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Isolation and Characterization of Steroids from Petroleum Ether Extract of Stem Bark of *Parinari curatellifolia* Planch ex. Benth (Chrysobalanaceae)

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Abstract

In our search for biologically active compounds, three steroids have been isolated from the stem bark of *Parinari curatellifolia* through series of column chromatography. Compound C_1, C_2 and C_3 where characterized as β-sitosterol (22,23-dihydrostigmasterol), stigmast-4-en-3-one and stigmasterol, respectively, on the basis of 1D-NMR (^{1}HNMR, ^{13}CNMR and DEPT-135), 2D-NMR (HSQC, HMBC, ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY), mass spectroscopy (MS) and IR spectroscopy. These compounds are reported for the first time as constituents in *Parinari curatellifolia.*

Keywords: *Parinari curatellifolia*, β-sitosterol, stigmast-4-en-3-one and stigmasterol

1. Introduction

For thousands of years, nature has been a rich source of medicinal agent. A remarkable number of potent drugs have been isolated from natural sources, particularly plants, many of which are well established in their use in traditional medicine (Cragg and Newman, 2005). Phytochemicals like terpenoids, alkaloids, flavonoids and steroids have been used either in their natural form as medicines (Salim *et al*., 2008). These compounds of plants, have been used either as starting materials or templates to synthesize more potent drugs with better activities. For example, diosgenin, a steroidal sapogenin obtained from the tubers of various *Dioscorea species* has been used as a starting material for production of progesterone, a hormone used as a female oral contraceptive. Progesterone is also a key intermediate in the production of the important anti-infammatory drug cortisone. *Parinari curatellifolia* (Planch ex. Benth) Chrysobalanaceae, is a plant used in Hausa traditional medicine in Northern Nigeria for treatment of cancer. It is locally called *'Ruura'*. The hot infusion of the bark of the plant is used in the treatment of pneumonia. A leaf decoction is either orally administered or used in a bath as a fever remedy. The crushed or pulped leaves are used in dressing fractures or dislocations, and for wounds, sores and cuts. After been stripped, the twigs can be used as chewing sticks (Sidi *et al*., 2006; Hines and Eckman, 1993; Orwa *et al*., 2009). The root and bark are used in the treatment of several diseases and snake bites. Teeth are washed with a root infusion for toothache (Sidi *et al*., 2006). It is also used in the treatment of diabetes (Ogbonnia *et al*., 2009). Traditionally the bark is used for washing clothes, vaginal douches, itchy scalp, dandruff and cough (Qasem and Abublan, 1996).

2. Methods and Materials

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 was obtained. It was powdered using pestle and mortar. 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 in 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 soxhlet extractor (Prashant, 2011). 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/ Sigma-Aldrich. They include: Petroleum ether (Sigma-Aldrich-St. Louis, MO, USA), Methanol (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 Dimethylsulphoxide ($DMSO\text{-}D_6$), Merck Germany, NMR tubes (5 mm in diameter) and Pasteur pipette.

2.6 Thin layer Chromatography

Several solvent systems were used to determine the separation profile of the petroleum ether fraction. Chloroform (100%), Chloroform / Ethyl acetate (4:1) and Chloroform / Hexane (4:1) were used. The extract was dissolved in chloroform and spotted on pre-coated silica gel TLC plates. The plates were allowed to dry and then developed in the specified solvent systems at different times. The plates were sprayed with 10% H_2SO_4, phosphomolybdic acid and/ or anisaldehyde and heated in an oven for 5 minutes at 105 ^oC.

2.7 Isolation Procedure - Column Chromatography

Silica-gel (200 g) was packed in a glass column (30 x 35 cm). The column was wet packed in petroleum ether. The extract (5 g) in a fine powdered form was loaded onto the packed silica-gel 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) were collected and monitored by TLC (chloroform 100%) and sprayed with 5% sulfuric acid. Similar fractions were pooled and concentrated *in vacuo*. Three major compounds were obtained and designated as C_1, C_2, and C_3. Further purification of the compounds was carried out by repeating the 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

The isolated compounds were weighed (10 - 25 mg) and dissolved in 0.5 ml of deuterated solvents (methanol, chloroform and dimethylsulphuroxide) 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 subjected to analysis.

