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Isolation of Metyhl- Piperate from n-hexane Extract of Fruit of Cabe Jawa (*Piper retrofractum* Vahl.)

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ABSTRACT

Cabe Jawa (*Piper retrofractum* Vahl.) is categorised in Piper's genus under family piperaceae. It is commonly reported to function as antimicrobial, antifungal and anti-inflammatory. The aim of this study is to obtain information on the isolation of secondary metabolites from n-hexane extract of Cabe Jawa fruits. The isolation stage began with the maceration method with n-hexane solvent, then continued with purification stage of fractination compounds which was conducted by using several chromatography techniques including thin layer chromatography (TLC), vacuum liquid chromatography (VLC), and radial chromatography. Methyl piperate compound was isolated from this extract. The structure of this compound was determined using spectroscopic ^{1}H NMR. Based on the results of spectroscopic, analysis of ^{1}H NMR was present at a chemical shift of 3.80 ppm singlet peak with integrity of 3H was the typical peak of a methoxy. At 6.12 ppm chemical shift indicated a signal peak for protons bound to C sp^2. Chemical shift at 6.96 ppm to 7.13 ppm for proton groups was attached to benzene ring. From the results of ^{1}H NMR spectrum assisted by biogenesis approach, it is concluded that the purified compound successfully isolated was a methyl piperate compound.

Keywords : Chromatography, methyl piperate, n-hexane extract, piper retrofractum

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INTRODUCTION

Indonesia is a tropical country which has various types of plants, empirically known to be potential phytopharmaca drug. Thus, these can be utilised as raw materials for the pharmaceutical industry, cosmetics and traditional medicine.

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Plant parts can be utilised as a source for obtaining secondary metabolite compounds. Secondary metabolite compounds are from groups of alkaloids, flavanoids, steroids and terpenoids. Plants are able to change a wide range of chemical compounds that have a variety of interesting bioactivity and this capability is also defined as a mechanism of self-defense against environmental threats. In this case the plant can produce chemical compounds that are pesticides, insecticides, antifungal or cytotoxic (Hernawan & Setyawan, 2003).

There are more than 700 species of genus *piper* of worldwide distribution. Species in this genus have high commercial and medicinal importance (Parmar et al., 1997). Phytochemical investigations of piperine show active physiologic compounds, including alkaloids, flavones, dihydrocaine, kawapyron, lignans, neolignan, profenilphenol, and terpenoids (Kubo et al., 2013). Plants of the genus *Piper*, such as *Piper nigrum, Piper methysticum, Piper auritum* and *Piper betle* have been known for a long time as agricultural commodities for herbs and medicines with high economic value. One of the plants belonging to the genus *piper* is *Piper retrofractum* Vahl. which is known as Cabe Jawa, used as one of Indonesia's traditional medicine ingredients in a mixture of herbs. Several studies have reported traditional herbs using Cabe Jawa as one of their formulations to have have very low bacterial contamination levels. This is due to the antibacterial and antifungal properties of Cabe Jawa. Traditionally, this plant is believed especially by Indonesians, to be able to treat asthma, bronchitis, hemorrhoids, fever, and abdominal pain as well as has stimulant effects on nerve cells that can increase body stamina. A literature survey informed that a number of biological studies have been carried out on this plant extract such as its antioxidant, anti-fungal, cytotoxic and α-glucosidase inhibitory activity (Banerji, et al., 1985; Banerji, Sarkar, Datta, Sengupta, & Abraham, 2002; Chansang, et al., 2005; Jong-Woong, et al.,1992; Luyen, et al., 2014; Muharini, Liu, Lin, & Proksch, 2015; Muharini, Liu, Wenhan, & Proksch, 2015).

Each plant has its own character in the growing environment where the plant is located. Thus, the differences in environmental conditions allow the emergence of certain characteristics, such as differences in plant morphology or different components of the compounds contained in the plant.

This paper focuses on isolation of methyl-piperate from n-hexane extract of fruit of *P. retrofractum*, and describes the isolation and structure elucidation of methyl-piperate. The discovery of these compounds is actually the first time for these species.

MATERIALS AND METHODS

The method of research conducted included several stages such as extraction, separation and purification, as well as the characterisation of compounds by ^{1}H NMR spectroscopy method.

General

^{1}H NMR spectra were recorded with a AGILENT 500 MHz operating at 500 (1H) MHz, using residual (δH 7.26) and deuterated solvent (δC 77.1) peaks of chloroform-*d* as reference standards. VLC (vacuum liquid chromatography) was carried out using Merck silica gel 60 GF254; for TLC analysis, pre-coated silica gel plates (Merck Kieselgel 60 GF_{254}, 0.25 mm thickness) were used. Solvents used for extraction and preparative chromatography were of technical grade and distilled before use.

