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Isolation and Structure Elucidation of Steroid from Leaves of *Artocarpus camansi* (Kulu) as Antidiabetic

Rosnani Nasution[1*], Tonel Barus[2], Pandapotan Nasution[2], Nurdin Saidi[1]

[1]Department of Chemistry, Faculty of Mathematic and Natural Sciences, Syiah Kuala University, Banda Aceh, Indonesia. 23111

[2]Department of Chemistry, Faculty of Mathematic and Natural Sciences, North of Sumatera University, Medan, Indonesia

[*]Corres. author: rosnani.unsyiah@gmail.com
Tel.: +62 085276921690.

Abstract: Research on plant leaves *Artocarpus camansi* (Kulu) was aimed to find a chemical compound with antidiabetic activity in male Swiss Webster mice. The study was began by preparing hexane, ethyl acetate and methanol extracts of the leaves of *A. camansi* plant. The antidiabetic activity assay revealed that hexane extract was most active extract. According to this, the hexane extract was further fractionated to give pure isolate. The pure isolate has a melting point of 77-80^0 C. Then the isolate was further characterized by ultra violet (UV), infra red (IR), one and two dimensional nuclear magnetic resonance (NMR) experiments such as ^{1}HNMR, ^{13}CNMR, distortionless enhancement by polarization transfer (DEPT), ^{1}H-^{1}H homo correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond coherence (HMBC), the result of the spectral analyses suggested that the isolate was *β*-sitosterol propionate. In the antidiabetic activity assays, *β*-sitosterol propionate showed, a greater ability to reduce blood glucose than crude extract (extract of hexane, ethyl acetate extract and methanol extract), that is,30 minutes after the administration of *β*-sitosterol propionate can reduce blood glucose of mice 87.67 mg/dL; after 60 minutes reduce the blood sugar as much as 89 mg /dL; and 90 minute, reduce blood sugar 22 mg /dL, in male Swiss Webster mice. Analysis of this variance were performed using ANOVA *one way Post hoc analysis* procedures, significant differences ($p<0.05$) and ($p<0.01$) conducted using Tukey.
Keywords: *Artocarpus camansi*; *β*-sitosterol propionate; antidabetic; glucose tolerance.

Introduction

Diabetes mellitus type 2 is a disease that is a global health problem because of the high morbidity and mortality caused by the disease. Data from the World Health Organization (WHO) in 2008 stated that there were approximately 180 million people with diabetes worldwide and this number is expected to increase by more than double by in 2030. According to WHO data in 2000, Indonesian state ranks fourth most cases of diabetes after India, China, and the United States, with a prevalence of 8.6 per cent of the total population. This trend is thought to occur due to various factors such as, lifestyle communities tend to be less movement, caloric intake is not balanced, and demographics (http://www.WHO.int/, 2009)[1].

Artocarpus camansi Blanco (breadnut) belongs to the family Moraceae (Mulberry family) is a plant with a height of 10-15 m (33-50 ft) or higher with the main branch along 5 m or more, gummy white on any

part of plant. In Indonesia, plant *A.camansi* often referred to as kulu, or kluih, this plant is native to New Guenea and possibly in Indonesia. *Artocarpus camansi*, has often been considered to be a form of seeded breadfruit, *A.altilis*, or syn: *A. communis*, so *A. camansi* often referred to by the name: *A. altilis, A.communis*, and *A.incisa*, breadfruit however, is separate species that originated from its wild seeded ancestor, breadnut [2]. However, research on *A. camansi* is very less, both of the chemical, as well as its potential as a drug (biological activity), while research on *A. communis* is relatively perfect [3]. Biological activity of *A.communis* traditionally can reduce blood sugar, and research on all parts of the plant have been carried out. The leaves of *A. communis* containing geranyl dihydrochalcone[4], and the bark of this plant contains lupeol acetate and *β*-sitosterol [5], while research on *A. camansi* has been conducted on the composition of the seeds [6]. Reports indicate that the pharmacological activities of *β*-sitosterol is considered in normalize blood sugar [7], therefore, the objective of this study was to investigate the antidiabetic activity of steroid derivative isolated and identified from the leaf of *A. camansi*, and the crude extract of (hexane, ethylacetate, methanol) of the leaf *A. camansi* also investigated.

