Trad. Med. J., September-December 2019
Vol. 24(3), p 204-209
ISSN-p : 1410-5918 ISSN-e : 2406-9086

Submitted : 26-07-2019
Revised : 09-11-2019
Accepted : 20-11-2019

Isolation and Identification of DPPH Radical (2,2-diphenyl-1-pikrylhidrazyl) Scavenging Active Compound in Ethyl acetat fraction of *Piper acre* Blume

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ABSTRACT

Piper acre Blume, known as Black Betel (local name), is a plant that is widely used by the people of East Kalimantan, especially in Samarinda, for the treatment of illness. Leaves (3-4 months old) are collected from Samarinda, extracted, fractionated, and monitored by DPPH antiradical activity. The isolation of the *Piper acre* Blume is performed on the active fraction, and the structure identification is based on spectroscopic data of the compound. The leaves were dried, pulverized, and macerated with MeOH. Dried MeOH extract was obtained upon evaporation of the solvent. The extract was then fractionated by vacuum liquid chromatography (vlc), eluted gradually by solvents having different polarities (n-hexane, ethyl acetate and methanol). The fractions obtained were monitored using TLC [n-hexane: ethyl acetate (3: 1 v/v)] that was visualized by $UV_{254\ nm}$, $UV_{366\ nm}$ and DPPH. The isolation was performed by preparative TLC [SiO_2, n-hexane: ethyl acetate (3: 1)] on ethyl acetate fraction that showed the highest DPPH antiradical value. A single compound was obtained, and it appeared as a round spot and pure according to TLC performances at 3 different solvent systems. The isolated *Piper acre* Blume compound displayed the IC_{50} value on the anti-radical DPPH (measured at λ 520 nm) as 10.41µg/mL. The IR spectrum (KBr) showed –OH band (3450 cm^{-1}), aliphatic bands [alkene, 3010 cm^{-1}; alkana 2900 cm^{-1}), an aromatic overtone bands (1900-200 cm^{-1}) and a strong C=O band (1725 cm^{-1}). The NMR (^1H- and ^{13}C-) (mono and 2D) indicated the present of a *p*-di-substituted aromatic signals (δ, 7.54 and 7.52, *d*, J =6 Hz, 1 H each), 2 methyl (δ, 0.96, *d*, J = 7.0 Hz, 6 Hs), a triplet signal (δ, 4.22 ppm). Other signals of CH- and CH_2 were shown as *m* signals at δ, 1.64 and 1.34 ppm. Based on those data, the compound was identified as isoamyl *p*-OH benzoate that is grouped as parabens used as a preservative in the pharmaceutical preparations. In conclusion, the anti-radical (DPPH) active compound present in the leaves of *Piper acre* Blume is identified as isoamyl *p*-OH benzoate, having IC50 value anti-radical DPPH 10,41µg/mL.

Keywords: anti-radical; DPPH; ethyl acetate; isoamyl *p*-OH benzoate; *Piper acre* Blume

INTRODUCTION

Piper acre Blume, a local name black betel found in Malinau district, North Kalimantan province, is used by local residents to maintain health condition. Therefore, black betel in local markets for sale at high prices and may be developed as a treatment medicinal purposes. Several studies on black betel have carried out, ethanol extract of black betel leaf are active as antioxidant having IC_{50} at 28.98 ppm (Rija'i *et al*., 2015a), while its ethyl acetate extract shows a little bit less IC_{50} value as 25.01 ppm (Rija'i *et al*., 2015b). Antioxidant value of dry leaf decoction showed IC_{50} of 28.98 ppm, fresh leaf decoction showed IC_{50} value slightly higher at 44.04 ppm, steeping of dry leaves displaying IC_{50} value of 31.54 ppm and fresh leaf steeping showing IC_{50} value of 55 ppm. (Rija'i *et al*., 2015a).

