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β- Sitosterol and Betulonic Acid from *n*-Hexane Extract the Stem Bark of *Tetracera indica*

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ABSTRACT. The *Tetracera indica* has been used traditionally to treat various diseases including skin medications, lower blood pressure, fever, headache, wound and gout medicine. The objective of the research was isolated and characterized the β-sitosterol (**1**) and betulonic acid (**2**) from *n*-hexane extract of the stem bark of *Tetracera indica*. The dry powder the stem bark (550 g) was maceration with *n*-hexane for 24 hrs. This extraction process was repeated three times. The *n*-hexane extract was separated and purified by chromatography technique. The structure of isolated compound was determined by spectroscopy data UV, IR, NMR and compared with reported data. Two compounds have been isolated and base on spectroscopy data the compounds is β-sitosterol (**1**) and betulonic acid (**2**). Betulonic acid is being reported for the first time from *T. indica,* mean while the β-sitosterol previously reported from MeOH extract of leaves of *T. indica* but is being reported for the first time from the stem bark of *T. indica*.

Keywords: Betulonic acid, β-sitosterol, *Tetracera indica*

INTRODUCTION

Indonesia is one country of the largest users of medicinal plants in the world along with other countries in Asia, such as China and India (Widjaja et al., 2014). The used of medicinal plants traditionally has been done by Indonesian people for generations. In previous studies, exploration and inventory of medicinal plants and thereused in people local community have been done. One of the medicinal plants is *Tetracera indica* (Yustian, Muharni, Sukarmi, Zulaicha, & Arbi, 2012). This species also known by the name *Tetracera assay* (Hoogland, 1953). The stem bark of *T indica* was used as a rope and the fruit used as vinegar in cooking. *T. Indica* in Malaysia is known locally name mempelas oil or mempelas rich and in Cina is know locally name caygiaychieu. Malay communities in Malaysia used dry powder of leaves to cure inflammation, while the Temuan Tribe in Selangor used the roots of *T. indica* to treatment of hypertensive patients and high fever. The Machang tribe in Kelantang used mixture the powdered of roots and leaves treat skin diseases (Faridah and Nurul huda, 1999). In Indonesia, especially the people of South Sumatra using the stem bark of the *T. indica* for the treatment of kidney stones (Muharni, Fitriya & Farida, 2016; Ong & Nordiana, 1999) and the leaves used for hypercholesterolemia (Samitra & Rozi, 2017). In India was used for the treatment of dysentery, hepatitis, diuretics, fever and fatigue (Lima, Lemos & Conserva, 2014).

The phytochemical test of the methanol extract of the stem bark of *T. indica* showed positive containing triterpenoid, steroid, saponin, flavonoid and phenolic compounds. The efficacy of a medicinal plant, closely related to secondary metabolites contained in these plants (Sangi, Runtuwene, Simbala & Makang, 2008; Yustian et al., 2012). Based on the literature study, flavonoid compounds in the form of 5,7-dihydroxy 8-metoxyflavone (wogonin), 5, 7,8-tryhydroxy flavone, isocutellarein methyl ether, kaemferol, quercetin, and tetrocrisin from the leaves of *T. indica* (Dogarai, 2011). Wogonin compound was reported active as antibacterial (Lima et al., 2014) and this compound also found from the fruit extract (Fitriya, Anwar & Sari, 2009). Another chemical compound that has been reported in the leaves is betulinic acid, β-sitosterol, and lupeol. Betulinic acid, also reported from the stem of *T.indica* (Dogarai, 2011; Abdullah, Ismail, Jamaludin & Hashim, 2013; Harrison, Sia & Sim, 1994). *T. indica* also reported having various biological activity such as anti-HIV, antidiabetic, antibacterial, and anti-inflammation (Lima et al., 2014; Hasan et al., 2017, Ahmed et al., 2012; Ragesh, Suja, Vilash, Aneeshkumar & Rajasekharan, 2016). Based on literature studies, information on the chemical content, especially in the stem of *T. indca* is still very limited. Muharni, Elfita, Adillah, Yohandini, & Julinar (2018) has been reported the

wogonin compound from the ethyl acetate extract. In this paper, we will report the isolation and characterization of two compounds from the *n*-hexane extract of stem bark of *T. indica*.

