **1** is a compound of 4-(2-ethylbutoxy)-3,5-dimethoxy benzaldehyde. Furthermore, the pure isolate showed increased antioxidant activity compared to its extracts, resulting in an IC_{50} of 221.41 ppm falling into the medium category. This increase is influenced by the presence of phenol groups substituted by methoxy and carbonyl groups in the structure of the compound.

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