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Isolation of flavonoid compound from ethyl acetate extract of fingerroot (*Boesenbergia Pandurata* (Roxb.) Schlechter) rhizome.

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ABSTRACT

Boesenbergia pandurata (Roxb.) Schlechter or fingerroot is a popular plant in Indonesia and has been used as one of ingredients of traditional *jamu* since long time ago. This plant is rich of flavonoid content. The isolation process was performed to obtain the flavonoid compound from fingerroot rhizome using extraction and purification method. A flavonoid compound was found as abundant in ethyl acetate fraction. It was precipitated after concentrating the extract so it was easily isolated with the simple process. Structure identification and analysis of flavonoid was performed by 2 dimension paper chromatography, spectrophotometer UV-Visible with shift reagents NaOH, NaOAc, NaOAc/H₃BO₃, AlCl₃, and AlCl₃/HCl, and NMR spectrometer (¹H, ¹³C, 2D-HSQC, and 2D-HMBC). Based on spectroscopic data and compared with published data, it was concluded that the isolated flavonoid was 5-methoxy-7-hydroxy flavanone or alpinetin

Keywords: *Boesenbergia pandurata* (Roxb.) Schlechter, flavonoid, temu kunci, alpinetin, jamu,

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INTRODUCTION

Boesenbergia pandurata (Roxb.) Schlechter or fingerroot (Fig. 1) is a plant belonging to Zingiberaceae family which is rich of flavonoid content. This plant has been used traditionally to treat several diseases. In Indonesia, the plant is known as *temu kunci* and a popular ingredient of Indonesian traditional medicines products so called *jamu*. It has been also used particularly as a spice for food purposes. Many products of *jamu* are often containing this plant as one of ingredient. This plant is also know with other botanical name as synonym such as *Boesenbergia rotunda* Linn *Gastrochilus panduratum* RIDL., and *Kaempferia pandurata* Roxb.[1-5]



Figure 1. Fingerroot (*Boesenbergia pandurata* (Roxb.) Schlechter (left) and its rhizome (right)

According to a review written by Cahyadi et. al. [1] many researches have been reported to prove the traditional used of the plant with various pharmacological activity such as antiulcer, inhibitory effect in melanogenesis biosynthesis, tyrosinase activity, anti-parasitic, anti obesity, anti-aging, anticancer, antiviral activity, anti-periodontal activity, antibacterial, antifungal, anti-inflammatory, and antioxidant, antifungal activities.it is also active against multispecies oral-biofilm [3-11]. In addition, secondary metabolites of fingerroot rhizome have been also reported. Flavonoid and essential oil are the most abundant secondary metabolites from this plants [1]. Three classes of flavonoid have been found exist in *B. pandurata* rhizome which are are chalcones, flavanones, and flavones. These flavonoid was also found in prenylated substitution in addition to unprenylated flavonoid. So far more than 51 flavonoid compounds from *B. pandurata* have been isolated and their structure was confirmed [12]. Production of flavonoid from *B. pandurata* was reported using cell culture technique. Panduratin A, cardamomin, and phytosterol can be produced trough callus and rhizomes formation of fingerroot. These thre secondary metaboliyes which are supposed to have pharmacological activities have been produced using cell culture of fingerroot *B. pandurata* with higher level compared to the original plants [12]. Other researchers have been also reported secondary metabolites produced plant tissue and cell culture of *B. pandurata*. The in vitro rapid regeneration of *B. pandurata* has been well performed through shoot-derived calli, shoot bud explants, somatic embyrogenesis and cell suspension cultures [13-15]. Acclimatization of plantlets in the soil were successfully achieved and the generated plants could grow very well which contain higher level of secondary metabolites. The cell suspension culture of *B. pandurata* was reported to produce some of the unprenylated and prenylated flavonoids which are different to wild type [16-17].