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Free radicals are atoms that have one or more unpaired electrons that makes the atomic molecule very reactive. The formation of free radicals occurs due to three stages including the stages of initiation, propagation and termination (Fessenden and Fessenden, 1994). Radicals are also chain reactions that occur in the body that can cause continuous damage, so free radical attacks occur mainly in normal cell metabolism of the endogenous defense system. The amount of free radicals can increase due to stress, radiation, cigarette smoke and environmental damage that causes the immune system, so the body needs additional antioxidants from outside that can protect against free radical attacks (Wahdaningsih *et al*., 2011).

Anti radical used today consists of 2 compounds namely DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS (2,2'-azino-bis-[3-ethylbenzothiazolin sulphonate]). Free radicals that are commonly used as models in the study of antioxidants or free radicals are 1,1-diphenyl-2-

picrylhydrazyl (DPPH) as the DPPH method is a simple, fast, and easy method for screening of anti-radical properties of several compounds. In addition this method is evident accurate, reliable and very practical (Molyneux, 2004).

METHODOLOGY

Materials and Equipments

Piper acre Blume obtained from a cultivation on the Nusa Indah street, Samarinda, East Kalimantan. The chemicals used were methanol, n-hexane, ethyl acetate, chloroform, acetone. Thin layer chromatography (Silica gel F_{254}) plates (E Merck), 1,1-diphenyl-2-picrylhydrazyl (DPPH) from Sigma Chem Co, anisaldehide. The tools used for extraction, fractionation and isolation are vessels, Buchner funnels, magnetic stirrers, erlenmeyer flasks, glass plates, sintered glass, pipettes, capillary pipes and glass columns.

Extraction and Fractionation

Piper acre Blume was macerated with methanol for 3 x 24 hours at room temperature. Filtration was done by a Buchner funnel and then the maceration was repeated twice. The filtrate obtained was combined and evaporated to produce a fractionated methanol extract with VLC (vacuum liquid chromatography) with solvents at different polarity such as n-hexane, ethyl acetate, and methanol. Upon evaporation of those fractions, dried n-hexane, ethyl acetate and methanol fractions were obtained respectively, Fraction ethyl acetate turned out to be the most anti radical active fraction among those other two fractions. Then the ethyl acetate fraction is further fractionated by column chromatography to simplify the fraction composition and then isolation of the active anti-radical DPPH compound with preparative TLC.

Column Chromatography

Before column chromatography was carried out, thin layer chromatography of the fractions obtained included n-hexane (9.3 g), ethyl acetate (7.3 g) and methanol (7 g). All extracts and fractions were analyzed by thin layer chromatography using n-hexane : ethyl acetate (3 : 1 v/v), the tlc pictures were observed by $UV_{254\ nm}$, $UV_{366\ nm}$ followed by visualization upon anisaldehyde reagent to see compounds that are undetected by UV light used. Column chromatography was carried out on ethyl acetate fraction. Ethyl acetate fraction (5 g) was diluted with small amount of chloroform and then dried up using small amount of silica gel 60 F_{254}. The column used was initially prepared with silica gel mixed with n-hexane to form like slurry, then put in a column and let stand overnight to precipitate the column. The powder fraction was placed on top of the silica and eluted with n-hexane: ethyl acetate (3:1 v/v) as the mobile phase. Fractions obtained were monitored by TLC with the similar mobile phase, and fractions showing similar tlc pictures were combined. As usual, visualization was performed under $UV_{254\ nm}$, $UV_{366\ nm}$ lights, anisaldehyde and also 0.2% DPPH solution.

