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Isolation and Characterization of Triterpenes from Petroleum Ether and Ethyl acetate Extracts of Stem Bark of *Parinari curatellifolia* Planch Ex. Benth *(Chrysobalanaceae)*

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Abstract

Parinari curatellifolia (Chrysobalanaceae) is a plant used in Nigerian folk medicine for cancer treatment. Through series column chromatography, betulin (lup-20(29)-en-3β,28-diol) and betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid) have been isolated from petroleum ether and ethyl acetate extracts of the stem bark of *Parinari curatellifolia* respectively. The compounds were characterized on the basis of 1D-NMR (^{1}HNMR, ^{13}CNMR and DEPT-45, 90 and 135), 2D-NMR (HSQC, HMBC, ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY), MS and IR spectroscopic studies. These two compounds are reported for the first time as constituents in *Parinari curatellifolia*.

Key words: *Parinari curatellifolia*, Chrysobalanaceae, Betulin, Betulinic acid and Triterpene

1. Introduction

Biologically active compounds isolated from plants have played an enormous roles in the development of new drugs. These compounds are synthesised by plants during their normal metabolic activities and sometimes they are synthesised when the plant needs to adapt to a particular change within its environment. These compounds have complex diverse chemical structures and they are often referred to as secondary metabolites. The terpenes are an example of a class of plant secondary metabolite. Researches have shown that they play an important role in exerting various physiological actions in humans and other animals (Mohammad, 2006; David, 2001). Terpenes have been used as drugs and a notable example is artemisinin. Artemisinin is a diterpene and artemisinin-based drugs are used today as first-line treatment against malaria (Christen and Veuthey, 2001). Another good example that demonstrates the clinical use of terpenes is taxol, a diterpenoid which is a potent anticancer agent. The volatile oils which have been used extensively in aromatherapy are composed mainly of terpenes.

Parinari curatellifolia (Planch ex. Benth) Chrysobalanaceae, is aplant used in Hausa traditional medicine in Northern Nigeria for treatment of cancer and different parts of the plant is used to remedy ailments and several other diseases. It is locally called '*Ruura*'. A hot infusion of the bark of the plant is used in the treatment of pneumonia and a leaf decoction is either orally administered or soaked in bathwater as a fever remedy. The crushed or pulped leaves are consumed to dress fractures and dislocations, and it is also used as an antiseptic to treat wounds, sores and cuts. After being stripped, the twigs can be used as chewing sticks for dental hygiene (Sidi *et al*., 2006; Hines and Eckman, 1993; Orwa *et al*., 2009). Teeth are also washed with the root infusion for toothache (Sidi *et al*., 2006). The root and bark are used in the treatment of several diseases and snake bites. The plant is also used in the treatment of diabetes (Ogbonnia *et al*., 2009).

The isolation and characterization of β-sitosterol, stigmast-4-en-3-one and stigmasterol from petroleum ether extract of the stem bark of *Parinari curatellifolia* (Planch ex. Benth) Chrysobalanaceae have been reported in the first part of this research (Halilu *et al*., 2013). The present paper, reports the isolation and characterization of two triterpenes from *Parinari curatellifolia*.

2. Materials and Methods

2.1 Collection and Identification of Plant Material

The leaves, flowers, fruits and root bark of the plant were collected from Zaria, Kaduna State, Nigeria, in September, 2011 and transported to the Herbarium Unit, Department of Biological Sciences, Faculty of Science, Ahmadu Bello University, Zaria, Nigeria for identification. Voucher number 903 was assigned to the herbarium specimen.

2.2 Drying and Preservation of Plant Material

The stem bark of the plant was shed dried for one week and repeatedly weighed until a constant weight. It was powdered using mortar and pestle. The powder was stored in an air-tight plastic container until required for use.

2.3 Extraction - Serial Exhaustive Extraction

The powdered stem bark was extracted serially with petroleum ether and ethyl acetate. The plant material (3 kg) was extracted (defatted) with 8 L of petroleum ether with the aid of a soxhlet extractor. The mark was allowed to dry and then extracted with 6 L of ethyl acetate. The extracts were filtered and concentrated at reduced pressure on a rotary evaporator.