2.11 Melting Point Determination/ Sample Preparation

About 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 the aid Soxhlet extractor using petroleum ether, ethyl acetate and methanol. The mass of the extracts obtained and the percentage yield are presented in Table 1.

3.2 Column Chromatography of Petroleum Ether

Three compounds were isolated from column chromatography of the petroleum ether extract of *P. curatellifolia*. Their masses and physical appearances are presented in Table 2.

3.3 Structure Determination of Compound C_1 - Mass Spectrometry of Compound C_1

The mass spectrum of compound C_1 was obtained by electron impact mass spectroscopy (EIMS). A characteristic molecular ion $[M]^{\cdot+}$ peak was observed at M/Z 414 with important fragment ions having M/Z 396, 381, 367, 313, 147 and 133. Other fragment ions also occur and are presented in Figure 1.

3.4 Proton Nuclear Magnetic Resonance Spectroscopy (1HNMR) of Compound C_1

Signals observed in the 1HNMR spectra of compound C_2 include δ_H (ppm) 5.33(m), 3.50(m), 2.24(m), 2.14(m),

1.97(m), 1.82(m), 1.64(m), 1.63(m) and 1.51(m). The spectrum showed a cluster of signals between δ_H 0.8 to 1.2, characteristic of methyl (CH_3) protons. Also other δ_H were observed between 1.2 to 1.5 which are characteristic of methylene (CH_2) protons. This is represented in Table 3.

3.5 ^{13}CNMR Spectrum of Compound C_1

Several signals were observed in the spectrum. They include δ_c (ppm) 140.7, 121.6 and 71.7. Other signals were observed between the range of δ_c (ppm) 10 to 55 which are characteristic of methyl (CH_3), methylene (CH_2), and methine (CH) carbons. This is represented in Table 3.

3.6 Infra Red (IR) Spectroscopy of Compound C_1

The IR spectrum of compound C_1 showed ν_{max} (KBr): 3431.36 cm^{-1} (aliphatic OH), 2928.06 cm^{-1} (CH_3 stretching), 1688.58 cm^{-1} (C=C stretching), 1452.47 cm^{-1}(CH_3 stretching), 1374.90 cm^{-1}.

3.7 Structure of compound C_1

From the MS, NMR and IR data, which compare well with available literature (Pateh *et al.,* 2009), the structure of compound C_1 is β-sitosterol (22, 23-dihydrostigmasterol). This is presented in Fig. 2.

3.8 Structure Determination of Compound C_2 - Mass Spectrometry of Compound C_2

Electron impact mass spectroscopy (EIMS) was used to obtain the mass spectrum of compound C_2. A characteristic molecular ion $[M]^+$ peak was observed at M/Z 412 and other fragment ion peaks at 398, 370, 327, 288, 257, 229, 187, 147 and 124 which is the most abundant ion (base peak). The fragmentation pattern is presented in Fig. 3.

3.9 Proton Nuclear Magnetic Resonance Spectroscopy (1HNMR) of Compound C_2

The proton NMR of compound C_2 showed signals at δ_H (ppm) 5.70 (1H, br.s), 2.36 (m), 2.00 (m), 1.82 (m) and other cluster of signals which ranges between δ_H (ppm) 0.8 to δ_H (ppm) 1.2 which are characteristics of methyl (CH_3) protons and others between δ_H (ppm) 1.1 to δ_H (ppm) 1.5 which are characteristic of methylene (CH_2) protons. This is presented in table 4.

3.10 ^{13}Carbon NMR Spectrum of Compound C_2

Signals seen in the spectra includes δ_c (ppm) 199.6, 171.7, 123, 55.9, 55.8 and many other peaks between 11.9 to 50.0 as presented in the table 4.

3.11 Infra Red (IR) Spectroscopy of Compound C_2

The IR spectrum of compound C_2 showed ν_{max} (KBr): 1734.26 cm^{-1} (C=O stretching), 2924.78 cm^{-1} (CH_3 stretching) , 1616.78 cm^{-1} (C=C stretching), 1462.86 cm^{-1}(CH_3 bending), 1262.14 (=C-H stretching).