Thin Layer Chromatography- Preparative

The active fraction that has DPPH anti-radical compound was then isolated using preparative TLC with a silent phased of silica gel F_{254} made with a thickness of 1 mm. Preparative plates were made by mixing 40 grams of silica gel F_{254} with 80 ml of distilled water in a covered flask (150 ml), shaken for 2 minutes, then sprinkled on a 20 x 20 cm glass plate, then silica plates were left to dried at room temperature and activated in the oven at a temperature set at 105°C for 60 minutes before use. Dry dishes were taken from the oven, leaving them at room temperature and ready for used. Dry plates were taken from the oven, let them stay at room temperature and they were ready for use. The solution of ethyl acetate fraction is spotted in line horizontally on the preparative tlc, and put into a tlc chamber containing a set up mobile phased. After development is completed, the plates were removed and leave them at room temperature till the rest of mobile phase evaporate from the plates. Locating the interest compounds was done by visualizing under $UV_{254\ nm}$ light and also spraying with suitable reagent on a small left side by covering the large side with a glass then the heating up with hair spray. The intended compound was then scrapped out, combined with those of the other plates, and extracted with a mixture of chloroform-methanol at equal amount. Upon filtration with sintered glass funnel, the filtrate obtained was evaporated to dryness giving residue of the intended compound (**1**).

Purity test

The **1** was dissolved with chloroform and then spotted on silica gel F_{254} plate, then the plate was developed in a chamber containing mobile phase. There were several mobile phases used in the purity checking by tlc, these were acetone 100%, acetone : n-hexane (10:1 v/v) and ethyl acetate 100%. As usual, visualization was under $UV_{254\ nm}$, $UV_{366\ nm}$, and anisaldehyde reagent.

Table I. DPPH Radical Scavenging Activity of **1** (IC_{50})

Concetration (µg/mL)	Absorbance	% Inhibition	IC50
1	0,788	34,67	
2	0,746	38,14	
4	0,744	38,30	10,41 µg/mL
8	0,606	49,75	
16	0,516	57,21	
Blanc		1.20	

Qualitative analysis of DPPH anti radical compounds

This test was carried out on isolates on the TLC plate sprayed with 0.2% DPPH solution in methanol. Chromatogram is examined 30 minutes after spraying. Active anti radical compounds will show yellowish-white spots with a purple background.

Quantitative analysis of DPPH anti radical compounds

The **1** obtained from the preparative TLC was tested for activity by diluting **1** in chloroform at concentrations of 1-16 µg/mL. Each concentration of DPPH 50 mM solution was added in chloroform and left at room temperature for 30 minutes. After being left at room temperature for 30 minutes, the remaining DPPH was measured spectroscophically at a wavelength of 520 nm. A blank (DPPH solution which does not contain test material) was also measured. The DPPH radical capture activity (%) is calculated by the following formula:

$$\% \text{ Inhibition} = \frac{Abs\ control - Abs\ Sample}{Abs\ control} \text{ x } 100\%$$

Data on anti radical activity DPPH of **1** and quercetin as positive control were analyzed, each of IC_{50} values were calculated through probit analysis. The IC_{50} is a concentration that inhibits 50% DPPH. The test was done in triplicates.

RESULTS AND DISCUSSION

Extraction, fractionation, isolation, identification and activity of DPPH anti-radical compounds from black betel leaf have been carried out. Extraction was carried out by maceration three times 24 hours at room temperature using methanol 3000, 2500 and 2000 ml. Filtration was carried out using a Buchner funnel and the residue was remasted twice in the same procedure. The filtrate was combined and evaporated with a rotary evaporator until a thick extract. Then fractionation was carried out with three different solvents of polarity in sequence to give n-hexane fraction (9.3 g), ethyl acetate fraction (7.3 g) and methanol (7 g). All of the following fractions with extracts were carried out TLC with eluent n-hexane:ethyl acetate (3:1 v/v) and then sprayed with anisaldehyde reagent.