EXPERIMENTAL SECTION

Materials

The materials : The stem bark simplicia of *T. indica*.The fresh stem bark of *T.indica* was collected in January 2018 from Musi Banyuasin South Sumatera Indonesia and identified by Dr. Laila Hanum (number specimen VIC 2702), Head of the Botany Laboratory, University of Sriwijaya. The stem bark (2 kg) is cut into pieces and then dried at room temperature after that the stem bark was ground until fine so that obtained the dried powder. Material for isolation: *n*-hexane, ethyl acetate, methanol, acetone pa, chlotoform p.a, cerium sulphate, silica gel G 60 (70-230 mesh), Thin Layer Chromatography (TLC) plate 60G F_{254} Merck (Art 5554). All technical quality solvents were distilled before used.

Instrumentation

Melting point was determined by Gallen Camp melting point apparatus. UV spectrum was recorded on spectrophotometer ultraviolet Beck DU-7500. The IR (KBr) spectrum was recorded on Perkin Elmer-FTIR spectrometer, Shimadzu UV-168A. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Agilent DD2 (^{1}H-NMR500 MHz, ^{13}C-NMR125 MHz),ultra-violet lamps used CAMAG 254 nm, Thin Layer Chromatography (TLC) was performed with silica gel 60G F_{254} using Merck (Art.5554) and spots were visualized by cerium sulfate vapors and ultra violet light.

Extraction and Isolation

The dried powdered plant material about 550 g was macerated with *n*-hexane for 24 hours The extraction process were repeated three times.The *n*-hexane extract was concentrated by *Rotary evaporator* at about 50°C and obtained crude *n*-hexane extract (250 mg). The crude *n*-hexane (250 mg) was dissolved in the of acetone and adsorbed on 500 mg silica gel (70-230 mesh), stirred evenly and the slurry formed was allowed to dry. Crude *n*-hexane extract was separated by column chromatography technique using silica gel G60 (70 -230 mesh) 60 g as stationary phase. The column (60 cm x 2 cm) was prepared by place cotton at the bottom of the column. The slurry of silica in *n*-hexane was poured into the column. The silica slurry were compacted by flowing *n*-hexane solvent repeatedly. The Extract has been preparate was charged into the column. The column was first eluted with *n*-hexane and then with the solvent *n*-hexane: ethyl acetate (9:1 to 3:7). The fraction of 10 mL were collected in bottles vial and evaporated.TLC analysis toidentify the component with UV lamp and cerium sulfate to the component viewer. Vials showed same TLC patters were combined into one fraction, and obtained 4 fractions, F1 (vials 1-2, 52 mg), F2 (vials 3-5.81mg), F3 (vials 6 -15.32 mg) dan F4 (vials 15-20.15 mg).

Fraction F1 (52 mg) was again separated and purified by open column chromatography using eluent *n*-hexane: ethyl acetate (9: 1 and 8: 2) 150 mL respectively. Base on TLC patters was combined into one sub fraction and obtained 2 subfractions $F_{1.1}$ (17 mg), and $F_{2.2}$ (22 mg). The subfraction F1.1 which on concentration yielded a residue which was after purification with *n*-hexane solvent was obtained pure compound white crystalline 9 mg (**1**). Fraction F2 after being separated and purified by open column chromatography method, based on TLC patters into combined one subfraction and obtained 3 subfractions F2.1 (28 mg), F2.2 (12 mg), and F2.3 (24 mg). Subfraction F2.1 after purification with *n*-hexane solvent was obtained colorless amorphous powder 22 mg (**2**). The TLC patters of the two isolated compounds using cerium sulfate showed one spot in various eluents.

Characterization of the compounds

The structure of compounds was determined the structure using UV, IR,^{1}H-NMR, ^{13}C-NMR and DEPT spectroscopy.