Material and Method

Plant Material

The old leaves that have fallen of *Artocarpus camansi* was collected in February 2012 in Aceh, Indonesia. The plant was identified at Department of Biology, University of North Sumatera, Medanense, Medan.

Animal

All experiment were carried out using breeding 4-6 week old male Swiss Webster mice chosen from animal colony of central animal research facility, University of North Sumatera, Medan. The colony was maintained under controlled condition soft temperature. The experimental protocol has been approved by North of Sumatera University, Mathematic and science Faculty Ethic Committee (Regd. No 317/KEHP-FMIPA/2013.

Spectroscopic Investigation

UV spectra were measured using a Varian Cary 100 Conc. and the IR spectrum was found using a Perkin Elmer Spectrum One FT-IR spectrophotometers. Mass spectra were measured using a Shimadzu GC-MS QP 2010 Ultra. The ^{1}HNMR (400 MHz), ^{13}CNMR (125 MHz), HSQC, HMBC, and COSY Spectra were recorded on a JEOL in CD_3Cl.

Testing Phytochemicals

The method used for testing of phytochemical can be found in : Phytochemical methods, Simplified Determination Method to Analyze plant[8].

Extraction and isolation of Steroid propionate from the leaves of *A. camansi*

The air dried of old leaves of *A. camansi* (1.7 kg) was extracted with hexane (40 L) for two times in percolator and filtered. The filtrate was evaporated in vacuo to give the dark brown residue, and the yield was 49.02 g. Then the sample was also extracted with ethylacetate and methanol solvent, and the yield was 41.58 g (extract of ethylacetae) and 50.2 g (extract of methanol) respectively. All of these extract were collected separately and preserved for bioassay test and analysis. Antidiabetic test results from all the three extracts, the most active is the hexane extract, so the isolation and testing activities was directed at the hexane extract.

Hexane Extract Fractionation

The hexane extract (30 g) was separated by column chromatography with silica gel (70-230 mesh, 150 g, Merck) as stationary phase and eluted with n-hexane gradually with ethylacetate to efford 59 fractions by TLC profile**.** Fractions (1-26) was none steroid, fractions 27-29 contained steroid, and 30-59 none of steroid. Fraction 27-29 (1,1 g) was further fractionated using column chromatography (70-230 mesh, 100 g, Merck) eluting with hexane to obtain *β*-sitosterol propionate from fraction 15-17 of 22 fractions.

Glucose Tolerance Test

Before use, the mice were acclimatized for 7 days in laboratory conditions as well as getting enough food and drinks. After 7 days, selected mice were healthy, characterized by weight stable or increased and did not show any abnormal behavior. Mice were divided into 6 groups, each of the groups contain three of mice, group I: diabetic control was given CMC-Na (0.5 %), group II: the standard drug glibenclamide was given orally at dose of 1 mg/kg), group III: treated with 50 mg/kg (effective dose) pure isolate leaf of *A. camansi*, group IV: treated with 50 mg/kg macerated hexanic extract of *A. camansi*, group V: treated with 50 mg/kg macerated ethanolic extract of *A. camansi*, dan group VI: treated with 50 mg/kg macerated methanolic extract of *A. camansi*.The extract and the pure isolate were suspended in CMC 1%.

Having fasted for 20-24 hours, the weight of mice were weighed, fasting blood glucose levels were measured and given treatment (above). After 30 minutes later, the entire group was given a dose of 3 g glucose/kg orally. Furthermore fasting blood glucose levels were recorded at 30, 60, and 90 minutes after glucose loading [9].

Blood Samples

Mice were put in a box modifications (restrainer), tail cleaned with a wet cotton so that the dirt is gone, then smeared with alcohol 70% v/v. Blood was drawn through the lateral tail vein, which was cut aseptically approximately 1-2 mm from the tip of the tail without anesthesia, blood droplets first removed, then the next drop of blood dripped on the strip One Touch Horizon.

