EPH - International Journal of Applied Science
ISSN (Online): 2208-2182
Volume 06 Issue 02-June, 2020

DOI: https://doi.org/10.53555/eijas.v6i2.101

ISOLATION AND FULL CHARACTERISATION OF TWO STEROL GLUCOSIDES FROM TEUCRIUM BARBEYANUM ASCHERS

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Abstract:-
The genus Teucrium is used in folk medicine for a long time as hypoglycemic and hypolipidemic agents, and showed a wide spectrum of interesting biological activities. In this study, the aerial parts of Teucrium barbeyanum which collected from Ras El-Hilal, Libya were subject of extraction with different organic solvents, followed by extensive columns chromatography and crystallization using various solvent systems in order to isolate compounds. Dichloromethane extract afforded two sterol glucosides which successfully identified as sitosterol-3-O-β-D-glucoside and the rare compound, clerosterol-3-O-β-D-glucoside. The protons, carbons and OHgroups were fully assigned by means of spectroscopic techniques including NMR (1H, 13C, DEPT-135, 90 and Q, COSY, HSQC and HMBC) and IR, and Electron Impact Mass Spectrometry.

Keywords:-*Teucrium barbeyanum, sterol glucosides, sitosterol-3-O-β-Dglucoside, clerosterol-3-O-β-D-glucoside, 1D and 2D NMR.*

INTRODUCTION

Teucrium genus which belongs to the *Lamiacea* family comprises approximately 200 species worldwide, most of them found in the Mediterranean region. In flora of Libya the genus is represented by 13 species, only five being endemic, including *T. barbeyanum*, which is dense branched ascending herb. *T. barbeyanum* is found in different locations in Al-Jabal Al-Akhdar, Cyrenaica Provence [1, 2]. EPH - International Journal of Applied Science | ISSN: 2208-2182The genus was known to have a wide spectrum of therapeutic use in traditional medicine, for instance as hypoglycemic and hypolipidemic agent and treatment of a variety of diseases in the digestive and respiratory systems. Consequently, the genus attracted wide interest among phytochemical scientist and was subject of extensive studies in last three decays, which resulted in confirming of several biological and pharmaceutical properties. It displayed antioxidant, anti-inflammatory, antimicrobial, anti-feed ant and cytotoxic activities [3, 4]. Numerous secondary metabolites were isolated from this germander including terpenes, iridoids, sterols, flavonoids, saponins, fatty acids, phenols and essential oils [2, 5]. In previous study, *T. barbeyanum* exhibited promising antioxidant activity of its extracts, which showed high phenolic and flavonoid contents. In addition, neoclerodane diterpenes, flavonoid, and phenolic compounds were isolated from different extracts [6, 7].

Isolation and identification of the phytochemical is the starting point of testing and evaluating of the biological activities of the isolate. It's common in the isolation processes to face structurally very close compounds which is unattainable to get them in pure form using ordinary technique. In these cases the challenge is to identify them in mixture rather than wasting time and efforts in separation. In the course of our investigation on Teucrium genus in Libya [8,9], the current work intended to isolate and identified an additional constituents of *T. barbeyanum* growing in Libya.

MATERIALS AND METHODS

Plant Material

The aerial parts of *Teucrium barbeyanum* Aschers were collected during the flowering season in May from "Ras El-Hilal" in "Gebel Akhdar", Cyrenaica region, Libya and were identified by Mr. Abdusslam *Elmogasapi*, College of Science, and University of Benghazi.

Extraction and Isolation

The plant was dried in shadow and grinded to small pieces with grinder. The plant material was then defatted using petroleum ether (40-60 °C) with Soxhlet apparatus. Finally, the defatted plant materials were exhaustively extracted with dichloromethane using Soxhlet to afford dichloromethane extract (DCM ext.), 23.2 g. The DCM ext. was subjected to silica gel (0.063-0.200 mm, 320 g) column chromatography. The column was eluted using gradient solvents with increasing polarity, started with 500 mL *n*-hexane, followed by 300 mL dichloromethane (100%), and then the solvent was sequentially changed with 20% increase in polarity (400 mL each) to EtOAC and MeOH. A total of 56 90 fractions were collected, which after TLC comparison and combination gave 10 main fractions (DA to DJ). Fraction DF (4.0 g) was chromatographed on 40 g silica gel (0.0400.063 mm), eluting with a solvent system of $CHCl_3$-EtOAc-MeOH (5:4:1) v/v (900 mL). Sub-fractions DF22-23 afforded **compound 1** (**Comp.1**) as precipitate, which was further purified by crystallization with solvent mixture MeOH-$CHCl_3$ to give a white amorphous precipitate with R_f value 0.47 [$CHCl_3$: EtOAc:MeOH (5:3.5:1.5)]. Sub-fractions DF22-23 also afforded **compound 2** in mixture (**comp. 2**) as white amorphous precipitate that repeatedly crystallized with MeOH-$CHCl_3$ and Me_2CO-*n*-hexane. The TLC (silica gel 60 F_{254}) with different solvent systems showed one spot of pure homogeneous compound.