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RESULTS AND SISCUSSION

Compound **1** is white crystalline, m.p 135 – 137°C. Spektrum UV (MeOH) indicates maximum absorption at λ_{max} 206 nm. The UV spectrum indicates that the isolated compound is a steroid group because there is no visible absorption for the chromophore group. IR absorptions bands appeared at IR Spectroscopic analysis, the observed absorption bands are bands appeared at (λ_{max} cm^{-1}) 3423 cm^{-1}(O-H stretching), 2935 and 2886 cm^{-1}(aliphatic C-H stretching asymmetric and symmetric), 1463 and 1377 cm^{-1} (cyclic$(CH2)_n$ bending), and the absorption frequency at 1056 cm^{-1} (C-O). The absorption frequencies in IR spectrum show similarity the absorption frequencies for β-sitosterol.

^{1}H-NMR ($(CD_3)_2CO$, 500 MHz) δH ppm and ^{13}C-NMR ($(CD_3)_2CO$, 125 MHz) δ_C ppm for compound **1** can see **Table 1**. The proton ^{1}H-NMR spectrum showed the proton of H-3 appeared as δ_H 3.30 ppm (1H,*m*), and signal for vinylic proton appeared at δ_H 5.20 (1H,*m*) indicating the presence of one methine group that is characteristic of β-sitosterol. ^{1}H-NMR spectrum of compound **1** showed the presence of 6 high intensity peaks at δ_H 0.71; 0.81; 0.83; 0.86; 0.96; and 1.02 ppm, indicating the presence of six methyl groups (**Figure 1**). From the 6 methyl proton signals, two between shown as double signal supported by ^{1}H-NMR spectrum and the DEPT spectrum which is only shows 6 methyl carbon signals. The ^{13}C-NMR spectra shown signals at δ_C 141.5 and 120.7 ppm recognizable as signal which

assigned at C-5 and C-6 double bonds respectively in β-sitosterol. Spectra ^{13}C-NMR compound **1** show twenty nine carbon signal. To determined the type of carbon, methyl methylene, methine or quaternary carbon can be used DEPT spectrum (**Figure 2**). Base on DEPT spectrum show consists of six methyls at δ_C11.3; 11.4; 18.3; 18.4; 18.9 and 19.20 ppm, nine methylenes at δ_C 37.4 ; 31.6; 42.4; 31.7; 22.9; 39.8; 24.1; 28.1; 20.9 ppm, eleven methines at δ_C 70.8; 120.7; 36.0; 50.3; 56.8; 56.0; 40.5; 45.8; 33.8; 25.9; 31.9 and three quaternary carbons at δ_C 141.5; 36.4 and 42.20 ppm. Signal carbon appeared at δ_C 70.3 characteristics for C-3.

Table 1. ^{1}H and ^{13}C NMR chemical shift values for compound **1**[a] (^{1}H-500 MHz, ^{13}C-125 Mhz, in $CDCl_3$) and β-sitosterol[b](^{1}H-400 MHz, ^{13}C-100 Mhz, in $CDCl_3$, ppm)(Pierre & Moses, 2015)

C No.	δC (ppm)[b]	δC (ppm)[a]	δH (ppm)[b]	δH (ppm)[a]	DEPT
1	36.8	37.4			CH_2
2	29.2	31.6			CH_2
3	78.6	70.8	3.51 (1H, *m*)	3.38 (1H,*m*)	CH
4	39.3	42.4			CH_2
5	140.4	141.5			C
6	121.2	120.7	5.31 (1H, *t*)	5.31(1H, *t*)	CH
7	31.4	31.7			CH_2
8	31.3	36.0			CH
9	49.6	50.3			CH
10	36.4	36.4			C
11	20.6	22.9			CH_2
12	38.3	39.8			CH_2
13	41.8	42.2			C
14	56.1	56.0			CH
15	23.8	24.1			CH_2
16	27.8	28.1			CH_2
17	55.4	56.8			CH
18	11.6	11.4	1.03 (3H, *s*)	1.02 (3H, *s*)	CH_3
19	19.1	19.2	0.71 (3H, *s*)	0.71 (3H,*s*)	CH_3
20	35.4	40.5			CH
21	18.6	18.9	0.91 (*d*)	0.96 (3H, *d*)	CH_3
22	33.3	33.8	4.98 (*m*)	5.01 1H, *dd*)	CH
23	25.4	25.9	5.14 1(H, *m*)	5.21 (1H, *dd*)	CH
24	45.1	45.8			CH
25	28.6	31.9			CH
26	19.7	18.4	0.80 (3H, *d*)	0.81 (3H, *d*)	CH_3
27	18.9	18.3	0.82 (3H, *d*)	0.83 (3H, *d*)	CH_3
28	22.1	20.9			CH_2
29	11.7	11.3	0.83 (3H, *t*)	0.86 (3H, *t*)	CH_3

