ISSN (Print 0974-2115) (Online 2349-8552)
www.jchps.com Journal of Chemical and Pharmaceutical Sciences

Bryononic Acid: Antibacterial Compound from Fruit Hulls of *S. koetjape* Merr Extract

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ABSTRACT

Triterpenoid Bryononic Acid was isolated from *Sandoricum koetjape* Merr plant collected from Serang district, West Java, Indonesia, in which its chemical structures were elucidated by spectroscopic analysis. The antibacterial activity was carried out using a microdilution method. The antibacterial evaluation of extracts and fractions from the *Sandoricum koetjape* hulls was active against *Salmonella enterica* with their Minimum Inhibitory Concentrations (MIC) value of 6.0. µg/mL. The results showed that the extracted plant indicated antibacterial activity with strong spectrum. Thus, identifying the chemical constituents responsible for this antibacterial activity may lead to this discovery and production of new antibacterial drugs against microbes.

KEY WORDS: *S. koetjape* Merr, *Salmonella enterica,* Antibacterial, Bryononic Acid.

1. INTRODUCTION

Various diseases caused by bacterial infection are suspected to have caused 50% of human deaths in tropical countries including Indonesia (Mahady, 2015). The efforts to overcome this problem have been progressed, and one of them is using antimicrobial drugs or antibiotics in the treatment. However, long term exposure of antibiotics to the human body has caused a new problem. The phatogens exposed to the same antibiotics were more resistant over time, which improves their survivability inside the human body – worsened the infection. Therefore, the dicovery of new antimicrobial/bacterial compounds for drug-resistent bateria is still an interesting topic (Arias and Murray, 2015). One common approach to obtaining antimicrobial compounds is to extract them from natural sources, including from plants.

Sandoricum koetjape Merr. (local name: Kecapi or Sentul) is a plant in the familly of Meliaceae. This plant is well known to grow over the tropical or subtropical climate and mostly found in Southeast Asia. In Indonesia as one of a tropical country, this plant is popular in before the 1980s, as one of fruit-producing plants. Besides, this plant is often used by local people to treat stomachache, diarrhea, vaginal discharge, and restore the condition of women after childbirth. However, this plant is recently neglected by because there are many alternative fruits, especially imported fruits (Heyne, 1987). The fruit itself contains sap dan has a bitter taste, which is often associated with the terpenoid compounds (Sim and Lee, 1972).

Phytochemically, the Meliaceae family plants contain a derivative of triterpenoid as its major secondary metabolites, known as limonoid compound (Roy, 2006; Fang, 2011; Tan and Luo, 2011). Limonoids in the Meliaceae have diverse and complex structure, due to the rearrangement of its skeletal frame and the oxidation rate of its higher carbon atoms (Roy, 2006).

Several pieces of literature indicate that phytochemical studies have been conducted on various parts of *Sandoricum koetjape* Merr. The discovery of sandorisin and 6-hydroxisandorisin from seeds in one of the examples. 3-oxo-12-oleanene-oic acid, caustic acid, ketonic acid, and sandorinic acid A-B from the twigs, also sandrapin A-B and the A-B syrup from the leaves of this plant are the another examples. These compounds are normal triterpenoids (oleanane and multiflorane skeletons), andirobin-type limonoids, and trijugin-type limonoids, respectively. The limonoids found from this plant have interesting molecular structures and various biological properties, such as antifeedant, insecticides, antimalarial, antivirals, antibacterial, and anticancer properties (Tan and Luo, 2011; Aisha, 2009).

Discovering new antibacterial drugs is very challenging, and therefore, based on the reasons mentioned above, it is very important and rational for the phytochemical study to be done through the isolation of the *Sandoricum koetjape* Merr fruit. It has been reported that the isolation of an oleane-type triterpenoid, bryononic acid from the stem bark of *S.koetjape* was done (Tukiran, 2006). The same compound is isolated throughout this study and studied for its potential as antibacterial compounds. The active compounds obtained are expected to be further developed as a potential compound in developing new drugs for treating bacteria-resistant infection.

2. MATERIALS AND METHODS

Materials: The plant materials used in this study are the fruit hulls of *Sandoricum koetjape* Merr. The chemicals used for the extraction and isolation are methanol, ethyl acetate, n-hexane, ethanol, acetone, and chloroform. The clinical isolates of *Salmonella enterica* were used as the bacteria for the antibacterial assay.

