

# POTENTIAL ANTIOXIDANTS OF SECONDARY METABOLITE ISOLATES ETHYL ACETATE FRACTION COLEUS AMBOINICUS LOUR. LEAVES

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**Submission date:** 31-Oct-2022 08:29AM (UTC-0700)

**Submission ID:** 1940494673

**File name:** Second\_Revision\_Final.docx (773.35K)

**Word count:** 3882

**Character count:** 23827

## POTENTIAL ANTIOXIDANTS OF SECONDARY METABOLITE ISOLATES ETHYL ACETATE FRACTION *COLEUS AMBOINICUS* LOUR. LEAVES

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*This aims of the study was to isolate and characterize secondary metabolites that have potential as antioxidants from the ethyl acetate fraction of the leaves of Coleus amboinicus, L. (C. amboinicus).*

**Materials and methods.** Purification of the ethyl acetate fraction of *C. amboinicus* using gravity column chromatography with a stationary phase (silica gel, Merck) and a mobile phase with a solvent ratio of *n*-hexane (Merck) and ethyl acetate (Merck). Examination of isolates includes physical (color, shape, and melting point). Qualitative purity detection by TLC at 254 nm and 366 nm wavelengths. Structural analysis of metabolites with UV-Vis spectrometer (Spectronic 3000, Genesis 10, Japan), FT-IR(KBr) (Shimadzu IR Prestige-21, Japan), NMR spectrometer (JEOL spectrometer, Japan) operating at 500 MHz (<sup>1</sup>H-NMR) and 125 MHz (<sup>13</sup>C-NMR), and Shimadzu's GC-MS (QP-2010S Shimadzu, Japan) and determination of potential antioxidant activity using the DPPH method.

**Results.** The secondary metabolite compounds were isolated in the form of yellow crystals with a melting point of 232-233°C, R<sub>f</sub> values of 0.86 and 0.56 which were monitored by TLC at a solvent ratio of *n*-hexane and ethyl acetate 6:4 and 8:2. Spectronic analysis with a UV-Vis Spectrometer showed two electron absorbances, namely a wavelength of 210 nm indicating methanol solvent and 272 nm isolate. The absorbance of functional groups at wave numbers 3379 cm<sup>-1</sup> (-OH; hydroxy), 2931 cm<sup>-1</sup> (-CH; aliphatic), 1735 cm<sup>-1</sup> (-C=O; carbonyl ketone), 1234 cm<sup>-1</sup> (-CO-; methoxy) and 1643 cm<sup>-1</sup> (-C=C-; alkene). GC-MS analysis obtained two absorbance peaks, (1) the first retention time of 6.658 minutes (3.95%) and (2) the second retention time of 9.001 minutes (96.05%). Structural analysis with <sup>1</sup>H&<sup>13</sup>C-NMR showed that 28 types of protons and 22 types of carbon. The antioxidant activity potential test showed an activity value (IC<sub>50</sub>) of 338.54 ppm.

**Conclusion.** The structure of the isolated secondary metabolite compound is 16-acetoxy-7 $\alpha$ -hydroxyroyleanone (syn. 16-acetoxyhorminone) and has potential as an antioxidant.

**Keywords:** Secondary metabolites, *Coleus amboinicus* Lour., DPPH, acetoxyhorminone

## 1. Introduction

Free radicals are atomic or molecular species that have unpaired electrons, thus making the atom in an unstable (highly reactive) state. Free radicals generally occur from reactive oxygen species (ROS) and reactive nitrogen species (RNS). Free radicals will be stable if they get electron donations or donate electrons to form atoms or molecules with paired electrons [1]. Free radicals play a dual role, in small concentrations involved in aerobic and metabolic reactions, while in large amounts they are detrimental to the body [2,3]. Free radical species are very easy to react to achieve stability and cause radical chain reactions [4]. The presence of free radicals in food will cause oxidative reactions that cause food to rot and if high concentrations in the body will trigger degenerative diseases [5]. To inhibit or delay the rate of spoilage of food and maintain health, it is necessary to add antioxidant compounds.