2.4 Chromatographic Materials and Reagents

The materials and the reagents/ solvents used were all of analytical grade and were obtained from Merck Chemical Company Germany and Sigma-Aldrich. They include: Petroleum ether (Sigma-Aldrich-St. Louis, MO, USA), Chloroform (Sigma-Aldrich-St. Louis, MO, USA), Ethyl acetate (Merck-Germany), Hexane (Merck-Germany), TLC silica gel pre-coated plates (Merck-Germany), Silica gel-60 for column (0.063-0.200 mm; 70-230 mesh) (Merck-Germany), Phosphomolybdic acid (PMA) (Sigma-Aldrich-St. Louis, MO, USA), Anisaldehyde (Sigma-Aldrich-St. Louis, MO, USA), Sulfuric acid (Sigma-Aldrich-St. Louis, MO, USA).

2.5 Equipment / Reagents for Structure Elucidation

GC-MS Agilent Technologies 6890N, USA, Perkin Elmer Spectrum RX FT-IR System, Germany, NMR Top spin 300 MHz and 400 MHz Bruker-Germany, melting point apparatus (Gallencamp, USA)., deuterated chloroform ($CDCl_3$), Merck- Germany, dichloromethane, Merck- Germany and deuterated dimethylsulfoxide ($DMSO\text{-}d_6$), Merck Germany.

2.6 Thin layer Chromatography

A mixture of chloroform / ethyl acetate (4:1) was used to determine the separation profile of the petroleum ether fraction. A mixture of ethyl acetate / hexane (4:1) was used to determine the separation profile of the petroleum ether fraction. The extracts were dissolved and spotted on pre-coated silica gel TLC plates. The plates were ran in specified solvent systems at different times. To develop, the plates were sprayed with 5% H_2SO_4 or phosphomolybdic acid (PMA) solution and heated in an oven for 5 minutes at 105 ^oC or heat gun.

2.7 Isolation Procedure - Column Chromatography

A petroleum ether slurry of silica-gel powder (200 g) was packed in a glass column (30 x 35 cm). The extract (5 g) in a fine powdered form was loaded onto the column and allowed to stabilize for 2 hours before elution commenced. The column was eluted in gradient profile. The elution began with petroleum ether 100% and chloroform was added gradiently from 0 to 100%. The elution continued with the addition of methanol from 0 to 100%. Several fractions (10 ml each) were collected and monitored by TLC (chloroform 100%) and sprayed with 5% sulfuric acid. Similar fractions were pooled and concentrated *in vacuo*. One major compound was obtained and designated as C_4. Further purification of the compound was carried out by repeating column chromatography. Another compound, $C_{5,}$ was isolated from the ethyl acetate extract using similar procedure a iterated in the section above (Section 2.7) . The ethyl acetate 4 g was loaded onto a column packed with 150 g of silica gel in hexane. The elution began with hexane 100% and ethyl acetate was added gradiently from 0 to 100%. Several fractions (10 ml) were collected and monitored by TLC (Ethyl acetate / Hexane 4:1) and stained with phosphomolybdic acid (PMA) solution. Similar fractions were pooled and concentrated *in vacuo* to give rise to the major compound, C_5 Further purification of the compound was carried out by repeating column chromatography.

2.8 GC – MS Sample Preparation/ Analysis

The isolated compounds were weighed (1 mg) and dissolved in 200 µL of dichloromethane in a glass vial and then injected into the GC - MS for analysis.

2.9 NMR Sample Preparation/ Analysis

The isolated compounds were weighed (10-25 mg) and dissolved in 0.5 ml of deuterated solvents (methanol, chloroform and dimethylsufoxide) and then subjected to 1D and 2D NMR analyses.