Extraction and Isolation: A total of 5 kg of the fruits hulls of *S. koetjape* were dried and finely ground. The extraction was performed by maceration method using methanol as solvent. The extraction was done three times for

each grounded plant sample to obtain the maximum amount of extract (24h each extraction). The methanol extracts were dried by evaporation under low pressure using rotary evaporator. Thin layer chromatography (TLC) analysis was performed to determine the estimated number of components to be isolated and the appropriate eluent at the fractionation stage. The dried methanol extracts were subsequently fractionated into n-hexane, chloroform, and ethyl acetate fractions. Each of these fractions will further fractionate using a vacuum liquid chromatography (VLC) method. The eluent was chosen based on the movement of components within the column at low pressure. The fractionation result was also monitored by TLC analysis and then purified by radial chromatography method using silica gel 60 with various mesh and ODS size to obtain each component. The purity of the isolates was determined based on the silica gel stationary phase TLC analysis. The structural determination of pure isolates was performed based on appropriate methodologies for the determination of the structure of natural compounds. The structural analysis of pure isolates was conducted using 1-dimensional NMR techniques: 1H, 13C and DEPT spectra and 2-dimensional NMR techniques: HMQC, HMBC and NOESY spectra. In addition IR spectrum data were measured to strengthen our NMR data analysis results. The interpretation of NMR data and other spectrum data were performed by our research group. The molecular structure will be proposed to the stereochemistry aspect.

Antibacterial activity assay: The antibacterial activity assay was performed *In Vitro* by disk diffusion method on *Salmonella enterica* as the bacterial target. The suspensions of *Salmonella enterica* was prepared following the standard method. In short, *Salmonella enterica* was bred for 24 hours at 37°C under aerobic conditions on agar medium. Bacteria are suspended in 0.9% NaCl (m/v) solution, with the concentration of 2 x 10^6 bacterial cells/mL according to standard McFarland reference. A stock of test solution containing our compound was made with concentration of 150 µg/mL in Mueller Hinton Broth (MHB) liquid medium. The concentration series of the test solution were prepared by dilution in a microplate of 96 wells with a concentration range of 0.02-50.00 µg/mL. A total of 200 µL of liquid medium was added to the first well. The solution concentration series was carried out by transferring 100 µL of test solution from first well to second well and diluted to 200 µL for both well. The same process was done from the second well to the third well and so on until the eighth well so that the amount of solution in each well was 200 µL. Furthermore, 10 µL of bacterial suspension was added to each well. The next two wells we are used as the control. The first control was filled with 200 µL of liquid medium and 10 µL of bacterial suspension (growth control), while only liquid media without bacterial suspension was used in the second control well (sterility control). The microplate incubated at 37°C for 24 hours. Bacterial growth was determined using a universal microplate reader at a 600 nm wavelength. The Minimum Inhibitory Concentration (MIC) values indicate the lowest concentration that can inhibit microbial growth.

3. RESULTS AND DISCUSSION

Twenty grams of dark chocolate-shaped methanol extract was obtained. 20 grams of MeOH extract of *S. koetjape* was extracted by chromatography technique, namely vacuum liquid chromatography (VLC) with n-hexane and ethyl acetate with different compositions as eluenty. This separation resulted in seventeen fractions, fraction A (67 mg), fraction B (68 mg), fraction C (1.26 g), fraction D (0.96 g), fraction E (0.39 g), fraction F (0.76 g), the fraction G (1.84 g), H fraction (0.72 g), I fraction (0.17 g), J fraction (93 mg), K fraction (85 mg), L fraction (0.34 g), M fraction (0.26 g), N fraction (0.19 g), O fraction (0.16 g), P fraction (0.29 g), and MeOH fraction (0.59 g). Compound (1) was obtained by adding MeOH into fraction H and fraction I was heated and then cooled and allowed to dissolve so as to produce a soluble fraction in MeOH (fraction H '= fraction I') and a pure sponge.

Compound 1, {11, $[\alpha]^{25}_D$ = +17,321 (c 0.31, $CHCl_3$)} is successfully isolated in the form of white powder and it is the main compound of the fruit hulls of 338 mg. The Absorption on the IR spectrum shows a presence of alkene and carbonyl groups (1695 cm^{-1}), -CH_3 and -CH_2 stretching (2933cm^{-1}), -C-O-H (1462 cm^{-1}), and -C-O stretching (1375 cm^{-1}).