Antioxidants are compounds or molecules that function to deactivate free radicals so that they can extend the lifetime of food and maintain health [6]. Antioxidants based on the source are divided into natural antioxidants and synthetic antioxidants. Antioxidants sourced from natural ingredients are considered safer and in abundant quantities that can replace the use of synthetic antioxidants [7,8]. Synthetic antioxidants are very effective, but the use of high concentrations for a long time will have side effects such as swelling of the liver, affecting the work of enzymes, and causing fatal bleeding in the pleural cavity and pancreas [9,10].

The leaves are called the local name of the plant species *Coleus amboinicus*, Lour (*C. amboinicus*) which contains secondary metabolites that have antioxidant, antimicrobial, antibacterial, antidiabetic, analgesic, and antifungal activities [11]. *C.*

*amboinicus* contains essential oils that have antimicrobial activity [12], antimalarial, and anti-inflammatory [13]. Phytochemical studies that have been carried out by this plant indicate the presence of various flavonoid compounds, monoterpenoids, diiterpenoids, triterpenoids, sesquiterpenoids, phenolics, flavonoids, alcohol, aldehyde esters of essential oils [11]. The content of phenolics and flavonoids that provides activity as an antioxidant [14]. The aim of this study was to isolate and analyze secondary metabolites from the ethyl acetate fraction of *C. amboinicus* leaves and to test their potential activity using the 2,2-diphenyl-1-picrylhydrazil (DPPH) method.

## 2. Planing (methodology) of research

*C. amboinicus* is one of the plants that is widely consumed by the community. Besides its nutritional content it also has the potential as an antioxidant, antimicrobial and etc. *C. amboinicus* leaves were extracted by maceration method with methanol solvent, then partitioned with *n*-hexane, chloroform and ethyl acetate as solvents. The ethyl acetate fraction of *C. amboinicus* leaves was continued in purification to obtain bioactive compounds in a pure state by gravity column chromatography using silica gel as the stationary phase and a combination of *n*-hexane and ethyl acetate as the mobile phase. The isolates obtained were continued in physical analysis and determination of their chemical structure as well as varying the concentration to determine antioxidant activity using the DPPH method (Fig 1).