2.10 IR Sample Preparation/ Analysis

The pure compounds were weighed (3 mg) and mixed with 5 mg of KBr and then ground to a very fine powder. The powder was compressed under high pressure using a press to produce pellets of the compounds to be analyzed. The pellets were then analysed.

2.11 Melting Point Determination/ Sample Preparation

Approximately 3 mg of the solid samples were loaded into separate capillary tubes and the melting points determined on an electronic melting point apparatus. The melting points were taken as a range of the beginning and total melting temperatures.

3. Results

3.1 Extraction of Plant Material

The powdered stem bark of *P. curatellifolia* was serially extracted with petroleum ether and ethyl acetate. The masses of the extracts and the percentage yields are presented in Table 1.

3.2 Column Chromatography of Petroleum Ether and Ethyl acetate Extracts

Two compounds were isolated by column chromatography of the petroleum ether and ethyl aceatate extracts of *P. curatellifolia*. Their masses and physical appearances are presented in Table 2.

3.3 Mass Spectrometry of Compound C_4

The molecular ion $[M]^+$ and other fragment ions were obtained by electron impact mass spectroscopy (EIMS). The mass spectrum showed the molecular ion at M/Z 442, with characteristic peaks of other fragment ions at M/Z 424, 393, 205 and 189. The M/Z 424, can be attributed to a loss in mass of 18 probably due to dehydration (H_20 = 18). The M/Z 393, is associated with loss in mass of 31 which is an equivalent mass to (CH_2OH), M/Z 189 and 234 may be as result of retro-Diels Alders fragmentation occurring in the molecule. The ion with the highest relative abundance is M/Z 85 which is the base peak. Several other fragment ions are also seen in Figure 1.

3.4 Proton Nuclear Magnetic Resonance Spectroscopy (^{1}HNMR) of Compound C_4

Characteristic signals in the ^{1}HNMR of compound $_4$ included δ_H (ppm) 4.70 (1H, br.s) 4.58 (1H, dd, *J=11.1*, Hz), 1.60 (1H, br,s) and 1.28 (1H, br.s). The spectrum also showed signals between δ_H (ppm) 0.73, 0.80, 0.95, 0.96, 1.60 and 1.62 which are the characteristics of methyl (CH_3) protons. Other signals due to methylene (CH_2) protons are also seen as presented in Table 3.

3.5 ^{13}C NMR Spectrum of Compound C_4

Several signals were observed in the spectrum. They included δ_c (ppm) 150.2 and 109.6 which are due to alkene, The signal at 78.9 is due to carbon holding the aliphatic OH group. Other signals were observed between the range of 10 to 55 which are characteristic of the methyl (CH_3),the methylene (CH_2) and the methine (CH) carbons (Table 3).

3.6 Infra Red (IR) Spectroscopy of Compound C_4

The IR spectra of compound C_1 showed ν_{max} (KBr): 3421.08 cm^{-1} (aliphatic OH), 2941.4 cm^{-1} (CH_3 bending), 2868.89 cm^{-1} (CH_2 stretching), 1688.58 cm^{-1} (OH stretching), 1453.17 cm^{-1}(C=C stretching), 1374.90 cm^{-1} (isopropyl).

3.7 Suggested Structure of Compound C_4

From the the MS, NMR and IR data which compared well with available literature, the suggested structure of compound C_1 is betulin (lup-20(29)-en-3β,28-diol) (Figure 2).

3.8 Structure Determination of Compound C_5

Compound C_5 was subjected to NMR and IR spectroscopy for characterization.

3.9 Proton Nuclear Magnetic Resonance Spectroscopy (^{1}HNMR) of Compound C_5

Prominent signals observed in the proton NMR spectra include δ_H (ppm) 12.02 (1H, br.s), which is characteristic of the hydrogen of the hydroxyl group of an carboxylic acid (COOH), δ_H (ppm) 4.67 (1H,s) and 4.54 (1H,s), which are the singlet hydrogens characteristic of alkene. Several clusters of signals occurred between the range of δ_H (ppm) 0.8 to 1.1, which are characteristic of methyl hydrogens (CH_3) and others between 1.1 to 1.5 which are characteristic of methylene (CH_2) protons. This data is presented in detail in Table 4.