3.12 Structure of Compound C_2

From the the MS data, NMR data and IR data which compare well with available literature, the structure of compound C_2 is stigmast -4-en-3-one as presented in Fig. 4.

3.13 Mass Spectrometry of Compound C_3 - Structure Determination of Compound C_3

Electron impact mass spectroscopy (EIMS) was used to obtain the mass spectrum of compound C_3. A characteristic molecular ion $[M]^+$ peak was observed at M/Z 412 and other fragment ion peaks at 394, 379, 351, 327, 255, 133, 83 and 55. This is presented in Fig 5.

3.14 Structure of Compound C_3

From the the MS data of compound C_3 , which compare well with the GC library data and the available literature, the structure of compound C_3 is stigmasterol as presented below in Fig. 6.

5. Discussion

Compound C_1 was isolated as a white crystalline powder, highly soluble in chloroform, with a melting point range of 136 – 140 ^oC. The mass spectra data showed molecular ion peak $[M]^+$ with M/Z 414, with characteristic peaks of other fragment ions at M/Z 396, 381, and 133 (Fig.1). The molecular ion peak 414 represents the intact molecule and also give the exact molecular weight of the compound. The M/Z 396 of the fragment ion is associated with loss in mass of 18 [M^+ – 18]. This loss in mass of 18 may be attributed to loss of water (H_2O). This also provides a useful information about the presence of a hydroxyl (OH) group in compound C_2. The M/Z 381, is associated with the loss in mass of 15 from M/Z 396. This can be associated to the loss of methyl (CH_3) group (M - CH_3). The M/Z 313, resulted from the loss in mass of 141 ($C_{10}H_{21}$) from the molecular ion [M^+-141]. This gives a useful information on the compound. This suggests the presence of a long external side chain. The presence of rings in the compound was confirmed by the presence of fragment ions with M/Z 133 and 147 (Fig.1). The ^{1}H-NMR showed six methyl (CH_3) signals at δ_H (ppm) 0.90 (3H), 0.91 (3H), 0.98 (3H), 0.99 (3H), 1.04 (3H) and 1.05 (3H) (Table 1) with different multiplicities indicative of different electronic environments. This suggests that there are six methyl groups present in the compound. The presence of down field signals at δ_H (ppm) 5.33 (1H, br.) suggest the presence of olefinic proton. The signal at δ_c (ppm) 121.69 confirms the presence of an olefinic carbon (C-5). The ^{13}CNMR gave 29 signals. This suggests that the compound may consists of 29 carbon atoms. The signal at δ_c (ppm) 140 indicates the presence of a quarternary carbon (C-6) atom. The carbon

at δ_c (ppm) 71 confirm the presence of carbon (C-3) bearing the hydroxyl group. This data is consistent with the earlier work published by Pateh *et al*., (2009) and it is a characteristic of β-sitosterol. The IR spectra of the compound showed ν_{max} (KBr): 3431.36 cm^{-1} (aliphatic OH), 2928.06 cm^{-1} (CH_3 stretching) , 1688.58 cm^{-1} (C=C stretching), 1452.47 cm^{-1}(CH_3 stretching), 1374.90 cm^{-1} (fig. 4.7). This information is also consistent with work of Parwaiz *et al* (2010) and Pateh *et al* (2009) and it is a characteristic of β-sitosterol. These data along with further NMR experiments, DEPT-135, COSY, NOESY, HSQC and HMBC support the structure of β-sitosterol (Fig. 2).