The research about isolation an active compounds from fingerroot rhizome was more likely performed from methanol or ethanol extract. Only a few research using specific polarity solvent as an extraction solvent. Hopefully, selecting a specific polarity solvent can pulled out certain compound which hard to extracted by methanol/ethanol and simplify the isolation process of that compound. So far, ethyl acetate was rarely used as a solvent to extract the fingerroot rhizome. So, the purpose of this research was to isolate flavonoid compound from fingerroot rhizome using ethyl acetate as extraction solvent.

MATERIALS AND METHODS

Plant Material and chemicals

The sample was dry powder of fingerroot rhizome from Manoko Training Garden, Lembang, West Java at January 2014. Alumunium plate coated silica gel Merck® 60 F₂₅₄ Thin Layer Chromatography. Analysis and

identification of isolated flavonoid were performed based on UV spectrum using Spectrophotometer UV-Visible Hewlett Packard® (HP). NMR Spectra measured using Spectrometer Agilent® series 500MHz.

Extraction

Dry powder of fingerroot rhizome (700 g) was macerated with ethyl acetate for 5 days, 5 times with 600 mL each and gently shaken few times. Ethyl acetate extract was combined and concentrated by vacuum rotary evaporator with temperature 50°C and 50 rpm, then resulted crude extract of 154 g in weight. Formed precipitate was separated and purified, then tested through various specific spray reagents

Purification and purity test

Precipitate (753 mg) was re-crystallized with methanol three times. It was dissolved in certain amount of methanol and slowly evaporated in room temperature. New crystal was formed and remaining methanol was carefully separated with small Pasteur pipette. This was repeated 3 times resulted pure colorless needle crystal (10,53 mg). The purity was checked using thin layer chromatography with three different elution system, 2 dimension thin layer chromatography using H₂SO₄ 10% as spray reagent, and determination of % AUC in TLC densitometry.

Structure analysis and identification

Isolated compound was determined by chemical reaction, UV spectrometry to find the maximum wavelength continuing with shift reagents. One mg isolated compound was dissolved with methanol as sample stock solution and divided into 4 parts. Some of the sample was measured its maximum wavelength (λ_{max}) with spectrometer UV-Visible. The second sample was added NaOH as shift reagent and measure its λ_{max} . Third sample was added a shift reagent sodium acetate (NaOAc) and measured its λ_{max} , then added boric acid (H₃BO₃) and measured its λ_{max} . Fourth sample was added aluminium chloride (AlCl₃) and measured its λ_{max} , then added HCl and measured its λ_{max} again. Further identification of isolated compound was performed using ¹H-, ¹³C- and HMBC NMR [18,19]. All data were analyzed and concluded the chemical structure of isolated compound

RESULTS AND DISCUSSION

Phytochemical screening showed that both the dried crude plant and ethyl acetate extract contain phenolic and flavonoid compounds, other secondary metabolites were not detected.

Maceration method was selected for extraction in order to protect extraction process from high temperature that could degrade the flavonoid compound. While semi polar solvent, ethyl acetate was used for solvent of extraction aimed to provide unusual flavonoid, so that the result of isolation process would be different to the usual flavonoid from fingerroot such as pinostrobin, pinoembrin, cardamonin that were found in previous research [12]. The monitoring extract with thin layer chromatography using n-hexane : ethyl acetate showed that a dominant band gave the white to yellow color fluorescence after visualization with citroboric spray reagent, 105°C in 5 min following with UV light. Other visualizations were done using FeCl₃ giving red color and TLC profile of isolated crystal that showed one node under UV 254 nm and 366 nm, and H₂SO₄ 10 % in methanol. These indicated that isolated compound belong to flavonoid compound group. Evaporation of ethyl acetate solvent from the extract using rotary evaporator with temperature 50°C and 50 rpm yielded the precipitation from slightly concentrated extract due to the solubility of compound in ethyl acetate. Further purification process of formed precipitate of ethyl acetate extract of fingerroot rhizome resulted colorless needle crystal (Fig. 4). Purity test using co-chromatography with three different TLC system showed only one node without any other contaminants. Solubility test was performed by dissolving the crystal using various solvent and showed that the isolate was soluble in ethyl acetate and methanol.