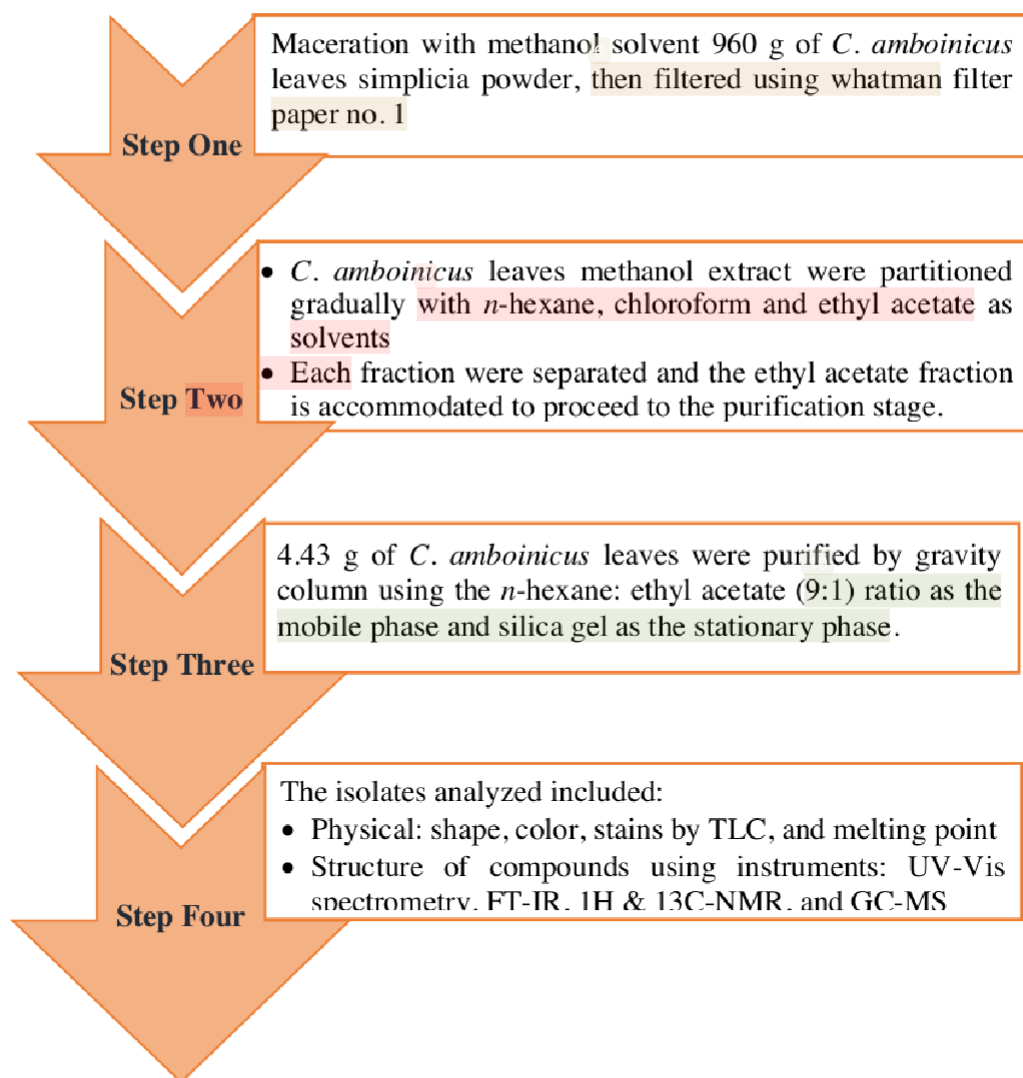


Fig. 1. Isolation scheme secondary metabolite ethyl acetate fraction from *C. amboinicus* leaves

### 3. Materials and Methods

As research materials, the tools used in this study include glassware, analytical balance, column chromatography, TLC chamber, UV lamp 254 & 366 nm, capillary tube, melting point measuring device (electrothermal 9100), UV-Vis spectrometer (Spectronic 3000, Genesis 10 made in Japan), FT-IR<sub>(KBr)</sub> (Shimadzu IR Prestige-21 made in Japan), NMR spectrometer (JEOL spectrometer, Made in Japan) operating at 500 MHz (<sup>1</sup>H-NMR) and 125 MHz (<sup>13</sup>C-NMR), and Shimadzu's GC-MS (QP-2010S

Shimadzu, Japan). Sample fraction of ethyl acetate, methanol (Merck), *n*-hexane (Merck), chloroform (Merck), ethyl acetate (Merck), silica gel TLC plate GF256 (Merck), silica gel 60 kieselgel 0.063-0.0200 mm (Merck), and DPPH (Merck).

*Plant sample preparation*, the ethyl acetate fraction used was extracted using methanol which was then fractionated in stages with *n*-hexane, chloroform, and finally ethyl acetate as solvent. The type of solvent used in the extraction and fractionation pro-analysis process with each step carried out three times. The ethyl acetate fraction was continued in the purification stage using gravity column chromatography. The eluent as the mobile phase was the ratio of *n*-hexane to ethyl acetate, starting with *n*-hexane as a solvent and then increasing the polarity of the solvent by adding ethyl acetate. Increasing the polarity of the solvent by a certain ratio at a volume of 100 mL. The eluate obtained is accommodated with a certain volume and a maximum of 10 mL using a vial. The eluate from column chromatography was evaporated at room temperature so that the isolate was in the form of crystals.

*Identification and analysis of isolates*, the obtained isolates were identified by physical form including the shape and color of the isolate, melting point, purity using thin layer chromatography with *n*-hexane and ethyl acetate as eluents. Structural analysis was performed using UV-Vis spectrometry, FT-IR<sub>(KBr)</sub>, <sup>1</sup>H&<sup>13</sup>C-NMR spectrometer, and GC-MS so that the molecular structure of the isolate can be determined [15].

*Determination of antioxidant activity*, the isolate was dissolved in methanol with various concentrations of 50, 75, 100, 125, and 150 ppm. DPPH was used as a free radical source with a concentration of 0.4 mM. The test was carried out by taking each variation of 250  $\mu$ L concentration into a 5 mL volumetric flask, then adding 1 mL of 0.4 mM DPPH solution and adding methanol to the 5 mL mark. The solution mixture was incubated for 30 minutes, then measured using UV-Vis spectrometry at a maximum wavelength of 515 nm. All measurements were performed with three repetitions [15,16].