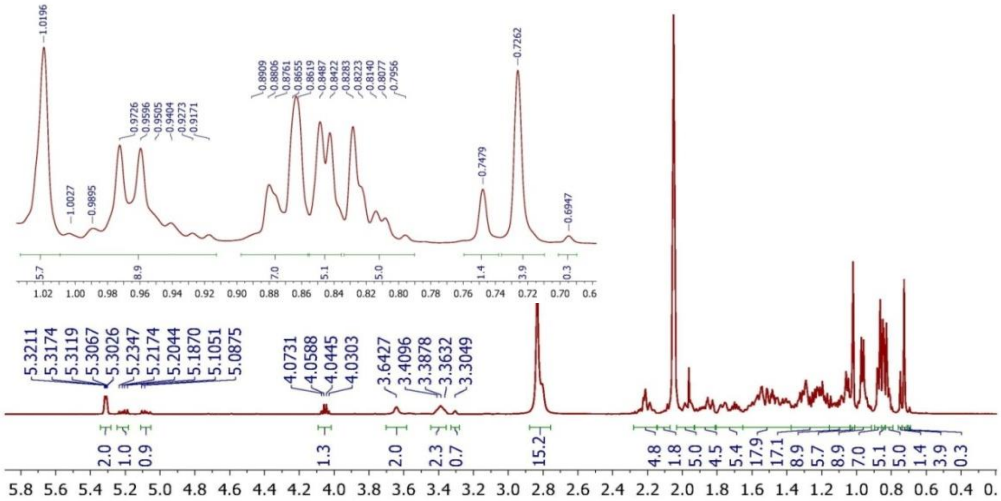

Figure1. Spectrum ^{1}H NMR β-sitosterol (**1**)

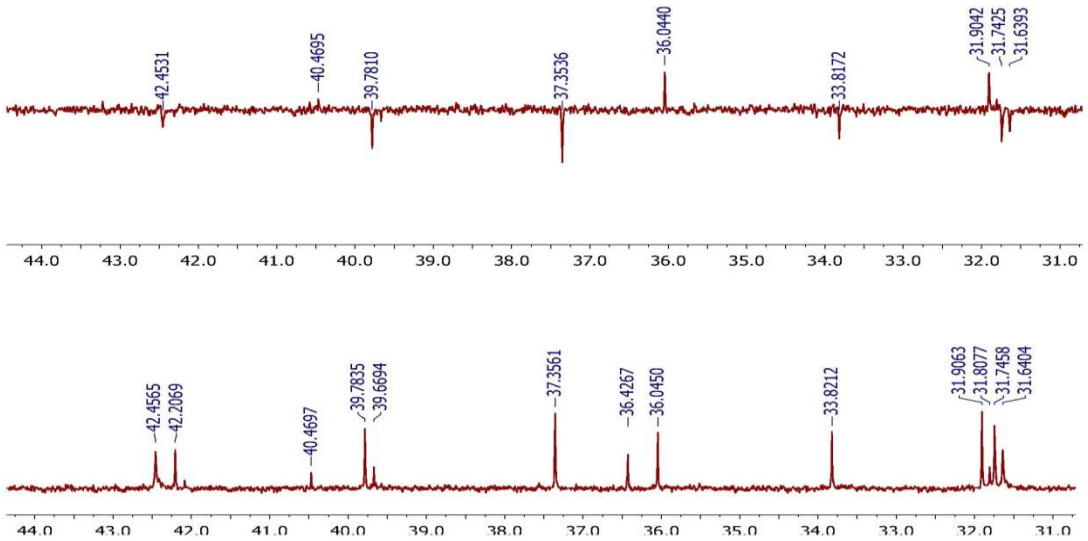

Figure 2. Spektrum DEPT β-sitosterol (**1**)