Statistical Analysis

Statistical analysis was performed using *Statistical Product And Service Solution* (SPSS) Program. Analysis of variance were performed using ANOVA *one way Post hoc analysis* procedures, significant differences ($p<0.05$) and ($p<0.01$) using Tukey.

Results and Discussion

Phytochemical test results

Phytochemical test results plant leaf *A. camansi*, containing secondary metabolites terpenoids, and steroids

Antidiabetic Activity

The effect of administration to pure isolate, hexane extract, ethyl acetate extract and methanol extract of lowering blood sugar levels in mice is shown in Figure 1 and Table 1. As can be seen in **Fig. 1 and Table 1,** pure isolate is significantly higher in lowering blood sugar levels on mice compared to others.

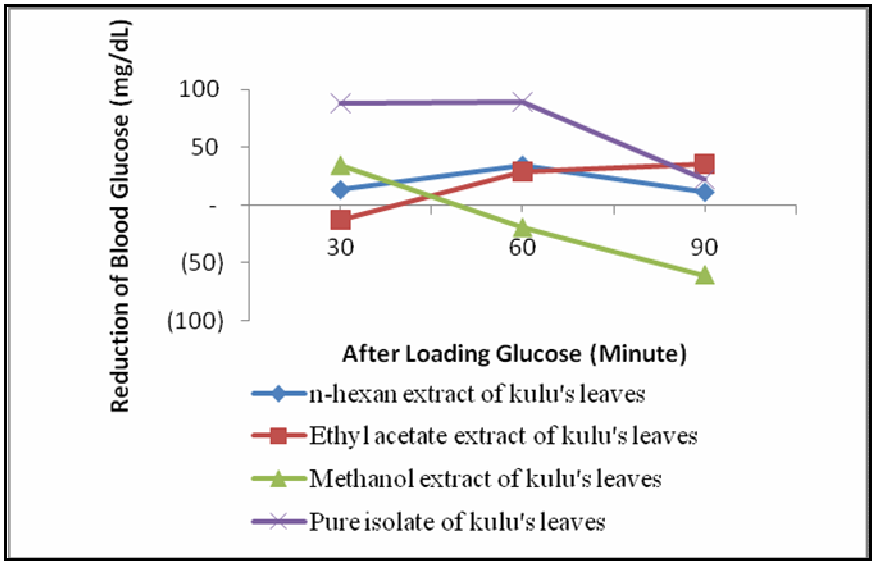

Fig. 1. Effects of extracts and pure crystals (isolates) decrease in blood sugar levels of mice

Pure isolate at 30 minute after glucose loading can decrease blood of mice as much as 87.67 mg/ dL, after 60 minutes can lowering blood sugar as much as 89 mg/dL, in the 90 minute, lowering blood sugar was only 22 mg /dL, the decrease is due to induced sugar has entered into a cell (in this case the diseased mice are not diabetic, induced diabetic only), as well as on hexane extract, at 30 minutes can lower blood sugar levels as much as 13.33 mg/dL, and increased in the 60 minute to be 34.33 mg/dL, and after 90 minutes the blood sugar levels of mice to be 11.33 mg/dL. In the ethyl acetate extract, a decrease in glucose levels by 13 mg/dL, to 28.33 mg/dL, and in the 90 minute, the ethyl acetate extract can lower blood sugar levels greater than hexane extracts and pure isolate. In the methanol extract at the minutes 30 can lower blood sugar levels as much as 34.33 mg/dL, but at minute 60 and 90, the extract is in fact an increase in blood sugar levels of mice (works antagonist) is -19.33 mg/dL and -60.33 mg/dL. It can also be seen from The Article Review [10] in which roots extract of *A.communis* can increase blood sugar levels.