Compound C_2 was isolated as a white crystalline powder, highly soluble in chloroform. The mass spectral data showed molecular ion peak $[M]^+$ with M/Z 412, with other peaks of fragment ions at M/Z 397, 370, 355, 271, 147and 124 (Fig.3). The molecular ion peak 412 represents the intact molecule and also gives the exact molecular weight of the compound. The M/Z 397 is associated with the loss in mass of 15 from the molecular ion. This can be associated with the loss of methyl (CH_3) group ($M^+ - CH_3$). The M/Z 370 arises from the loss in mass of 29. This is associated with the loss of ethyl group (C_2H_5) from M/Z 397. The M/Z 355 resulted from loss in mass of 15(CH_3) from M/Z 370. The M/Z 271 resulted form loss in mass of 141 ($C_{10}H_{21}$) from the molecular ion. This gave a vital information about the presence of a bulky side chain in the molecule. This is a characteristic of steroidal fragmentation pattern. The M/Z 147 and 124 confirms the presence of rings and it resulted from the decomposition M/Z 271 (Fig.3) (Alexander *et al*., 2010). The ^{1}H-NMR (Fig. 4.20) showed six methyl (CH_3) signals at δ_H (ppm) 0.70 (3H), 0.81 (3H), 0.83 (3H), 0.90 (3H), 0.97 (3H) and 1.17 (3H). This suggests that there are six methyl groups present in the compound. The presence of down field signals at δ_H (ppm) 5.70 (1H, br.s) suggest the presence of oleifinic proton. The ^{13}CNMR gave 29 signals (Table 4) suggesting that the compound consists of 29 carbon atoms. The appearance of signal at δ_c (ppm) 199.6 gave a vital information about the primary functional group in the compound. This carbon (C-3) appeared in the region usually occupied by ketone and aldehyde carbonyl groups. The carbon is not coupled to any hydrogen and this confirms the presence of a ketonic carbonyl function in the compound. The presence of carbon δ_c (ppm) 123.0 confirms the carbon that holds the olefinic proton at δ_H (ppm) 5.70 (1H, br.s). The signal at δ_c (ppm) 171.0 confirm the presence of a quarternary carbon (C-6). This data together with COSY, NOESY and HMBC are characteristic of stigmast-4-en-3one. The IR spectra of the compound showed ν_{max} (KBr): 1734.26 cm^{-1} (C=O Stretching), 2924.78 cm^{-1} (CH_3 Stretching) , 1616.78 cm^{-1} (C=C stretching), 1462.86 cm^{-1}(CH_3 bending), 1262.14 (=C-H stretching) (fig. 4.27) . These data are consistent with the published work (Tzong-Huei lee *et al*., 2005 and Asli *et al.,* 2005). This data further confirms the information derived from the above experiments and is consistent with stigmast-4-en-3one (Fig. 4).

Compound C_3 was obtained as a white crystalline powder. It was identified on the basis of GC-MS spectral libraries which was searched for matches. The mass spectra of the compound showed molecular ion peak $[M]^+$ at M/Z 412 which represent an intact molecule and also give the molecular weight of the compound. Other fragment ion peaks occur at M/Z 394, 379, 369, 351, 327, 288, 271, 255, 133, 83 and 55 (Fig.5). The fragmentation pattern of the compound C_3 and the library generated spectra were the same. One of the major similarity between compound C_3 and the library spectra is that both have the same molecular ion peak with M/Z 412 (Fig.6). The two spectra also compared well at M/Z 351, 300, 271, 255, 133, 833 and 55. From the spectrum (Fig.6), the M/Z 394 occurred from loss of water (mass of 18) from the molecular ion $[M^+ - H_2O]$. This gave a vital information about the presence of hydroxyl (OH) group in the molecule. The M/Z 255 occurred as a result of loss in mass of 141 ($C_{10}H_{21}$) from M/Z 394. This suggests the presence of bulky side chain in the compound and this is a characteristic of steroids. The M/Z 379 resulted from loss in mass of 15 (CH_3) from 394. M/Z 133 and 146 occurred as a result of decomposition that occurred as a result of the presence of rings. These data are characteristic and consistent with stigmasterol (Fig.6).

5. Conclusion

On the basis of chromatographic and spectroscopic techniques β-sitosterol, stigmast-4-en-3-one and stigmasterol have been isolated, characterized and reported for the first time as phyto-constituents of *P. 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_1	134.0	white powder
2.	C_2	9.8	white powder
3.	C_3	4.0	white powder