1H-NMR Spectrum (chloroform-*d6*, 500 MHz), δH (ppm): 2,43 (1H, *br s*, H-1, ax) & 2,51 (1H, *m*, H-1, eq); 1,49 (1H, *br s*, H-2, eq) & 2,00 (1H, *br m*, H-2, ax); 1,56 (1H, *br s*, H-5, ax); 1,41 (1H, *d*, 5,1, H-6, ax) & 1,59 (1H, *br s*, H-6, eq); 1,81 (1H, *br m*, H-7, ax) & 2,09 (1H, *br s*, H-7, eq); 1,93 (2H, *br d*, H-11, ax); 2,26 (1H, *br s*, H-12, eq) & 1,64 (1H, *br d*, H-12, ax); 1,29 (1H, *br s*, H-15, eq) & 1,44 (1H, *d*, 4,6, H-15, ax); 0,87 (1H, *br d*, H-16, eq) & 2,01 (1H, *br m*, H-16, ax); 1,49 (1H, *br s*, H-18, eq); 1,62 (1H, *br m*, H-19, ax) & 2,41 (1H, *br s*, H-19, eq); 1,35 (1H, *d*, 4,1, H-21, ax) & 2,17 (1H, *br s*, H-21, eq); 1,28 (1H, *br m*, H-22, eq) & 1,67 (1H, *br d*, 5,4, H-22, ax); 1,02 (3H, *s*, Me-23); 1,05 (3H, *s*, Me-24); 0,99 (3H, *s*, Me-25); 0,93 (3H, *s*, Me-26); 0,81 (3H, *s*, Me-27); 1,00 (3H, *s*, Me-28); 1,19 (3H, *s*, Me-30).

13C-NMR Spectrum (chloroform-*d6*, 125 MHz), δC (ppm): 34,36 (C-1); 35,36 (C-2); 218,22 (C-3); 47,08 (C-4); 51,13 (C-5); 20,50 (C-6); 27,68 (C-7); 134,87 (C-8); 132,67 (C-9); 30,82 (C-10); 20,57 (C-11); 29,90 (C-12); 42,14 (C-13); 37,04 (C-14); 25,16 (C-15); 34,22 (C-16); 37,43 (C-17); 44,44 (C-18); 30,43 (C-19); 40,34 (C-20); 29,46 (C-21); 36,81 (C-22); 21,11 (C-23); 26,74 (C-24); 31,20 (C-25); 21,61 (C-26); 18,09 (C-27); 19,42 (C-28); 185,32 (C-29); and 32,66 (C-30).

The ^{13}C NMR spectrum showed 30 carbon signals, which consisted of: 26 carbon signals in the alkyl region (δC 0-60 ppm), 2 carbon signals in the alkene region (δC 134.87 and 132.67 ppm), 1 signal -COOH (δC 185.32 ppm), and 1 signal - C = O (δC 218,22 ppm). The 1H NMR spectrum shows the signals are located in the aliphatic region (0.7-2.6 ppm).

Based on the spectrum of DEPT-135 and HSQC, it is known that the number of units is 8-CH_3, 12-CH_2, 4-CH, and 6-Cq, with 8 DBE consisting of 3 DBE clusters function (1 alkenes, 1 -COOH, and 1-C = O) and 5 DBE basic frames (cyclic). If it is returned to its origin, then the number of units is 8-CH_3, 12-CH_2, 4-CH, and 6-Cq. There are seven basic frameworks that fit the system. These skeletons are: oleanan, taraxeran, multifloran, glutinan, stictan, friedelan, and grammaceran. Two carbon alkenes are –Cq, and therefore it is known that the origin of the skeleton will have two adjacent CHs. Finally, possible basic frameworks are reduced to three: oleanan, multifloran, and glutinan. Based on the HMBC spectrum, the -C = O group is correlated with 2-CH_2 and 2 -CH_3, while the -COOH group is correlated with 2 -CH_2 and 1-CH_3. It then can be determined that the position -C = O in the A ring at C-3 and position -COOH in the E ring at position C-29. Correlated CH3-units with clusters -C = O, -COOH, and = Cq different from each other which means the position between clusters does not correlate two to three bonds so it can be inferred that the base skeleton is not glutinan. The HMBC correlation shows that H-23, H-24, and H-25 are correlated with C-5, then H-5 is correlated with C-9 alkene. Based on the above analysis, it can be concluded that the skeleton of this compound is a multiflorane with the functional group position -C = O in C-3, alkene in C-8 / C-9, and -COOH in C-29.

HMBC spectroscopy data (Table.1) and 1H-1H COSY also strengthened our structural assumptions of compound 1, where some important HMBC correlations and 1H-1H COSY can be seen in Figure.1.

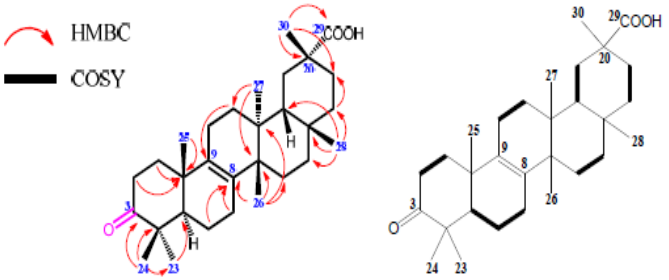

Figure.1. Correlation of HMBC (1H? 13C) and 1H-1H COSY of Compound 1

The relative configuration of compound 1 is determined by the correlation of NOESY (Fig.2). The H-18 beta orientation is evidenced by the correlation of NOESY with H3-25, H3-26, H3-28, and H3-30; H-5 alpha orientation is proved by NOESY correlation with H3-23.