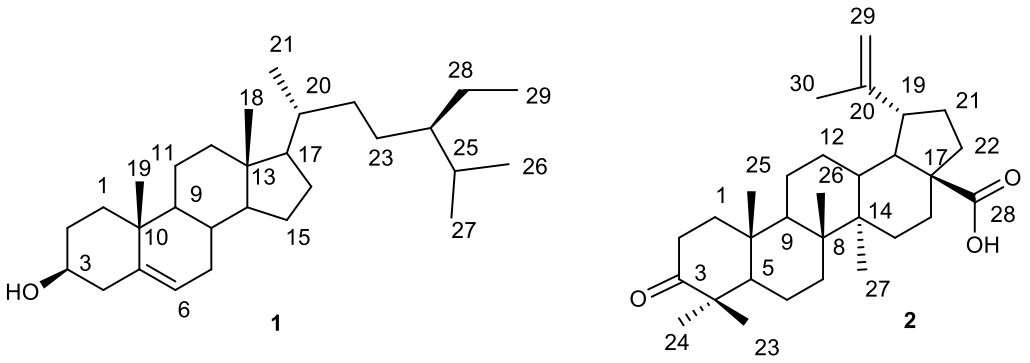

Figure 3. Structure β-sitosterol (**1**) and betulonic acid (**2**)

The UV, IR, ^{1}H NMR, and ^{13}C NMR spectra data of compound **1** has been comparison with literature data of β-sitosterol and show the structure of compound **1** is β-sitosterol. (**Figure 3**). The β-sitosterol has also been previously reported from MeOH extract leaves of *T.indica* but for the first time reported from the extract of stem bark of *T. Indica*.

Compound **2** is colorless amorphous powder m.p 291-293^{0}C. UV spectrum showed no patterns above λ_{max} 210 nm. IR spectrum appeared absorption band at 3338 cm^{-1}(O-H streching), which is strengthened with the absorption frequency at 1037 (C-O). IR spectrum also appeared absorbtion at 2935 and 2886 cm^{-1} (C-H aliphatic streching), 1641 cm^{-1} (C=C isolated) 1456 cm^{-1} (cyclic(CH2)$_n$ bending and 1381 cm^{-1} (gem dymethyl -CH(CH$_3$)$_2$). In the IR spectra of this compound the present several vibrations characteristic of the triterpenoid compound.

The ^{1}H-NMR spectrum of the isolated compound **2** (**Figure 4**) shows signal peaks that accumulate below the δ_H 3 ppm area and also the peak for the dominant methyl signals. This indicates that the isolated compounds are triterpenoid or steroid groups. The widening of the ^{1}H- NMR spectrum in the area of δ_H 0.85-1.80 ppm showed six signal methyl groups at δ_H 0.95 ppm (3H,*s*); 1.00 (6H,*s*); 1.04 (6H,*brs*); δ_H 1.70 ppm (3H,*s*). The signal at 4.59 (1H, *brs*) and 4.72 (1H,*brs*) for two signal vinylic protons. The ^{13}C-NMR spectrum (**Figure 5**) shows a characteristic spectrum for triterpenoids where the signals appear to accumulate below δ_C 60 ppm which is a signal for C, CH, CH$_2$ and CH$_3$ from cyclo aliphatic from triterpenoid.