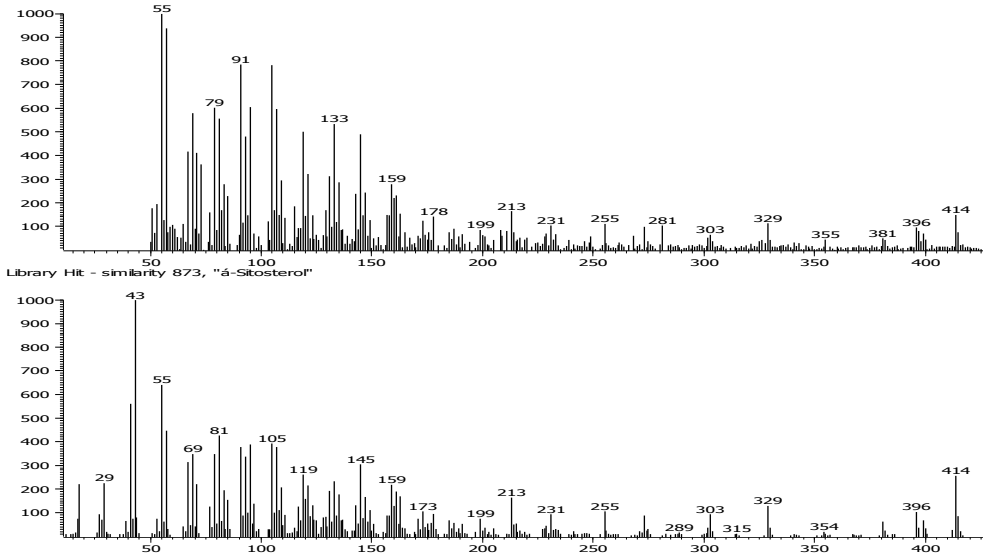


Figure 1. *Mass Spectrum of Compound C_1*

Table 3. Comparative ^{1}H and ^{13}C-NMR Chemical Shifts of Compound C_1, in $CDCl_3$, 400 MHz with a known compound.

H-Position	δ_c (ppm)	δ_H (ppm)	δ_c* (ppm)	δ_H* (ppm)	No. of H, Multiplicity, J (Hz)
1	37.2	1.13; 1.13	37.3	1.38; 1.13	2H, m
2	31.6	1.59; 1.24	31.6	1.56; 1.31	2H, m
3	71.7	3.51	71.8	3.25	1H, m
4	40.4	2.24; 1.97	42.2	2.23; 1.98	2H, m
5	140.7	-	140.8	-	-
6	121.6	5.33	121.7	5.35	1H, m
7	33.4	2.14; 1.97	31.9	2.04; 1.79	2H,
8	31.8	1.49	31.9	1.45	1H, m
9	50.1	1.45	51.2	1.44	1H, m
10	39.6	-	36.5	-	-
11	22.6	1.51; 1.23	21.1	1.52; 1.27	2H, m
12	39.7	1.56; 1.47	39.8	1.56; 1.31	2H, m
13	42.1	-	42.3	-	-
14	56.0	1.42	56.8	1.40	1H, m
15	26.0	1.63; 1.46	24.3	1.60, 1.35	2H, m
16	24.3	1.63; 1.46	28.3	1.60; 1.35	2H, m
17	56.7	1.47	56.0	1.47	1H, m
18	12.0	1.04	11.9	1.04	3H, s
19	19.0	1.05	19.4	1.30	3H, s
20	36.1	1.64	36.2	1.64	1H, m
21	19.3	0.98	18.8	0.92	3H, d, J=6.4Hz
22	33.9	1.25	33.9	1.25	2H, m
23	26.0	1.25	26.1	1.25	2H, m
24	45.8	1.46	45.9	1.46	1H, m
25	29.6	1.81	29.2	1.82	1H, m
26	21.0	0.91	19.8	0.91	3H, d, J=6.12 Hz
27	21.1	0.99	19.3	0.91	3H, d, J=5.11 Hz
28	23.0	1.49	23.1	1.55	2H, m
29	12.2	0.90	12.2	0.90	3H, m