3.10 ^{13}Carbon NMR Spectrum of Compound C_5

Signals observed in this spectra included δc (ppm) 177.2, 150.2, 109.6, 76.7 as characteristic signals and others as presented in the Table 4. The quarternary carboxylic carbon chemical shift is observed at 177.2 while the alkene is observed at 150.2 and 109.6. The signal at 76.7 is due to carbon holding the aliphatic OH group.

3.11 Infra Red (IR) Spectroscopy of Compound C_5

The IR spectra of the compound showed ν_{max} (KBr): 3427.05 cm^{-1} (aliphatic OH), 2941.72 cm^{-1} (CH_3 bending), 2868.79 cm^{-1} (CH_3 stretching), 1687.79 cm^{-1} (C=O stretching), 1452.0 cm^{-1} (OH caboxylic acid) and 1376.0 cm^{-1} (isopropyl).

3.12 Suggested Structure of Compound C_5

From the NMR and IR data, which compare well with available literature, the suggested structure of compound C_5 is betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid) (Figure 3).

4. Discussion

Compound C_4 was isolated as a white powder, soluble in chloroform, with a melting point of 255- 257 ^oC. The mass spectral data showed molecular ion peak $[M]^{\cdot+}$ with M/Z 442, with characteristic peaks of other fragment ions at M/Z 424, 393, 234 and 189 (Figure1) The molecular ion peak 442 represents the intact molecule and also give the exact molecular weight of the compound. The M/Z 424 of the fragment ion is associated with the loss in mass of 18 [$M^{\cdot+}$ – 18]. The decrease in mass of 18 may be attributed to a loss of the element of water (H_2O) molecule. It gives a useful information about the type of functional group present in the molecule. The loss of a water molecule in the compound suggests the presence of a hydroxyl (OH) group. The M/Z 393, is associated with the loss in mass of 31 , It also gives vital information about another functional group present in the molecule. The loss in mass of 31 in a molecule, generally correspond with the loss of a -CH_2OH, thus suggesting the presence of a t -CH_2OH group in the compound. The M/Z 189 and 234 were produced as a result of retro-Diels- Alders fragmentation that occurred in the molecule (Assimopoulou and Papageorgiou, 2005). This information provides a worthwhile evidence of the structural arrangement of the compound, suggesting that the compound is composed of rings. Generally, retro-Diels Alders fragmentation occurs in triterpenes and steroids which biogenetically originates form the same precursor (biogenetic isoprene). The decomposition usually takes place in ring C of both triterpenes and steroids. The presence of M/Z 189 and 234 from M/Z 424 suggest that the compound is a triterpene (Figure 1). The ^{1}H-NMR (Table 3) showed six methyl (CH_3) signals at δ_H (ppm) 0.73 (3H), 0.80 (3H), 0.95 (3H), 0.96 (3H), 0.60 (3H) and 1.62 (3H) respectively. This suggests that there are six methyl groups present in the compound. The presence of the down field signals at δ_H (ppm) 4.70 (1H, br.s) and 4.58 (1H, br.s) suggest the presence of exocyclic methylene olefinic protons of the lupane triterpenes (Ayotollahi *et al*., 2011), which are attached to carbon-29. The occurrence of two broad singlets at δ_H (ppm) 1.60 (1H, br.s) and 1.88 (1H, br.s) indicates the presence of two hydroxyl groups in the compound respectively. The ^{13}C-NMR gave signals at δ_c (ppm) 78.98 (C-3) δ_c (ppm) 56.2 (C-28) which are the hydroxylated carbons, δ_c (ppm) 150.4 (C-20), a quarternary carbon and δ_c (ppm) 109.66 (C-29), the carbon to which the olefinic protons are attached. The ^{1}H-NMR and ^{13}C-NMR of the compound are in agreement with the published literature (Seyed *et al*., 2009) (Table 3). The IR (KBr) spectra of the compound showed characteristic peaks at ν_{max} (ν, cm^{-1}) 3421.08 cm^{-1} (aliphatic OH), 2941.4 cm^{-1} (CH_3 bending) , 2868.89 cm^{-1} (CH_2 stretching), 1688.58 cm^{-1} (OH stretching), 1453.17 cm^{-1}(C=C stretching), 1374.90 cm^{-1} (isopropyl). The IR data agrees with the earlier reported work (Elvira *et al*., 2009) and Prince *et al*. (2010). The data acquired from the MS, NMR and IR are most characteristic of betulin which belongs to lup-20(29)-ene type triterpenes.