UV-Visible spectrum of isolated compounds shows maximum absorbance at λ_{max} 285 nm and shoulder at λ 300 – 320. This absorbance was occurred due to presence of chromophore group in the flavonoid structure. The shoulder region at λ 300 – 320 nm and 2D paper chromatography results indicated that the

flavonoid was flavanon (absence of C2-C3 double bond) [18]. Further identification using shift reagents in spectrophotometer UV-Visible clearly supported the structure of isolated compound. After adding shift reagent of NaOH the maximum wavelength was shifted to longer wavelength for +38 nm (bathochromic shift) and stay stable after 5 min. Adding a shift reagent NaOAc yielded bathochromic shift for +37 nm and stay stable after 5 min, this showed the existing of hydroxyl group (OH) at the C7, adding H₃BO₃ into NaOAc treated sample did not give any shift. Adding a shift reagent AlCl₃ following HCl did not give a shift on wavelength, this indicated there was any o-dihydroxy group at A or B ring, Taken together, all the UV spectrum of sample in methanol and adding several shift reagents showed that the isolated compound had hydroxyl group at C7 without free hydroxyl at C3 and C5 (Fig.2, Table 1).

Spectrum data from ¹H-NMR (500 Mhz, MeOH-d₄) (Fig. 3) showing one proton methoxy signal at δ_H 3,82 ppm (3H, s). There was 2 proton signal with meta coupling constant in aromatic proton region at δ_H 6,05 ppm (H, d, J=2 Hz) and δ_H 6,10 ppm (H, d, J=2 Hz). One proton at δ_H 5,42. Around δ_H 7,35 – 7,47 ppm contained 5 proton which indicated phenyl-monosubstituted group. Two proton signal at δ_H 2,71 ppm (H, m) and 2,98 ppm (H, m) indicated aliphatic carbon proton in the flavonoid structure.

Table 1: Shifting of maximum wavelength of isolated compound spectrum after treatment with several shift reagents .

Shift reagents	λ max of peak 2 (nm)	Δ maximum wavelength on peak 2 compare to the spectrum in methanol	Note
Methanol	285	-	-
NaOH	323	+38	New peak on 250 nm
NaOH (5 min)	322	+37	New peak on 250 nm
NaOAc	322	+37	-
NaOAc (5 min)	321	+36	-
NaOAc / H ₃ BO ₃	286	+1	-
AlCl ₃	285	0	-
AlCl ₃ / HCl	285	0 compared to AlCl ₃	-

Table 2: Chemical Shift Data¹H, ¹³C, and 2D HBMNMR of Isolated Flavonoid

δ ¹³ C (ppm)	δ ¹ H (ppm)	HMBC Correlation	Interpretation
80.43	5,42 (H,dd, J = 3 Hz, J = 12,7 Hz)	-	C2
45,73	2,71 (H,m; 2,98 (H,m)	(C 192,01) ; (C 80.43; C 140,87; 192,01)	C3
192,01	-	-	C4
166,82	-	-	C5
97.43	6,05 (H,d, J = 2 Hz)	C 94,56; C 105.05; C 166,82	C6
167.33	-	-	C7
94,56	6,10 (H,d, J = 2 Hz)	C 97.43; C 105.05; C 164,45; 167.33	C8
164,45	-	-	C9
105.05	-	-	C10
140,87	-	-	C1'
127,52	7,47 (2H,d, J = 7,5 Hz)	C 80.43; C 127,52; C 129,77	C2' dan C6'
129,77	7,40 (2H,dd, J = 7,5 Hz)	C 129,93; C 140,87	C3' dan C5'
129,93	7,35 (H,dd, J = 7,7 Hz)	C 127,52	C4'
56,48	3,82 (3H,s)	C 166,82	C metoksi

Note: δ refer to chemical shift as ppm; s : singlet; d : doublet; dd : double doublet; m : multiplet ; J : coupling constant.