RESULTS AND DISCUSSION

Identification of Compound 1

The **Comp.1** was obtained from DCM extract as white powder. The spectral data of **Comp.1**, indicated the presence of sterol skeleton. The EI-MS of **Comp.1** (Appendix 1) showed a weak molecular ion peak $[M]^+$ at m/z = 576.5, (calculated, 576.4389) which is in agreement with the molecular formula of $C_{35}H_{60}O_6$, having six degrees of unsaturation (one double bond and five rings). The spectrum of EI-MS also showed an ion peak at m/z = 414.4, which is a characteristic of the loss of a glucose moiety $[M-C_6H_{10}O_5]^+$. Upon losing a water molecule gave base peak at m/z = 396.4. The IR spectrum of **Comp.1** (Appendix 2) showed absorptions consistent with the presence of the hydroxyl group at 3385 cm^{-1} (O−H stretching), olefinic at 1456−1368 cm^{-1} (C=C stretching) and saturated moiety at 2932 cm^{-1} (sp^3 C−H stretching). The full assignments of ^1H and ^{13}C signals of the compound were accomplished by combination of different techniques of NMR (^1H, ^{13}C, DEPT-135 and 90, ^1H-^1H COSY, ^1H-^{13}C HSQC and HMBC). The ^1H NMR spectrum (Fig. 1, Table (1) of **Comp.1** showed signals of oxymethine proton at δ_H 3.46 attributed to H-3, olefinic proton at δ_H 5.33 belonging to H-6, two methylene protons at δ_H 2.37 (dm, J = 13.4 Hz) and δ_H 2.12 (t, J = 11.1 Hz) for H-4. The spectrum also showed distinguished signals of sugar moiety in the middle of the spectrum at δ_H 3.68-2.85. The coupling constant (d, J = 7.8 Hz) associated with the anomeric proton at δ_H 4.22 established β-configuration of the sugar moiety. The ^1H-^1H COSY spectrum (Appendix 3) showed the coupling between H-6 at δ_H 5.33 and H-7 (δ_H 1.91 and 1.41).

Among the sugar moiety, H-3′ (δ_H 3.12), H-4′ (δ_H 3.01) and H-5′ (δ_H 3.07) showed coupling constants of 8.7, 9.0 and 9.6 Hz, respectively, characteristic to the axial configuration, establishing the glucose identity of the sugar. Moreover, the COSY spectrum exhibited strong cross peaks of three OH-protons at δ_H 4.90-4.85 with H-2′, H-3′ and H-4′, and of a OH-proton at δ_H 4.42 with H-6′, suggesting the four hydroxyl protons of the sugar moiety. More COSY correlations in Comp.1 are displayed in Appendix 3. The ^{13}C NMR spectrum (Fig. 2) of **Comp.1** represented 35 carbons, 29 of which belonged to the sterol skeleton and six were of sugar moiety. These carbons with the assistance of DEPT 135 and DEPT 90 (Fig. 3) showed 14 methine, 12 methylene, six methyl and three quaternary carbons. Three deshielded signals at δ_C 140.32,

121.12 and 100.64 belonged to the olefinic (C-5, C-6) and the anomeric (C-1′) carbons, respectively. The full assignment of ^{13}C values is shown in Table (1).

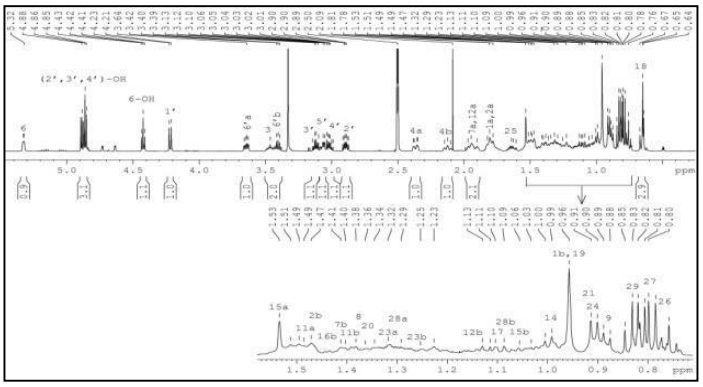


Figure 1: ^{1}H NMR spectrum of Compound 1 (DMSO-d_6, 500 MHz)