Compound C_5 was isolated as a white crystalline powder, soluble in dimethylsulfoxide (DMSO), with melting point of 297- 299 ^oC. The ^{1}HNMR (Table 4) showed six signals due to methyl (CH_3) protons respectively at δ_H (ppm) 0.83 (3H), 1.00 (3H), 1.06 (3H), 1.22 (3H), 1.80 (3H) and 1.79 (3H). There is also a signal at δ_H (ppm) 12.02 (1H, br.s), which is characteristic of the hydrogen of the hydroxyl group of carboxylic acid (COOH). This confirms the presence of an carboxylic acid functionalilty in the compound. The signals at δ_H (ppm) 4.67 (1H,s) and 4.54 (1H,s) are characteristic of the exocyclic olefinic protons which confirm the presence of an unsaturated bond in the compound. The ^{13}CNMR showed thirty carbon atom signals in the compound (Table 4). The appearance of a signal at δ_c (ppm) 177.19 which occurred in the region usually occupied by the acid carbonyl group, confirm the presence of acid functionality in the compound. The appearance of signal at δ_c (ppm) 109.6 confirm the presence of the carbon bearing the olefinic protons. The ^{13}CNMR and ^{1}HNMR data obtained agrees with the spectral results reported by Ayotollahi *et al* (2011) for betulinic acid (Table 4). The information is further supported by the IR spectral results. The IR spectra of the compound showed ν_{max} (KBr): 3427.05 cm^{-1} (aliphatic OH), 2941.72 cm^{-1} (CH_3 bending), 2868.79 cm^{-1} (CH_3 stretching), 1687.79 cm^{-1} (C=O stretching), 1452 cm^{-1} (OH caboxylic acid) and 1376 cm^{-1} (isopropyl) (fig. 4.39). The IR data agrees with the earlier work

carried out by Elvira *et al*.(2009) , Prince *et al*.(2010) , Soek *et al*.,(2010) and Ayotollahi *et al*. (2011 for betulinic acid.

5. Conclusion

On the basis of column chromatography and spectroscopic studies (MS, NMR and IR), betulin and betulinic acid have been isolated and characterized. These compounds are reported for the first time as constituents of *Parianri curatellifolia*.

6. Acknowledgement

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Table 1. Mass and Percentage Yield of Extracts

S/No.	Extracts	Mass obtained (g)	% Yield
1	Pet. Ether extract	10.12	0.33
2	Ethyl acetate extract	24.49	0.82

Table 2. Masses and Physical Appearances of Compounds Isolated

S/No.	Compounds	Mass (mg)	Physical Appearance
1.	C_4	50.0	white powder
2.	C_5	48.7	white powder