#### 4. Research results



### *Purification of the ethyl acetate fraction of *C. amboinicus* leaves*

Purification of the ethyl acetate fraction by gravity column chromatography using eluent ratio of *n*-hexane: ethyl acetate where the eluent polarity is gradually increased. The first eluent was 100 mL of *n*-hexane (non-polar 100%), then the polarity was increased by the ratio of *n*-hexane: ethyl acetate (9:1) eluent and obtained isolates in the form of yellow crystals weighing 50.1 mg.

### *Physical characterization of isolate*

Melting point of the isolate using electrothermal 9100 and obtained a melting point range of 232-233°C. The results were observed using Thin Layer Chromatography (TLC) under UV light at wavelengths of 254 nm and 366 nm ( $R_f$  value 0.56 and 0.86) with an eluent ratio of *n*-hexane: ethyl acetate (8:2 and 6:4) under one color luminescence can be seen in Fig. 2. The solubility test showed that the crystals were completely soluble with methanol, ethyl acetate and chloroform.

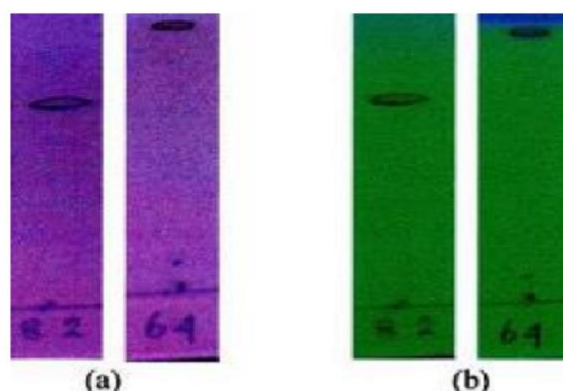


Fig. 2. Crystal analysis results with TLC; (a)  $UV_{\lambda 254 \text{ nm}}$  (b)  $UV_{\lambda 366 \text{ nm}}$

### *Characterization of isolate structure*

Characterization of the structure of the isolates using UV spectrometry. The isolates were dissolved in methanol and analyzed in the UV range (200-400 nm). The results of the analysis showed two absorbance peaks, namely max 210 nm and 272 nm. The absorbance data isolate at max 210 nm showed the absorbance of methanol

(solvent) [15] and max 272 nm showed the absorbance of active isolates. The absorbance at max 272 nm shows the electronic transition from  $n \rightarrow \pi^*$  and at wavelength 270-350 shows the electronic transition from  $n \rightarrow \pi^*$  and  $\pi \rightarrow \pi^*$  [17].

The results of functional group analysis using FT-IR<sub>(KBr)</sub> showed a typical functional group absorbance (Table 1). The results of the GC-MS analysis obtained the first peak with a retention time of 6.658 minutes (3.95%) and the second peak with a retention time of 9.001 minutes (96.05%).

Table 1

Determination of the main functional group absorbance of isolates

Wavenumber (cm <sup>-1</sup> )	Functional group absorbance
3379	-OH hydroxyl
2931	-CH aliphatic
1735	-C=O carbonyl
1234	-O- methoxy
1643	C=C alkene

The results of the structural analysis using <sup>1</sup>H-NMR Fig. 3 and <sup>13</sup>C-NMR are shown in Fig. 4. The results of the interpretation of the chemical shift data for isolates are described in Table 2. Proton analysis with <sup>1</sup>H-NMR showed 28 types of protons and carbon analysis with <sup>13</sup>C-NMR showed 22 types of carbon. The solvent used for proton and carbon analysis is CDCl<sub>3</sub>.