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

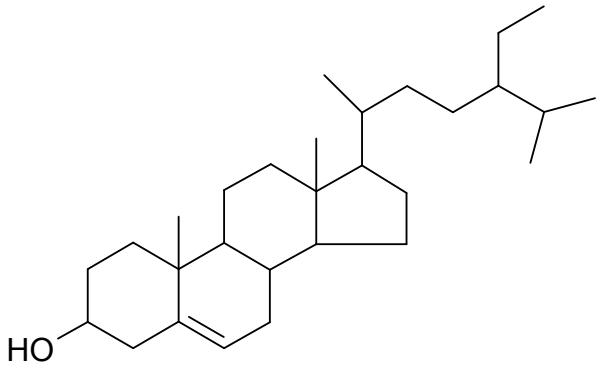
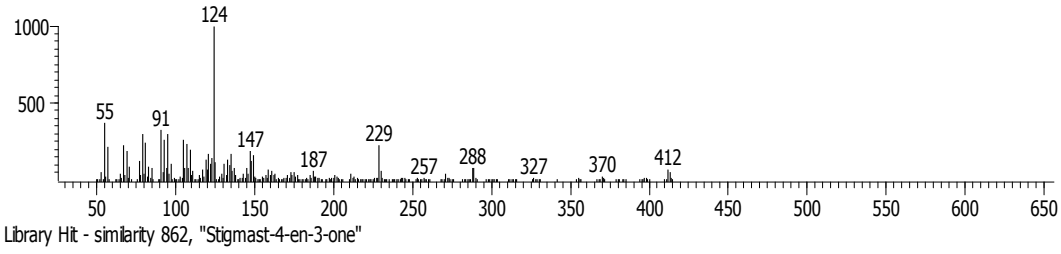


Figure 2. *Structure of compound C_1*



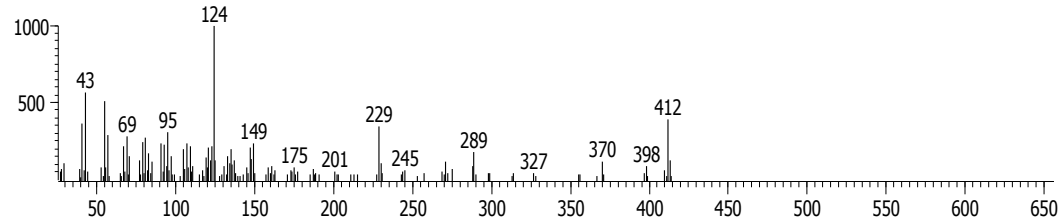


Figure 3. *Mass Spectrum of Compound C_2*

Table 4. Comparative ^{1}H and ^{13}C-NMR Chemical Shifts of Compound C_2, in $CDCl_3$, 400 MHz with a known compound.

H/C-Position	δ_c (ppm)	δ_H (ppm)	δ_c*(ppm)	δ_H* (ppm)	No. of H, Multiplicity, J (Hz)
1	35.6	1.48; 1.23	35.9	1.49; 1.24	1H, m, each
2	33.9	2.36; 2.21	34.1	2.99; 2.89	1H, m, each
3	199.6	-	199.8	-	-
4	123.7	5.7044	123.9	5.72	1H, br, s
5	171.7	-	171.8	-	-
6	32.0	2.00; 1.90	32.6	2.01;1.91	(1H, m) each
7	29.3	1.17; 1.41	29.9	1.17; 1.42	(1H, m) each
8	35.6	1.35	35.8	1.41	1H, m
9	53.8	1.32	54.0	1.41	1H, m
10	39.6	-	39.1	-	-
11	21.0	1.54; 1.26	21.2	1.52; 1.27	(1H, m) each
12	39.6	1.55, 1.31	39.8	1.56; 1.31	(1H, m) each
13	42.8	-	42.6	-	-
14	55.9	1.02	56.1	1.40	1H, m
15	24.1	1.61; 1.33	24.4	1.60; 1.35	(1H, m) each
16	33.8	1.61; 1.33	33.1	1.60; 1.35	(1H, m) each
17	55.8	1.15	56.2	1.47	1H, m
18	11.9	0.70	12.1	0.70	3H, s
19	17.3	1.17	17.6	1.17	3H, s
20	36.1	1.64	36.3	1.64	1H, m
21	186.9	0.97	18.9	0.91	3H, d, J=6.6 Hz
22	35.6	1.26; 1.26	34.1	1.25; 1.25	(1H, m) each
23	26.0	1.25; 1.25	26.3	1.25; 1.25	(1H, m) each
24	45.8	1.47	46.0	1.46	1H, m
25	28.1	1.83	28.4	1.82	1H, m
26	19.8	0.83	20.0	0.83	3H, d, J=6.6Hz
27	19.0	0.81	19.2	0.81	3H, d, J=6.6 Hz
28	23.0	1.54; 1.54	23.3	1.55; 1.55	(1H, m) each
29	14.1	0.90	12.1	0.90	3 H, t, J=6.6 Hz