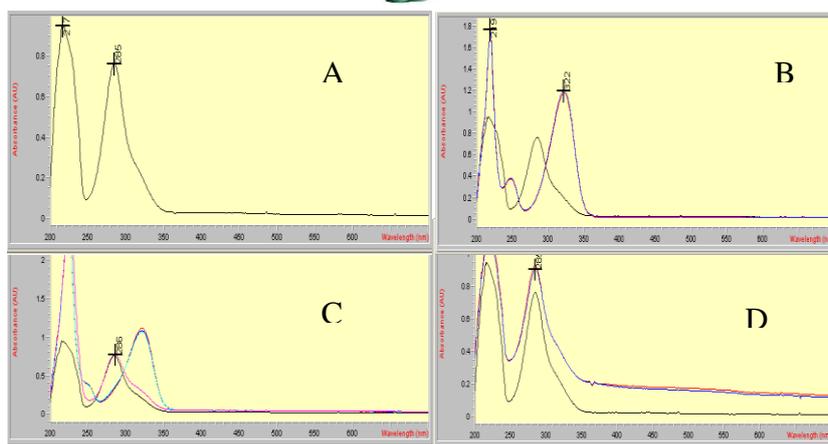


Figure 2. UV Spectrum of isolated compound in methanol (A) in several shift reagents; sodium acetate/boric acid, NaOAc/H₃BO₃ (B), in sodium hydroxide, NaOH, (C), Aluminium chloride/hydrochloride, AlCl₃/HCl (D)