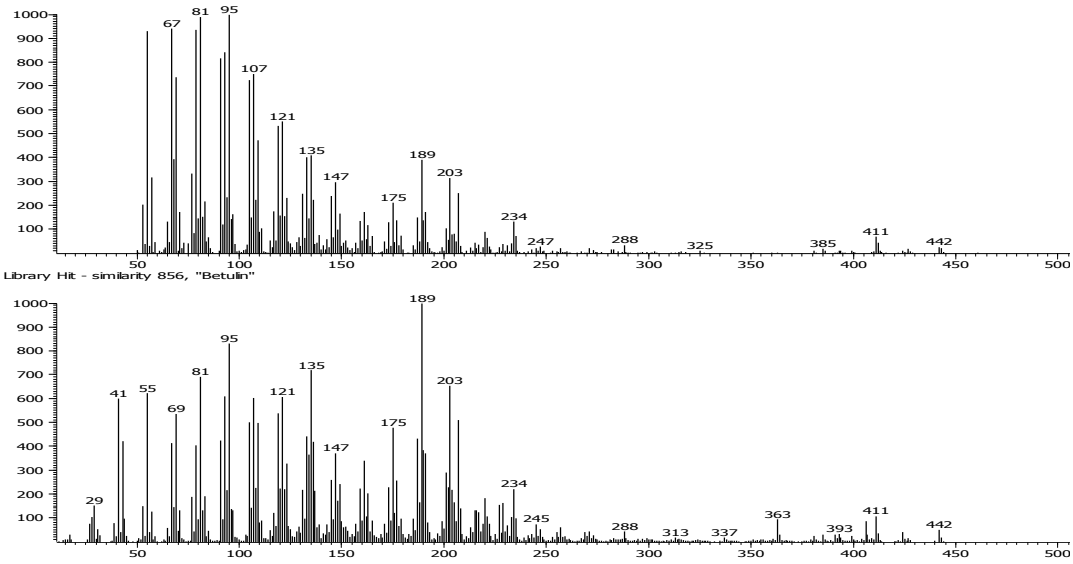

Figure 1. *Mass Spectrum of Compound C_2*

Table.3 ^{1}H and ^{13}C-NMR Chemical Shifts of Compound C_4 in $CDCl_3$, 300 MHz

H/C -Position	δ_c (ppm)	δ_H (ppm)	δ_c* (ppm)	δ_H* (ppm)	No. of H, Multiplicity, *J*(Hz)
1	38.37	0.88; 1.64	38.69	0.88; 1.63	2H, m
2	27.38	1.56; 1.52	27.37	1.57; 152	2H, m
3	78.98	3.19	78.96	3.18	1H, (dd, *J=11.1, 4.58* Hz)
4	38.85	-	38.86	-	-
5	55.33	1.38	55.28	0.67	1H, m
6	16.07	1.51; 1.27	18.28	1.51; 1.37	2H, m
7	34.31	1.38; 1.38	34.22	1.38; 1.38	2H, m
8	40.67	-	40.94	-	-
9	50.49	1.26	50.30	1.26	1H, s
10	37.19	-	37.14	-	-
11	20.83	1.42; 1.23	20.81	1.42; 1.24	2H, m
12	25.48	1.06, 1.62	25.19	1.01; 1.63	2H, m
13	38.71	1.62	37.29	1.62	1H, m
14	42.42	-	42.73	-	-
15	27.97	1.66; 1.30	27.03	1.67; 1.03	2H, m
16	29.68	1.92; 1.18	29.15	1.92, 1.19	2H, m
17	46.87	-	47.76	-	-
18	49.25	1.55	48.75	1.56	1H, m
19	42.42	2.15	47.83	2.37	1H,
20	150.42	-	150.46	-	-
21	30.92	1.92; 1.41	29.73	1.91; 1.42	2H, m
22	34.31	1.55; 1.30	33.95	1.02; 1.82	2H, m
23	20.83	0.95	27.96	0.95	3H, s
24	20.83	0.73	15.34	0.74	3H, s
25	16.07	0.80	16.09	0.80	3H, s
26	18.27	1.06	15.97	1.00	3H, s
27	15.32	0.96	14.74	0.96	3H, s
28	56.28	1.56; 1.31	60.50	1,56, 1.31	2H, s
29	109.66	4.70; 4.58	109.67	4.66; 4.56	2H, br,s
30	19.36	1.62	19.36	1.66	3H, s

*Seyed *et al*., (2009), coupling constant (*J*), singlet (s), Doublet (d), doublet-doublet (dd), multiplet (m), broad singlet (br.s)