Asli *et al* (2006)* coupling constant (J), singlet (s), Doublet (d), doublet-doublet (dd), multiplet (m), broad singlet (br.s)

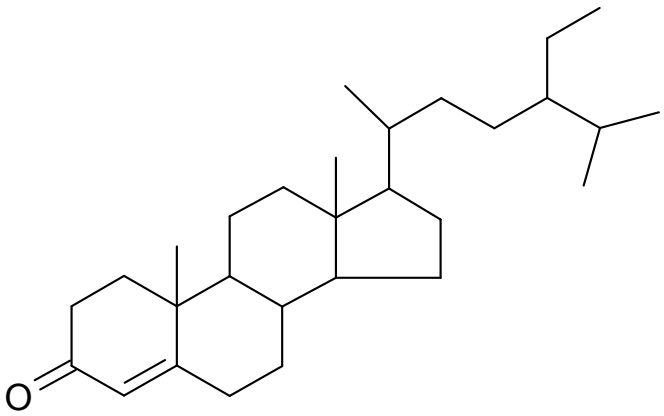


Figure 4. *Structure of compound C_2*

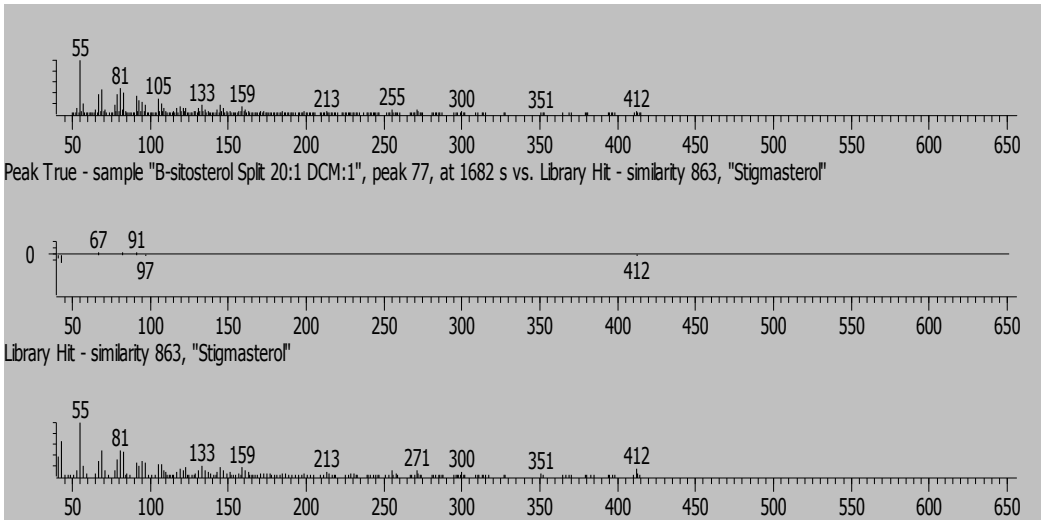


Figure 5. *Mass Spectrum of Compound C_3*

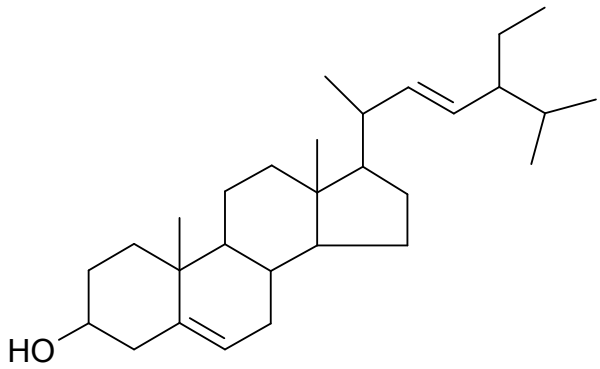


Figure 6. *Structure of compound C_3*