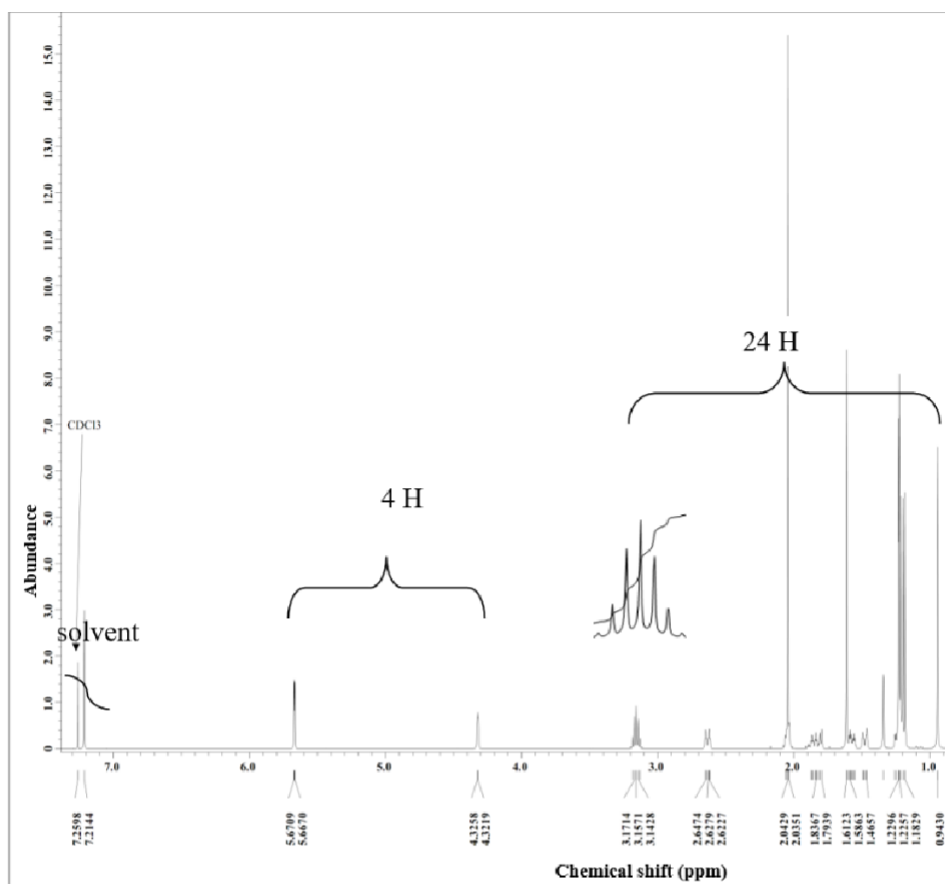


Fig. 3. Proton shift ( $^1\text{H}$ -NMR) isolate

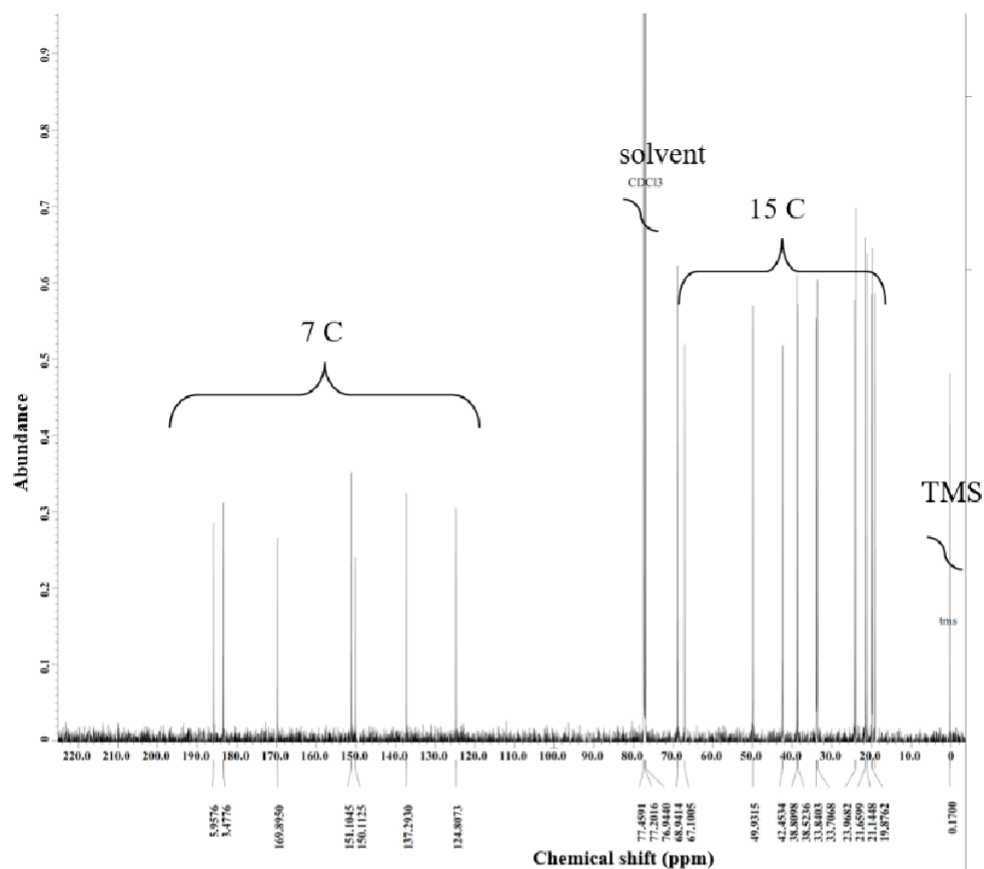


Fig. 4. Carbon shift ( $^{13}\text{C}$ -NMR) isolate

The structural analysis of the correlation of  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR proton chemical shift spectra was described in Table 2, the molecular structure of the analysis results was shown in Fig. 5.

Table 2

Chemical shift data for  $^1\text{H}$  &  $^{13}\text{C}$ -NMR isolate

Carbon Position	Chemical shift	
	$\delta_{\text{C}}$ (ppm)	$\delta_{\text{H}}$ (ppm)
1	38.52	2.61-2.64 <i>tt</i> , ( $^3J_{\text{CH}}$ 5.2 Hz; 4.6 Hz)
2	20.01	1.47-1.50 <i>tt</i> , ( $^3J_{\text{CH}}$ 5.2 Hz; 4.6 Hz)
3	42.45	1.55-1.60 <i>tt</i> ( $^3J_{\text{CH}}$ 5.2 Hz; 4.6 Hz)

4	33.84	
5	49.93	1.79-1.87 <i>ttd</i>
6	21.66	1.61 <i>s</i>
7	67.10	4.32 <i>t</i> ( $J = 2$ Hz)
8	137.29	
9	150.12	
10	38.81	
11	183.48	
12	151.11	
13	124.81	
14	185.96	
15	24.33	3.13-3.19 <i>m</i>
16	68.94	5.67