Table 1. Comparison of Blood Glucose Levels Decrease Between Leaf Extract and Pure Isolate *A. camansi* (Kulu)

	Decrease Control Of Blood Glucose Levels After Glucose Loading (mg/dL)								
	30 minutes			60 minutes			90 minutes		
Extract/ pure isolate	average	SD	p	average	SD	p	average	SD	p
Hexane	13.33	3.06	0.015*	34.33	0.58	0.004*	11.33	3.51	0.196
Ethyl Acetate	-13	39	0.02*	28.33	13.28	0.002*	35.33	8.62	0.091^
Methanol	34.33	3.51	0.074^	-19.33	3.51	0.000*	-60.33	1.53	0.000*
Pure isolate	87.67	21.22	-	89	22.52	-	22	7	

* = Significantly different than the pure isolate ($p < 0.05$)
^ = Significantly different than the pure isolate ($p < 0.1$)

Structure Determination of Steroid Compound

Steroid compound, was obtained as a colourless amorphous powder from hexane extract, the compound has melting point of 77-80 ^{0}C. Analysis of the IR spectroscopic data showed a sharp absorption band at 1735.93 cm-$^{-1}$ of C=O group, chelated presenting as ester. Absorption band at 1631 cm^{-1}, indicated the presence of isolated C=C group (1620-1680), are strengthened by the absorption band at 725 cm^{-1}. Absorption band is at 1172 cm^{-1}, of CO bond. $OCOCH_2CH_3$ group on β-sitosterol is domiciled ekuitorial, supported by the absorption peaks at 1068, 56 {uptake is greater than 1025-1031[11]}. Ultra violet spectroscopic data showed, absorption band at, λ_{maks}^{MeOH} nm (log ∈) 275 (2.66), 240 nm (2.76). Characterization by Mass-Spectroscopy of Gas Chromatography-Mass Spectroscopy (GC-MS) showed that this compound is similar with β-sitosterol fragmentation patterns. MS spectrum of steroid compound in the Fig. 2.

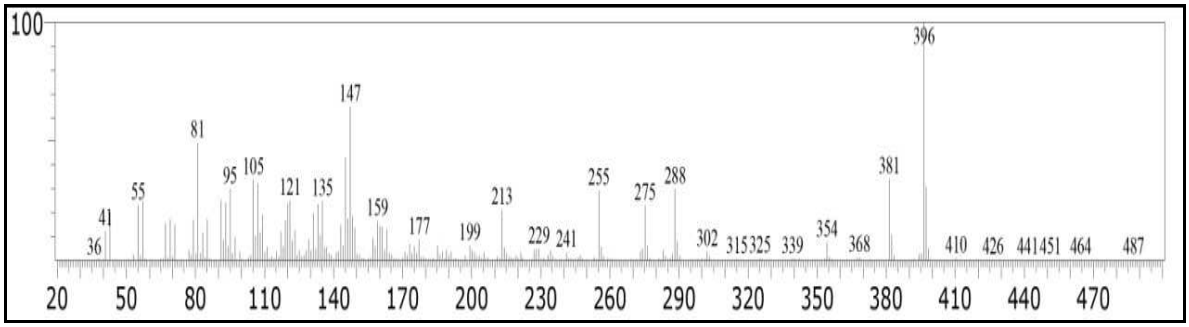

Fig. 2. MS spectrum of Steroid Compound

MS spectrum of steroid compound is shown in Figure 2, characterization for possible β-sitosterol are: peak at 396, is the $(M\text{-}CH_3\text{-}CH_2\text{-}C{=}O\text{-}H_2O)^+$, the peak is 381 $(396\text{-}Me)^+$, 275 (side chain, C10H21), 213 (255-termination ring D), 199 (213-Me).