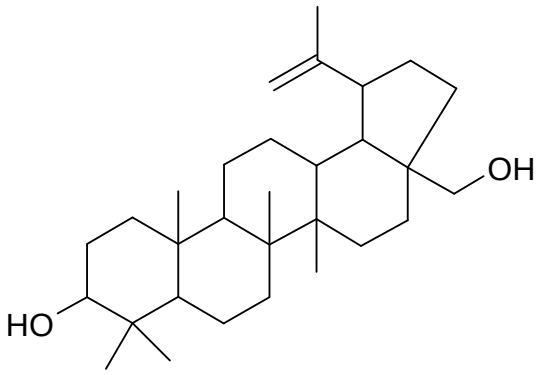

Figure 2: *Structure of Compound C_4*

Table. 4: ^{1}H and ^{13}C-NMR Chemical Shifts of Compound C_5 in DMSO, 300 MHz

H/C-Position	δ_c (ppm)	δ_H (ppm)	δ_c*(ppm)	δ_H*(ppm)	No. of H, Multiplicity, J(Hz)
1	39.22	0.98; 1.63	39.3	0.99; 1.67	1H, m, each
2	28.23	1.83; 1.46	28.3	1.85; 1.47	1H, m, each
3	76.75	3.31	78.1	3.45	1H, t (J=7.2 Hz)
4	39.50	-	39.5	-	-
5	55.38	0.82	56.0	0.82	1H, m
6	18.92	1.56; 1.38	18.8	1.56; 1.38	1H, m, each
7	33.90	1.43; 1.39	34.9	1.45; 1.38	1H, m, each
8	41.20	-	41.1	-	-
9	49.92	1.37	51.0	1.38	IH, s
10	37.56	-	37.6	-	-
11	21.05	1.43; 1.21	21.2	1.43; 1.21	1H, m, each
12	25.05	1.21; 1.84	26.2	1.21; 1.94	1H, m, each
13	38.66	2.90	38.7	2.74	1H, m, each
14	41.97	-	42.9	-	-
15	30.37	1.26; 1.84	30.3	1.26; 1.88	1H, m, each
16	32.68	1.55; 2.90	32.9	1.55; 2.63	1H, m, each
17	55.39	-	56.6	-	-
18	49.92	1.77	49.8	1.77	1H, t , (J= 11.5 Hz)
19	47.00	2.11	47.8	2.18	1H, m
20	150.27	-	151.3	-	-
21	31.69	1.53; 2.21	31.8	1.53; 2.24	1H, m, each
22	37.56	1.57; 2.20	37.6	1.57; 2.25	1H, m, each
23	29.18	1.22	28.7	1.22	3H, s
24	16.98	1.00	16.3	1.00	3H, s
25	17.95	0.83	16.4	0.83	3H, s
26	17.95	1.06	16.4	1.06	3H, s
27	18.92	1.08	14.9	1.07	3H, s
28	177.19	-	178.8	-	-
29	109.60	4.67; 4.54	109.9	4.95; 4.77	2H, s, each
30	20.44	1.79	19.5	1.79	3H, s

Robert and Samir (2004)* coupling constant (*J*), singlet (s), Doublet (d), doublet-doublet (dd), multiplet (m), broad singlet (br.s)

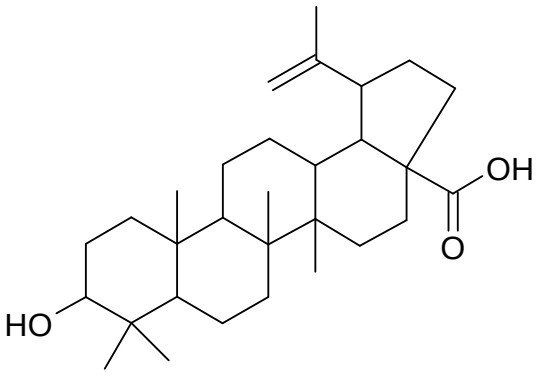

Figure 3: *Structure of Compound C_5*