17	19.15	1.19 <i>d</i> ( $J = 7.15$ )
18	33.71	1.23 <i>d</i> ( $J = 1.95$ )
19	24.33	
20	19.88	0.94 <i>s</i>
1'	169.90	
2'	21.15	2.04 <i>s</i>
	77.5	CDCl <sub>3</sub> (solvent)

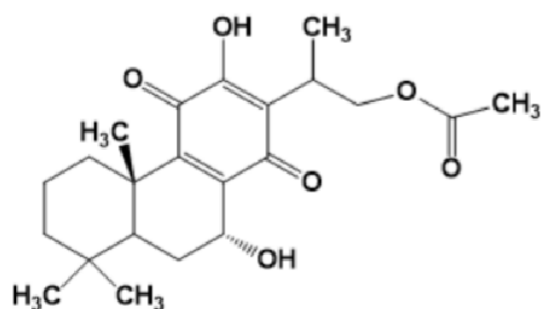


Fig. 5. Structure of 16-acetoxy-7 $\alpha$ -hydroxyroyleanone isolate (syn. 16-acetoxyhorminone)

### *Determination of antioxidant activity*

The antioxidant activity test was carried out using the DPPH method measured at the maximum absorbance of lambda DPPH 515 nm. The antioxidant activity test data is presented in Table 3. The DPPH method was chosen because the processing process is relatively fast, inexpensive and has better sensitivity compared to other methods. The measurement results obtained a linear regression equation, namely:  $y = 0.15440x - 2.27$ ;  $R^2 = 0.9453$  (Fig. 6). The value of the antioxidant activity of the isolates obtained from the measurement results was 338.54 ppm. The antioxidant activity of the isolate was categorized as weak as an antioxidant.