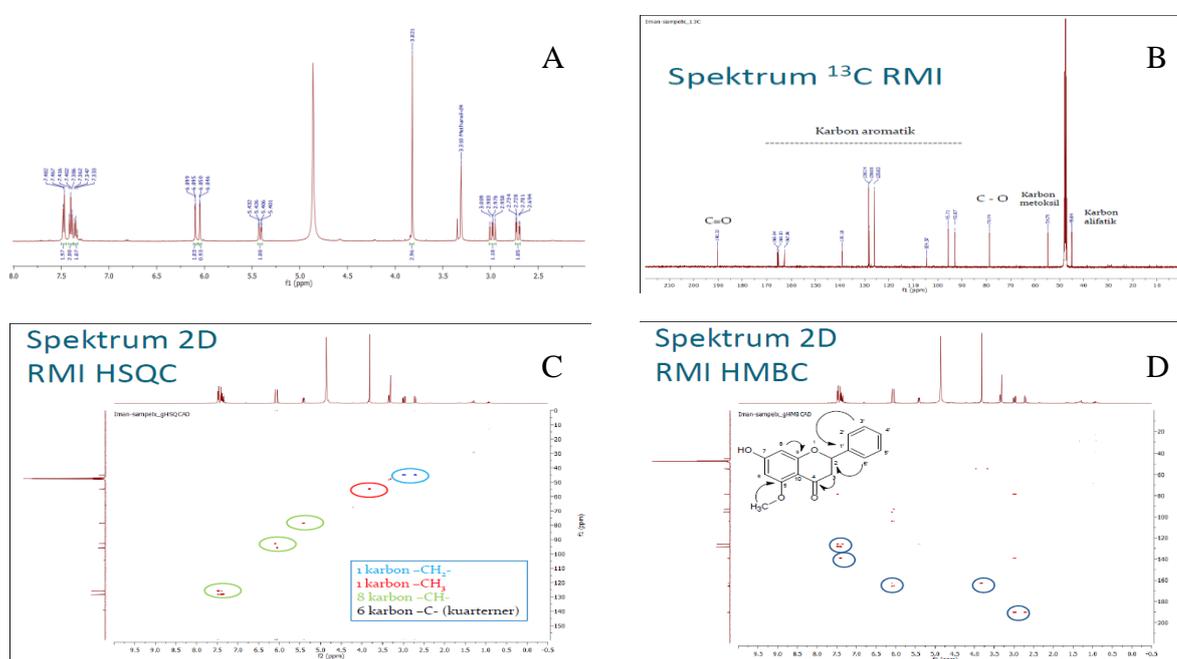


Figure 3. Nuclear Magnetic Resonance (NMR) Spectrum of isolated compound, ¹H-NMR (A), ¹³C-NMR (B), 2D HSQC (C) and 2D HMBC (D)



Figure 4. Chemical Structure of 5-methoxy-7-hydroxy flavanone (alpinetin) and pure isolated compound and its crystal form

Spectrum of ¹³C-NMR (125 MHz, MeOH-d₄) (Fig. 2) showing 14 carbons with different surroundings. One methoxy signal detected at δ_c 56,48 ppm and methylene aliphatic carbon signal at δ_c 45,73 ppm. One carbonyl (C=O) signal was detected at δ_c 192,01 ppm. Three O-substituted carbon aromatic signal at δ 164,45 ,

166,82, dan 167,33 ppm. Nine carbon aromatic signal was seen at δ_c 94,56, 97,43, 105,05, 127,52 (2C), 129,77, 129,93 (2C), and 140,87 ppm, also one single bond carbon-oxygen signal (-HC-O-) at δ_c 80,43 ppm.

In 2D-HSQC NMR (Fig. 2), by pulling a correlation line between each proton from ^1H NMR towards ^{13}C NMR spectrum, it shows six carbons with unpaired electron signal (Quaternary Carbons). Two proton signal at δ_H 2,71 and 2,98 ppm which correlated to one carbon signal at δ_c 45,73 ppm (Methylene group). The dots scheme of the signals is specific for H3-cis and H3-trans proton from Flavanone structure. [3]. This HSQC data was gave information that the flavonoid structure had one methyl (CH_3) at δ_c 56,48 ppm; one methylene group (CH_2) at δ_c 45,73 ppm; eight methyngroup (CH) at δ_c 80,43; 94,56; 97,43; 127,52 (2C); 129,77 (2C); and 129,93 ppm; and six quaternary carbon at δ_c 105,05; 140,87; 164,45; 166,82; 167,33; and 192,01 ppm.

In 2D-HMBC NMR (Fig. 2), it was seen that methoxy proton recognizing carbon at 5th position (δ_H 166,82 ppm). Protons at δ_H 2,71 and 2,98 ppm recognizing carbonyl signal at 4th position (δ_c 192,01 ppm), thus that proton belongs to C3 (flavanone structure). Two signal of aromatic proton at δ_H 7,47 ppm is recognizing C2 carbon (δ_c 80,43 ppm) so that aromatic proton was C2' and C6' whereas two another aromatic signal at δ_H 7,40 recognizing one quaternary carbon at δ_c 140,87 ppm, thus shows that the protons is belongs to C3' and C5' which recognize C1' carbon.

To confirm the interpretation data of flavonoid structure from ^1H , ^{13}C , 2D HSQC, dan 2D HMBCNMR, it was compared with literature data of alpinetin [1] and it didn't gave a significant difference. So, the flavonoid compound which successfully isolated is 5-methoxy-7-hydroxy flavanone or alpinetin.

CONCLUSION

The flavonoid compound was successfully isolated from ethyl acetate extract of fingerroot *Boesenbergia pandurata* (Roxb.) Schlecht rhizome and identified as 5-methoxy-7-hydroxy flavanone or alpinetin. The isolation procedure was performed with simple extraction using maceration method and simple re-crystallization process for purification. Although the isolated compound is not a new compound, but the isolation method with simple procedure was not reported before.

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