The ^{13}C-NMR spectrum shows 30 signal (**Table 2**), 4 signal appeared on δ_C 100 ppm at δ_C 110.0; δ_C 151.6 ppm for C=C and two signal at δ_C 177.5 ppm (C=O acid) and δ_C 216.4 ppm (C=O ketone). In the ^{13}C-NMR spectrum no signal appears at δ_C 73−75 ppm which the characteristics for signal C-3 which bind OH which is commonly found in triterpenoid compounds.To determine the type of carbon determined from the DEPT 135 spectrum so that it can be determined the amount of C

quaternary, CH (methine), CH_2 (methylene), and CH_3 (methyl). In the DEPT 135 spectrum, the CH_3 and CH signals appear upward, the CH_2 signal downwards and the C quartener signal does not appear. DEPT Spectrum show 6 signal methyl at 14.9; 16.3; 16.4; 19.5; 21.3 and 30.1 ppm), 11 methylene signal at δ_C (20.4; 22.2; 26.4; 30.5; 31.3; 32.7; 34.4; 34.5; 37.5; 40.2 and 110.0 ppm), 5 methine signal at δ_C (39.1; 47.9; 49.8; 50.6; and 55.5 ppm) and 8 signal C quaternary at δ_C (37.6; 41.5; 43.3; 47.7; 56.8; 15.6; 177.5; and 216.4 ppm).

The UV, IR, ^{1}H-NMR, and ^{13}C-NMR spectra data and their comparison with those described in literature (Liu et al., 2012) showed in **Table 2**. Base on data in **Table 2** the structure of compound **2** is betulonic acid (**Figure 3**). Comparison of ^{13}C-NMR data in **Table 2** shows that the isolated compound show a chemical shift value that is almost the same as the comparison data, while there is a slight difference in chemical shift value because the solvent used to isolated compound of solvent $(CD_3)_2CO$ which is different from the literature data that used solvents $CDCl_3$.

Liu et al., (2012) reported that betulonic acid compound from root extracts of *Belamcanda chinensis* (L.) which had biological activity as antitumor. According to Shabana, El Sayed, Yousif & Sleem, (2011) in *Harpephyllum caffrum* leaves also contain betulonic acid which is active as an antioxidant and antitumor. Betulonic acid alsowas found by Zhang, Li, Fong, & Soejarto, (2016) in leaves of *Vatica cinerea* King (Dipterocarpaceae) which showed anti-HIV activity. Betulonic acid has been widely reported from other species but for the first time reported from the extract of stem bark of *T. indica*.

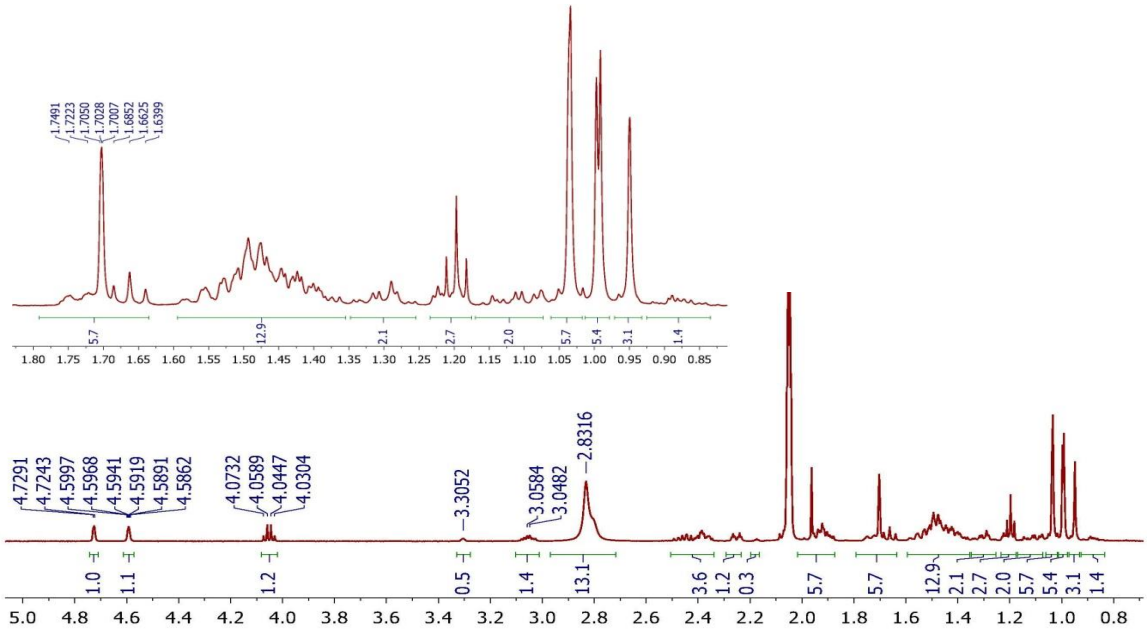

Figure 4. Spectrum ^{1}H NMR betulonic acid (**2**)