Isolation process was carried out on ethyl acetate fraction by column chromatography and preparative TLC. Silica gel F_{254} was used on column chromatography by comparison with fractions (1:25). From the isolation results obtained 51 fractions, observed on TLC profiles with the same fraction. Eight fractions of combined fractions were obtained there are fr I (F.1-4), fr II (F.4-11), fr III (F.12-16), fr IV (F.17-23), fr V (F .24-28), fr VI (F.29-35), fr VII (F.36-44), and fr VIII (F.45-51). Each fraction was observed TLC profile in the mobile phased of n-hexane: ethyl acetate (20: 1 v / v) then visualized at UV_{254} nm, UV_{366} nm, sprayed with anisaldehyde and 0.2% DPPH solution. Positive results were shown in fraction I which had DPPH anti-radical compound activity which was then carried out preparative groups to obtain isolates. One spot shown in each of the three TLC solvent systems indicated that the compounds obtained (**1**) was pure with TLC.

Anti radical compound activity test using UV-Vis spectrophotometer was measured by DPPH (50 mM solution) at λ 520 nm, performed on a 3 ml solution of isolates with concentrations of 1, 2, 4, 8 and 16 µg/mL mixed with 1 mL of DPPH. The presence of anti-free radical compounds was expressed by the value of percentage of inhibition concentration (IC_{50}), which means that 50% of DPPH free radical inhibition on the solution concentration of isolates. The test results showed the IC_{50} value **1** was 10.41 µg/mL (Table I; Figure 1).

The IR spectrum of **1** (KBr) (Figure 2) showed –OH band (3450 cm^{-1}), aliphatic bands [alkene, 3010 cm^{-1}; alkane 2900 cm^{-1}), an aromatic overtone bands (1900-2000 cm^{-1}) and a strong C=O band (1725 cm^{-1}). Mass spectrum of **1** was not quite informative, but other information related to this mass spectrum was able to obtain from other