Table 3

Testing the potential antioxidant activity of isolates using the DPPH method at a maximum wavelength of 515 nm

[ ] ppm	Absorbance			%inhibition			Means
	I	II	III	I	II	III	
Blank	0.83	0.83	0.83				
50	0.78	0.78	0.78	5.67	5.67	5.67	5.67
75	0.75	0.75	0.74	10.13	9.77	10.37	10.09
100	0.72	0.73	0.72	12.67	12.42	12.91	12.67
125	0.71	0.71	0.71	14.84	14.84	14.84	14.84
150	0.64	0.64	0.64	22.44	22.56	22.80	22.60

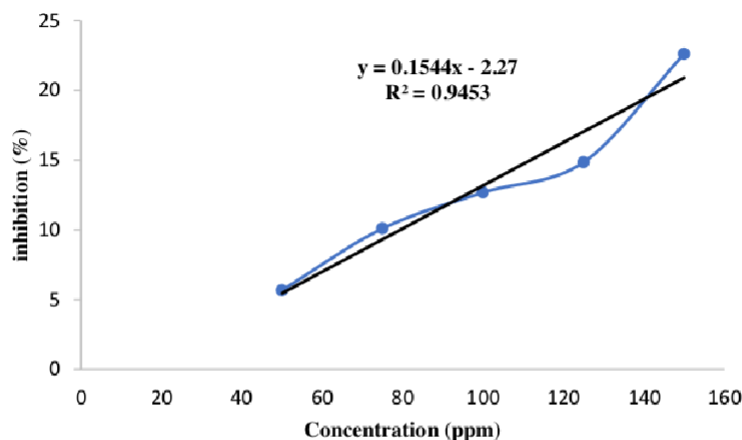


Fig. 6. Curve for determining the antioxidant activity of isolate

## 5. Discussion of research results

Silica gel in purification by reverse phase chromatography is generally used considering the polar active site and the use of relatively low polar eluents. Silica gel with a polar active surface in the presence of a cyanol group (Si-OH), which makes the interaction between bioactive compounds in the fraction and the stationary phase not strong, so that at this ratio the eluent is eluted faster through the stationary phase [18] and has a higher economic value than ordinary. The results of the analysis using TLC under UV light at 254 and 366 nm showed a single Rf point with 0.56 and 0.86, which indicated that the bioactive compounds were qualitatively in a pure state, supported by a melting point value of 232-233°C in a small range.

The results of the isolation of bioactive compounds from the ethyl acetate fraction of *C. amboinicus* leaves using conventional methods using gravity column chromatography were identified with the name 16-acetoxy-7 $\alpha$ -hydroxyroyleanone (syn. 16-acetoxyhorminone) and have potential as an antioxidant. This compound belongs to the diterpene group. Previous researchers reported that diterpene group compounds have potential activity as antibacterial, antitumor, anticancer and other potential [19-21]. Compounds isolated in the future need to be continued in testing various pharmacological potentials, especially in testing potential activities as anticancer, tumor and antibacterial for their use as active drug ingredients.

**Research limitations.** During purification process, the success in obtaining bioactive compounds in a crystalline state is largely determined by the selection of the solvent that we will use as the mobile phase. Determination of antioxidant activity was still very limited to testing with the DPPH method, so testing with a different method is needed.

**Prospects for further research.** The further research to be carried out is determining antioxidant activity with different methods and conducting *in vitro* and *in vivo* tests on the potential of isolates as anticancer in accordance with previous information which reported that the diterpene group of compounds has potential as anti-bacterial, anti-tumor, anti-cancer and other potentials.

## 6. Conclusion

Prior to maceration, the first step must be to ensure that the sample used is free from water and is in powder shapes. The purpose of drying and powdering is to optimize the process of extracting metabolites contained in the sample and to facilitate the process of separating solvents from the extract.

The success of purification of the ethyl acetate fraction of *C. amboinicus* leaves lies in the accuracy of determining the eluent as the mobile phase and filling silica gel as the stationary phase in gravity column chromatography. In addition, the observation of the separation process in the eluate reservoir that passes through the stationary phase with the eluent is also an important part.

The isolated compound obtained is a yellow crystal with a melting point of 232-233°C and the results of chemical structure analysis are isolate 16- acetoxy-7 $\alpha$ -hydroxyroyleanone (syn. 16-acetoxyhorminone) isolate and its potential as an antioxidant. The isolation and purification method of a fraction from natural materials can be used as a reference method for obtaining secondary metabolites.

## Conflicts of interest



The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this paper.

### Financing

The study was financed by the Indonesian government through the Lembaga Pengelola Dana Pendidikan (LPDP) who are willing to be a funding donor for this research.

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