The ^{1}H NMR spectrum of Steroid compound, measured in $CDCl_3$ and presented in Table 2, displayed a one proton downfield multiplet at 5.31-5.33, d, J=4.28 assigned to C-6 olefinic proton. A one proton broad

multiplet at 3.45-3.54,m, was ascribed to C-3. The methyl protons were appeared as three proton broad signal at δ 0.64 (Me-18) and 0.97 (Me-19). Four doublets at δ 0.73 ppm and δ 0.75 ppm (J=6.4), doublet at δ 0.79 ppm and δ 0.81 (J=7.32) were ascribed corresponding to H-21, and H-27. Doublet at δ 0.82 and δ 0.84 ppm corresponding to H-26, and doublet at δ 0.88 ppm and 0.90 ppm (J=6.72) corresponding to H-29. One peak broad multiplet at 3.45-3.54, m showed the presence of 3α-methine proton (axial) (Jaa = 10 Hz ; Jae = 5Hz) showed that H - 3 is attached to CO. Quartet at 2.72 - 2.79 ppm, showed for CH_2 (C2') of propionic groups, and triplet at δ 2.49 to 2.53 showed the presence of CH_3 of propionic groups (C3').

Based on the ^{13}C-NMR spectrum of steroid compound , the compound have 32 carbon atoms and shows the group C=O (178.69), ena (140.68 and 121.69). Characterization by DEPT, it was known that steroid compounds having carbon methyl 7, 9 metin, and 12 methylene, while 4 quaternary C cannot be shown by this spectrum.

^{1}H and ^{13}C chemical shift assignment and 1H/13C correlation of steroid compound as determined from HSQC spectrum can be seen in Table 2.

Table 2. ^{1}H and ^{13}C NMR Spectral Data of *β*-sitosterol propionate (from HSQC)

Carbon number	^{1}H, δ	^{13} X, δ	Carbon number	^{1}H, δ	^{13} X, δ
1	1.01-1.06, m 1.80-1.85, m	37.4078	17	1.01-1.06, m	56.01
2	1.79-1.83, m 1.92-2.00, m	31.5255	18	0.64, s	11.82
3	3.45-3.54, m	71.78	19	0.97, s	19.36
4	1.90-2.00, m	42.1597	20	1.27-1.38, m	36.11
5	-	140.6836	21	0.73, 0.75 d, J=6.40	18.74
6	5.31-5.33, d, J=4.28	121.6954	22	0.95-1.01, m 1.20-1.26, m	33.90
7	1.38-1.44, m 1.79-1.83, m	31.86	23	1.01-1.10, m	26.00
8	1.38-1.44	31.86	24	0.83-0.88, m	45.78
9	0.86-0.91, m	50,08	25	1.61-1.66, m	29.06
10	-	35.45	26	0.82; 0.84 d, J=6.72	19.79
11	1.40-1.53, m	21.044	27	0.79; 0,81d, J=7,32	19,00
12	2.21-2.26, m	39.735	28	1.20-1.28, m	23.01
13	-	42.28	29	0.88; 0.90, d, J=6.72	11.94
14	0.90-1.01, m	56.72	1'	C=O	178.69
15	1.44-1.53, m	24.27	2'	2.72-2.79, q	33.90
16	1.79-1.83, m	28.22	3'	2.49-2.53, t	15.96

Heteronuclear Multiple Bond Coherence (HMBC) spectrum of the steroid compound: protons at atom H-19 is correlated by atom C-1, C-5, C-9, C-10. There is correlation between proton H-18 to atom C-12, 13, 14, 17. Correlations between protons H-26 with atom C-24, C-27, C-28. Correlation between proton H-27 with atom C-26, correlation proton H-22 with atom C-21 and C-23. Correlations between protons H-24 with C-22 atom. correlation between the protons of 14 with atom C-17, the correlation between the protons of H- 20 with atom C-21 and C-23 atoms. Correlations between protons H-25 with atom C-22, the correlation between protons H-28 with atom C-29 and 25, the correlation between protons H-4 with atom C-3 and C-5, proton correlation H-7 with C-14 atom. Correlation between H-1 to C- 3, the correlation between protons 2' with the atom C-3, C-3', 1', and the correlation between the protons H-3' with the atom C-1 '(C = O).

This correlation can be drawn on the structure of compound in Fig 3.