Plant Material

Fruit samples of Cabe Jawa were collected from Lembang, West Java, Indonesia in January, 2017. The plant was identified by the staff members at Herbarium Bagoriense, LIPI- Bogor Plantation Cansevation Center, and the voucher specimen deposited at the herbarium.

Extraction and Isolation

The dried fruit of Cabe Jawa (500 g) was macerated using n-hexane solvent. The maceration extract was filtered using a buchner funnel and the filtrate was concentrated using a vacum rotatory evaporator.

Separation and Purification

The separation and purification steps of compounds in this study were conducted through two stages of liquid vacuum chromatography (VLC) and radial chromatography. About 15 g of total hexane extract in VLC (n-hexane-ethyl acetate = 9:1 until 0:10) obtained the combined fractions, then separated by chromatotron.

Characterisation

The process of characterising compounds with NMR 1H spectroscopy was performed at the Bandung Institute of Technology, Indonesia.

RESULTS AND DISCUSSION

The extraction process of Cabe Jawa with n-hexane solvent obtained 21.5 g of n-hexane extract. Separation and purification was done by vacuum liquid chromatography (VLC) method. Firstly, it analysed using thin layer chromatography (TLC). This analysis aimed to determine the solvent to be used at the time of separation with VLC. The chromatogram pattern in TLC shows the separation pattern that occurs in VLC.

The first separation was carried out using VLC, the solvent used was an organic solvent which increased its gradient polarity. In this separation, n-hexane and ethyl acetate solvents were used. Based on chromatogram analysis of TLC, eluent hexane and ethyl acetate were used with several compositions of 100% n-hexane (3 elution), n-hexane and ethyl acetate 9: 1 (3 elution), 8: 2 (4 elution), 7: 3 (2 elution), 1: 1 (2 elution), 100% ethyl acetate (1 elution) and methanol 1 elution with 100 ml volume at each elution. From the VLC results, 15 fractions were obtained.

Fractions having the same chromatogram patterns are combined to achieve four combined fractions. The mass of each

fraction is fraction A (1-5) obtained as much as 9.8 g, fraction B (6-9) as much as 3.4 g, fraction of C (10-11) as much as 0.4 g, and fraction D (12-15) as much as 2.7 g. The targeted compound is a nonpolar compound with good separation, so further separation is taken from Fractions A and B. The fractions in VLC return to separate the compounds contained in the fraction.

The second process VLC was done the same way as the previous one, so that 15 fractions were obtained. Based on chromatogram analysis of merging VLC fractions based on similar pattern of stain according to polarity level, three main fractions were obtained among the fractions of A2, as much as 4.6 g, B2 fraction as much as 1.9 g and C2 fraction as much as 1.4 g. The combined fractions were analysed by TLC using 100% dichloromethane eluent. The mass and chromatogram patterns of each fraction were taken into consideration to determine which fractions would be further separated against the C2 fraction which was further purified by using radial chromatography (chromatotron). The result of chromatotron to fraction C2 was obtained by pure compound whose purity with TLC obtained as much as 25.8 mg. The isolated compounds were then analysed using 1H NMR spectroscopy.

Determination of the structure of isolated compounds was analysed using 1H (proton) NMR spectroscopy. Spectroscopic analysis was performed to obtain an idea of the various types of hydrogen atoms present in the isolated compounds. The proton NMR spectrum provides information on the chemical environment of the H atom, the number of H atoms in each environment and the cluster structure adjacent to each H atom. The 1H NMR spectra of isolated compound is shown in Figure 1.