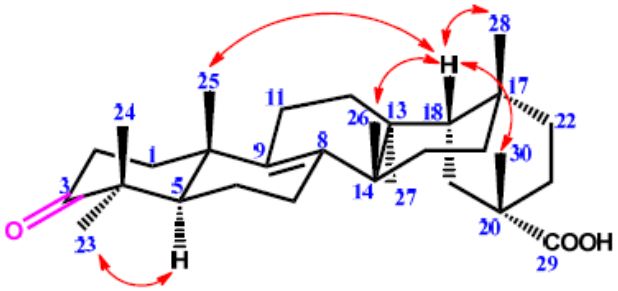

Figure.2. Correlation NOESY of Compound 1

To reinforce the assumption that the isolated compound is a Bryononic Acid (1), the NMR spectroscopic data of the isolated compound is compared with literature data (Table.1), which shows the harmony of data with Bryononic Acid (1) from Kosela (1995).

Compound 1 with the name of Bryononic Acid or 3-oxy-D acid: C-friedoolean-8-en-29-oat has been found previously on the fruit hulls of *S. koetjape*. In addition, compound 1 has also been found in the Cucurbitaceae family (Kongtun, 2009).

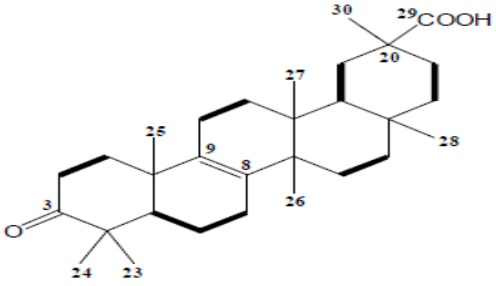

Figure.3. Structure of Bryononic Acid

Table.1. ^{1}H and ^{13}C NMR Bryononic Acid Spectroscopic Data

No. C	Bryononic acid (1) (chloroform-d_6) (Kosela, 1995)		Isolated bryononic acid (1) (chloroform-d_6)	
	δH (integration, multiplicity, J [Hz])	δC (ppm)	δH (integration, multiplicity, J [Hz])	δC (ppm)
1	**Not assigned**	34,2	2,43 (*br s*) & 2,51 (*m*)	34,36
2		35,4	1,49 (*br s*) & 2,00 (*br m*)	35,36
3		218,2	-	218,22
4		47,1	-	47,08
5		51,1	1,56 (*br s*)	51,13
6		20,5	1,41 (*d*) & 1,59 (*br s*)	20,50
7		27,7	1,81 (*br m*) & 2,09 (*br s*)	27,68
8		134,9	-	134,87
9		132,7	-	132,67
10		30,8	-	30,82
11		20,6	1,93 (*br d*)	20,57
12		29,9	2,26 (*br s*) & 1,64 (*br d*)	29,90
13		42,2	-	42,14
14		37,0	-	37,04
15		25,2	1,29 (*br s*) &1,44 (*d*)	25,16
16		34,4	0,87 (*br d*) & 2,01 (*br m*)	34,22
17		37,4	-	37,43
18		44,4	1,49 (*br s*)	44,44
19		30,4	1,62 (*br m*) & 2,41 (*br s*)	30,43
20		40,4	-	40,34
21		29,5	1,35 (*d*) & 2,17 (*br s*)	29,46
22		36,8	1,28 (*br m*) & 1,67 (*br d*)	36,81
23	1,06 (*s*)	21,1	1,02 (*s*)	21,11
24	1,09 (*s*)	26,8	1,05 (*s*)	26,74
25	1,03 (*s*)	19,4	0,99 (*s*)	31,20
26	1,96 (*s*)	21,6	0,93 (*s*)	21,61
27	0,84 (*s*)	18,1	0,81 (*s*)	18,09
28	1,03 (*s*)	31,2	1,00 (*s*)	19,42
29	-	185,5	-	185,32
30	1,22 (*s*)	32,7	1,19 (*s*)	32,66

Antibacterial bioactivity of compound 1 (bryononic acid) from the fruit hulls of *S. koetjape* against *Salmonella enterica* was obtained with the MIC value of 6.0 μg/mL.

4. CONCLUSION

An active antibacterial compound contained in *S. koetjape* Merr from fruit hulls successfully isolated and identified as bryononic acid which also has very strong bacterial activity against *Salmonella enterica* with MIC value of 6.0 μg/mL.

5. ACKNOWLEDGEMENT

The authors appreciate DPRM Dikti for awarding a Post-Doctoral Research Grant. We also thank the Herbarium-LIPI of Bogor, Indonesia for identification of the plant specimen and ITB for NMR measurements.

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