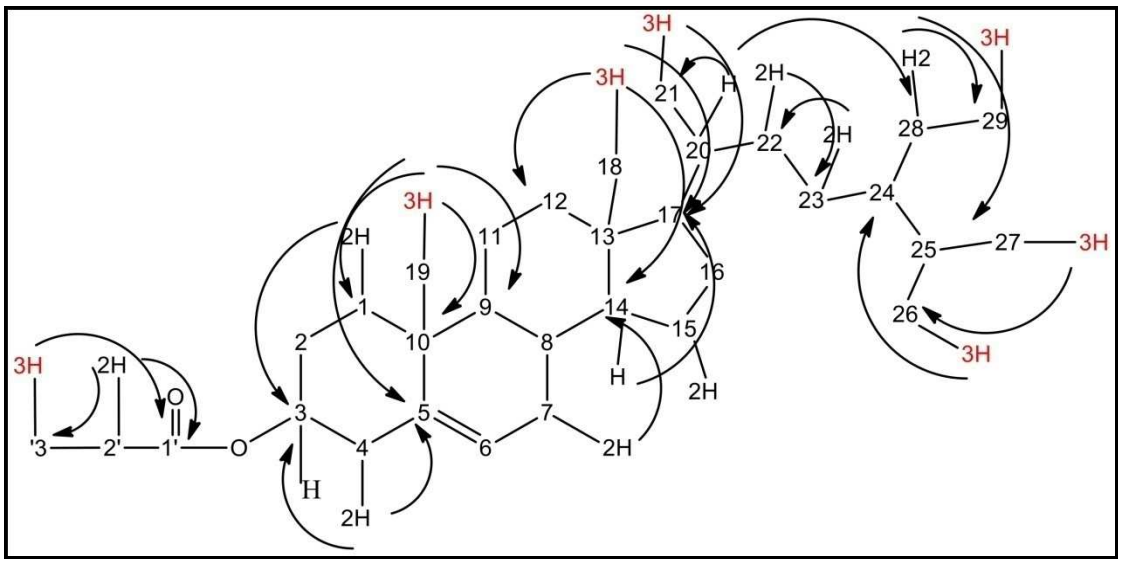

Fig. 3. Correlation between H Atom and C atom in long distance (HMBC)

Characterization the steroid compound with a COSY: proton H-12 (δ1.97, m) cross peak with H-11 (1.46 m), proton H-16 (1.79 to 1.83, m) cross peak with H -17 (1.03 to 1.06 m). Proton H-20 (1.27 m), cross peak with methyl protons H-21 (0.82); Proton H-25 (1.61 m) cross peak with the methyl protons of CH_3-26 (0.82 d) and CH_3-27 (0.79 d). Proton H-28 (1.20 m) cross peak with the methyl protons of CH3-29 (d, 0.88). Proton H-2' cross peak with the proton H-3', suggesting a bond between C-2 and C-3.

The chemical sift of this steroid matched the report values in ^{13}C-NMR[12,13]. The test and characterization results indicate the possibility of the steroid compound in this study is the structure of *β*-sitosterol propionate.

4. Conclusions

The results shown that the pure isolate is a steroid compound (with Liebermann Burchards positive bluish green), in the form of a white crystalline compound with a melting point of 77-80^0 C. Based on the analysis of the IR spectrum, UV, ^{1}H-NMR, ^{13}C-NMR, DEPT, and supported by HMBC, HSQC, and COSY, steroid compound possibility is *β*-sitosterol propionate. *Artocarpus camansi* plant leaf extracts may reduce blood sugar levels of the mice, which was conducted by glucose tolerance, but the most active extracts reduce blood sugar levels of mice were hexane extract. In addition, pure isolate (possibility *β*-sitosterol propionate) from hexane extract has a greater antidiabetic activity of the extract three types, ie 30 minutes after loading can reduce blood glucose of mice as much as 87.67 mg / dL, after 60 minutes will decrease blood sugar as much as 89 mg/dL, at minute 90, lowering blood sugar was only 22 mg / dL, which was conducted on male mice.

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