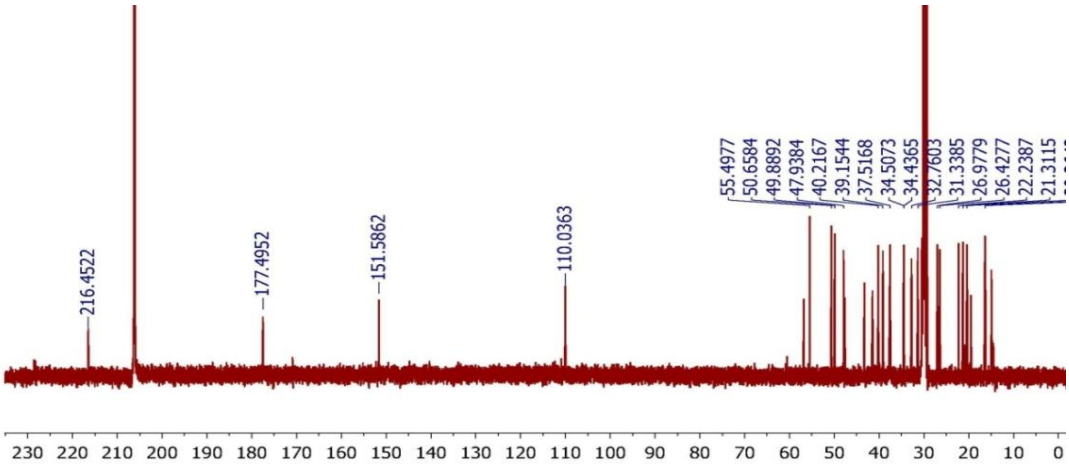

Figure 5. Spectrum ^{13}C NMR betulonic acid (**2**)

Table2. ^{1}H and ^{13}C NMR chemical shift values for compound **2**[a] (^{1}H-500 MHz, ^{13}C-125 MHz, in $(CD_3)_2CO$) and betulonic acid[b] (^{1}H-400 MHz, ^{13}C-100 MHz, in $CDCl_3$, ppm)(Liu et al., 2012)

C No.	δC (ppm)[b]	δC (ppm)[a]	DEPT
1	39.6	40.2	CH_2
2	34.1	34.1	CH_2
3	218.5	216.4	C
4	47.4	41.5	C
5	54.9	55.5	CH
6	19.6	20.4	CH_2
7	33.6	34.4	CH_2
8	40.6	43.3	C
9	49.9	50.7	CH
10	36.9	37.7	C
11	21.4	22.2	CH_2
12	25.5	26.4	CH_2
13	38.5	39.1	CH
14	42.5	47.7	C
15	29.7	30.5	CH_2
16	32.1	32.7	CH_2
17	56.4	56.8	C
18	49.2	49.9	CH
19	47.0	47.9	CH
20	150.4	151.6	C
21	30.6	31.3	CH_2
22	37.1	37.5	CH_2
23	26.7	26.7	CH_3
24	21.0	21.3	CH_3
25	15.8	16.3	CH_3
26	16.0	16.4	CH_3
27	14.6	14.9	CH_3
28	182.4	177.5	C
29	109.8	110.0	CH_2
30	19.4	19.5	CH_3

CONCLUSIONS

Two compounds β-sitosterol (**1**) and betulonic acid (**2**) were successfully isolated and identified from the *n*-hexane extracts of stem bark of *Tetracera indica*. betulonic acid was first reported from *T. indica*.

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