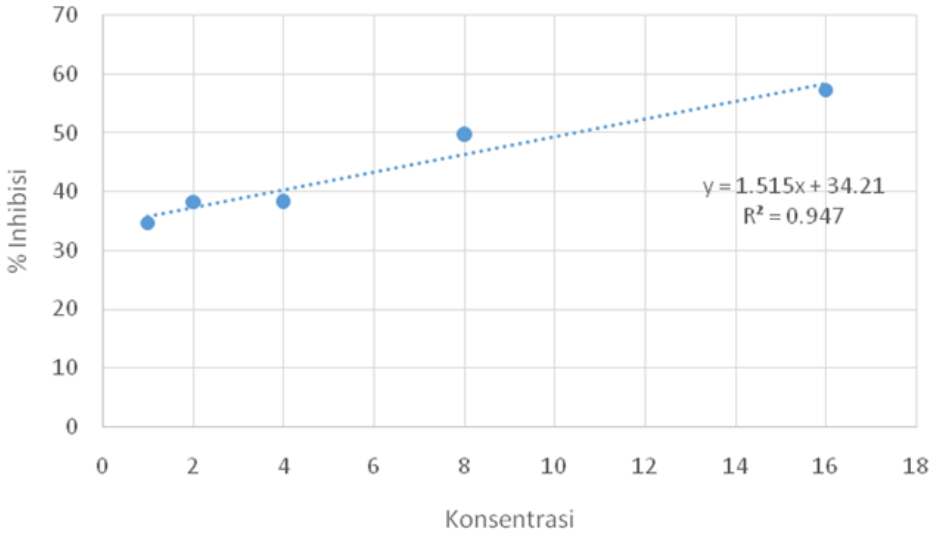

Figure 1. DPPH Radical Scaveging of **1**

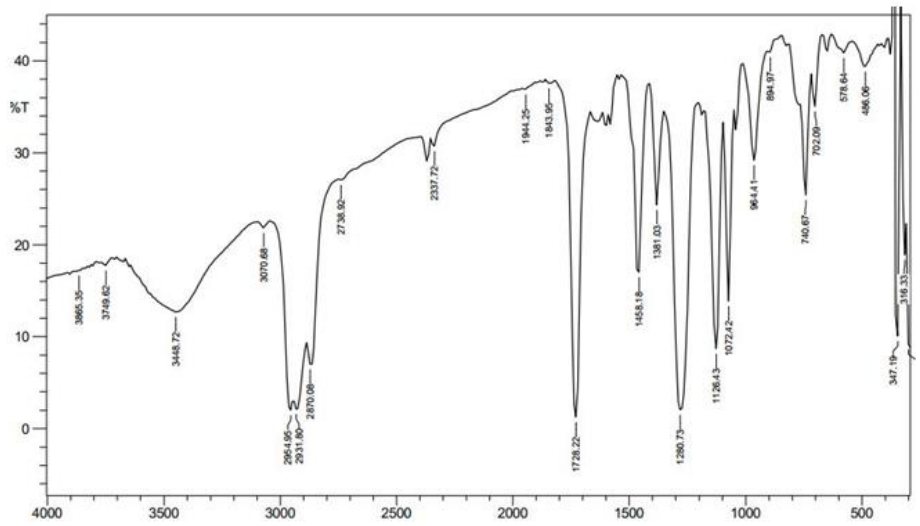

Figure 2. Infrared spectrum of **1** (KBr)

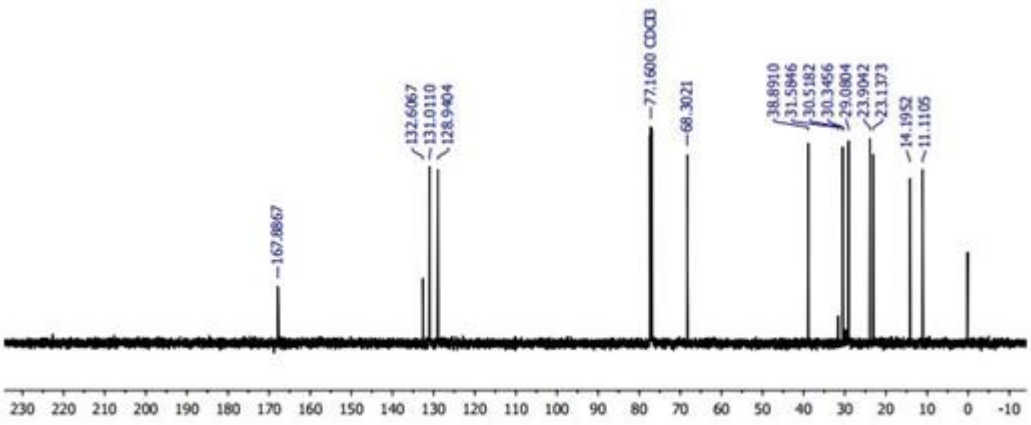

Figure 3. ^{13}C- NMR (500 MHz, $CDCl_3$, δ) spectra of **1**.