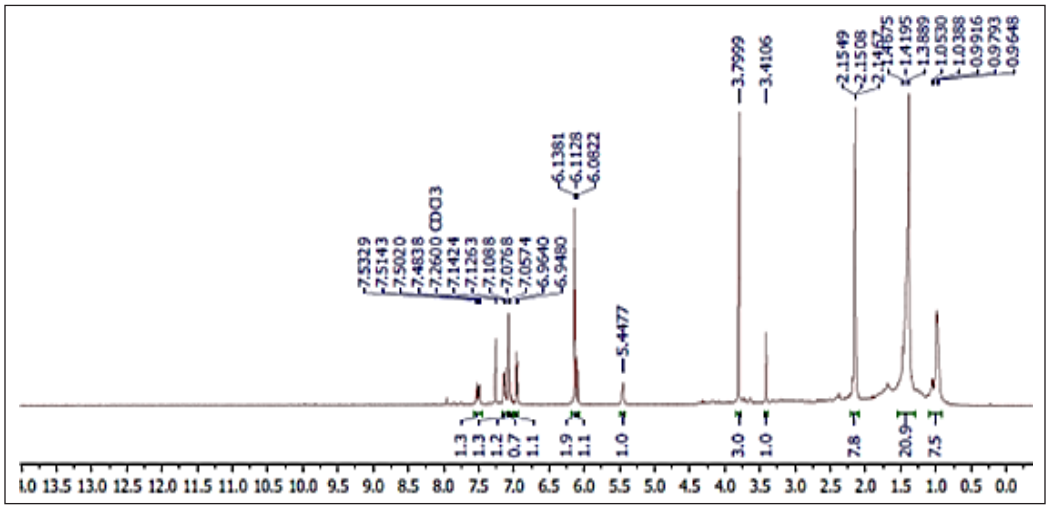

Figure 1. The ^{1}H NMR spectra of isolated compound

Based on the proton NMR spectrum, the number of protons identified was 12 protons. Chemical shift of 3.80 ppm singlet peak with 3H integrity is the typical peak of a methoxy. At 6.12 ppm chemical shift indicates a signal peak for protons bound to C sp^2. The chemical shift at 6.96 ppm to 7.13 ppm is a chemical shift for proton groups attached to the benzene ring. All ^{1}H NMR data of isolated compounds and comparison with the standard of methyls piperate can be found in Table 1.

Table 1
Data ^{1}H NMR spectra methyl piperate

No C	δ_H isolated compound (Multiplisitas, J, Integrasi) (ppm)	δ_H metil-piperat (Multiplisitas, J, Integrasi) (ppm)
1	-	-
2	6,08(d, J=15,3Hz;1H)	5,95 (d,J=15,2 Hz;1H)
3	7,51 (dd, J=6,15,J=4,9;1H)	7,36 (dd, J=15,25, J=11Hz, 1 H)
4	7,02 (dd, J=16; J=11 Hz, 1H)	6,67 (dd, J=15,3, J=10,95Hz; 1H)
5	7,07 (d, J=9,7;1H)	6,76 (d, J=15,4 Hz;1H)
6	-	-
7	7,13 (d, J=9,7;1H)	6,87 (d, J=8,05Hz;1H)
8	6,96 (d, J=8,1H)	6,74 (d,J=8 Hz; 1H)
9	-	-
10	-	-
11	7,11 (s, 1H)	6,95 (s,1H)
$-OCH_3$	3,80 (s, 3H)	3,72 (s, 3H)
$-OCH_2-O$	6,13 (d, J= 12,65 Hz,2H)	5,94 (d, J=1,8;2H)

In the proton NMR spectrum analysis, there is a chemical shift similar to the methyl piperate compound (compound contained in the genus piper), so further analysis compares the value of NMR proton chemistry of the isolated compound with standard NMR proton methyl piperate. Based on the results of the proton NMR spectrum comparison, there is a similar shear peak to indicate the isolated compound is a methyl piperate compound. The integrity of the number of protons is equal to the number of standard methyl piperate protons of 12 protons. Compared to the conclusion that the compound successfully isolated was a methyl piperate compound shown in Figure 2.

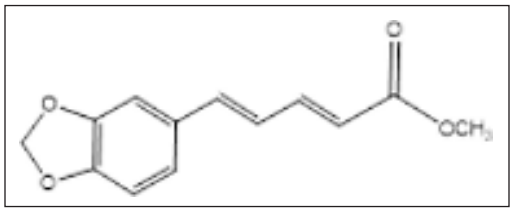

Figure 2. Structure of methyl piperate compound

Characterisation of compounds other than using NMR analysis showed there is also traced biogenesis approach. The biogenesis approach aims to analyse structures that become precursors in the main framework of methyl piperate compounds.

The formation of methyl piperate starts from the precursor L-tyrosine which produces 4-cumaric acid through the enzyme tyrosine ammonia liase (TAL). The reduction process is catalysed by enzymes with high substrate specificity and NADPH. The substitution pattern with meta-methoxy and para-hydroxyl cyclisation on the sinamoil backbone by extension of acetyl-CoA or malonyl CoA chain via Claisen reaction yields keto-ester. The keto-ester subsequently was reduced by NADPH, followed by dehydration for piperoil-CoA and subsequently methylated to give a methyl piperate compound (Dewick, 2002).

CONCLUSION

Methyl piperate was successfully isolated and identified from n-hexane fraction of *Piper retrofractum*. Spectroscopic analysis of 1H NMR showed a chemical shift of 3.80 ppm singlet peak with integrity of 3H confirming the typical peak of a methoxy. At 6.12 ppm, chemical shift indicates a signal peak for protons bound to C sp^2. The chemical shift at 6.96 ppm to 7.13 ppm is a chemical shift for proton groups attached to the benzene ring. From the results of NMR 1H spectrum and assisted by biogenesis approach, it is concluded that the purified compound successfully isolated was a methyl piperate compound.

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