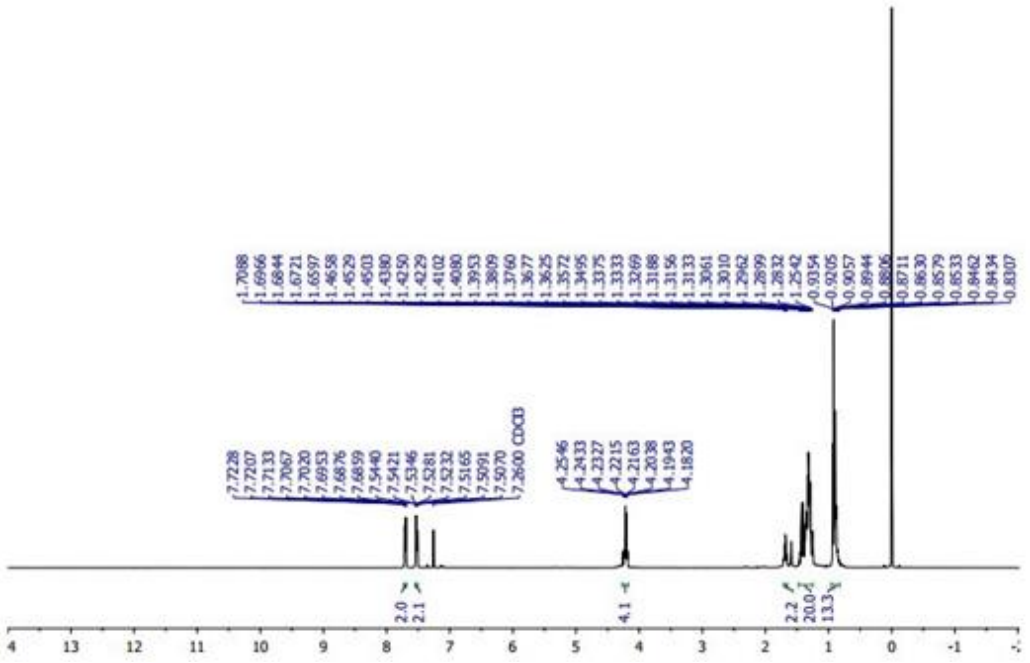

Figure 4. ^{1}H- NMR (500 MHz, $CDCl_3$, δ) of **1**

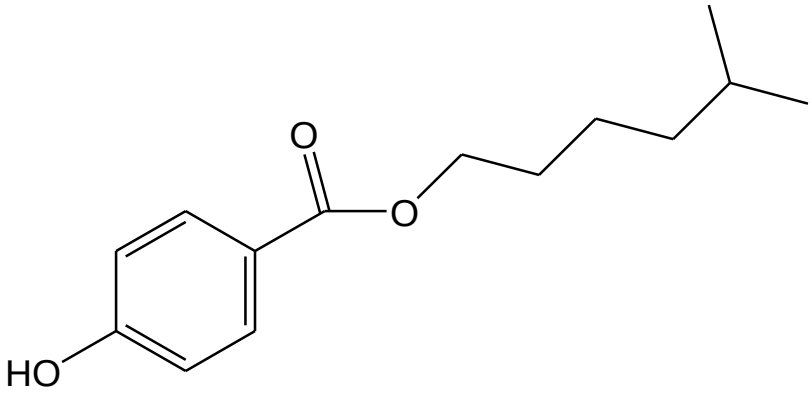

Figure 5. Structure of *p*-OH isoamyl benzoate (**1**)

data such as the number and character of carbons (^{13}C), number and character of protons (^{1}H-). The ^{13}C- NMR spectrum of **1** (500 MHz, $CDCl_3$) showed the presence of 14 carbon atoms (Figure 3), 1 Carbon was identified as a C=O (δ, 168 ppm), 6 carbons were identified as aromatic carbons in which two of them were substituted aromatic carbons (δ, 128.9 and 132.6 ppm). The 7 other carbons were identified as saturated alkane carbons, in which 1 of them was clearly an oxygenated carbon (δ, 68.3 ppm), 3 of homolog CH_2 (Figure 4), and a gem dimethyl signals. The ^{1}H-NMR of **1** (500 MHz, $CDCl_3$) (Figure 4) indicated the present of proton signals specific of a *p*-di-substituted aromatic signals (δ, 7.54 and 7.52, *d*, J =6 Hz, 1 H each), 2 methyl (δ, 0.96, *d*, J = 7.0 Hz, 6 Hs), a triplet signal (δ, 4.22 ppm, J=7 Hz).

Based on the data above then **1** was identified as *p*-OH isoamyl benzoate (Figure 5). This compound is an analog or derivative of *p*-OH benzoic acid that is commonly grouped of parabens and used excessively as preservative in the food production. The present of isoheptanol or the alcohol portion of this ester in plant is quite interesting, it could be a subject of further study.

CONCLUSION

The anti radical DPPH compound from isolation and identification of *Piper acre* Blume obtained isoamyl *p*-OH benzoate with IC_{50} value of 10.41 µg/ml.

ACKNOWLEDGMENTS

Thank you to the Faculty of Pharmacy Gadjah Mada University and Mulawarman University for funding this research.

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