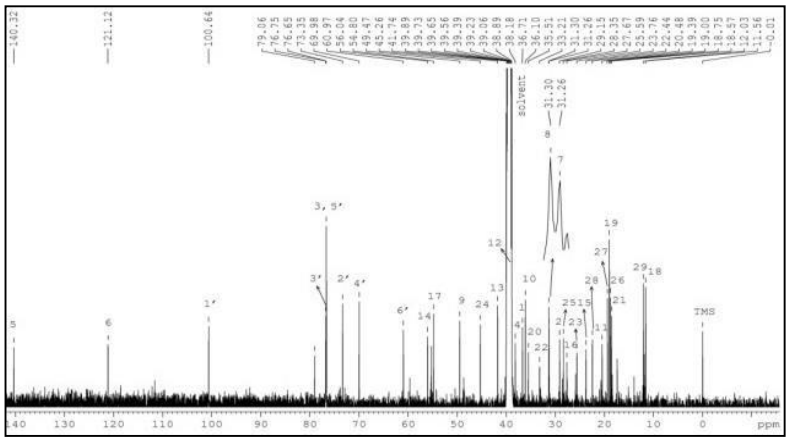


Figure 2: ^{13}C NMR spectrum of Compound 1 (DMSO-d_6, 125 MHz)

^{1}H-^{13}C HSQC spectrum of **Comp.1** (Appendix 4) showed the attachments of the sugar protons to their respective carbons including C-1′ (δ_C 100.64) attached to H-1′ (δ_H 4.22) and C-6′ (δ_C 60.97) attached to H-6′ (δ_H 3.64 and3.40). Moreover, this HSQC spectrum confirmed the signals at δ_H 4.90-4.85 and δ_H 4.42 as the hydroxyl groups of the sugar moiety as no direct attachment were observed between these protons and carbons. ^{1}H-^{13}C HMBC spectrum of **Comp.1** (Appendix 5) showed long range correlations of H-1a (δH 1.79), H-2a (δH 1.82) and H-4a (δH 2.37) with C-3 (δC 76.65). In turn, H-3 exhibited correlation with C-1′, establishing connectivity of the sugar moiety at C-3, which was further confirmed by the correlation of H-1′ with C-3. HMBC spectrum also showed cross peaks of H-2a, H-4a with the olefinic C-5 and C-6, confirming the double bond at C-5/C-6. Detailed HMBC correlations of Comp.1 are showed in Appendix 5. On the basis of the spectroscopic data of Comp.1, it was identified as sitosterol-3-O-β-Dglucoside (stigma-5-en-3-O-β-glucoside), commonly known as daucosterine. The spectroscopic data of Comp.1 were in agreement with those reported for the sitosterol-3O-β-D-glucoside [10].

Table 1: ^{1}H and ^{13}C NMR (500/125 MHz) Assignments* of Compound 1 in DMSO-d_6

Pos.[a]	^{1}H		^{13}C
	δ_{ppm}, multiplicity[m] (J in Hz)		δ_{ppm} (type)[†]
	Ha	Hb	
1	1.79	1.08-0.90	36.71 (CH_2)
2	1.83	1.50	29.15 (CH_2)
3	3.46		76.65 (CH)
4	2.37,dm (13.4)	2.12,t (11.1)	38.18 (CH_2)
5			140.32 (C)
6	5.33		121.12 (CH)
7	1.93	1.53	31.26 (CH_2)
8	1.40		31.30(CH)
9	0.88		45.26 (CH)
10			36.10 (C)
11	1.49	1.41	20.48 (CH_2)
12	1.96	1.13	39.65 (CH_2)
13			39.89 (C)
14	0.99		56.04 (CH)
15	1.53	1.06	23.76 (CH_2)
16	1.82-1.79		27.67 (CH_2)
17	1.07		49.80 (CH)
18	0.65		11.56 (CH_3)
19	0.97		19.00 (CH_3)
20	1.36		35.51 (CH)
21	0.92		18.57 (CH_3)
22	1.28	0.93	33.21 (CH_2)
23	1.33-1.26		25.59 (CH_2)
24	0.91		41.74 (CH)
25	1.64		28.35 (CH)
26	0.77		18.75 (CH_3)
27	0.81		19.39 (CH_3)
28	1.34-1.27		22.44 (CH_2)
29	0.84		12.03 (CH_3)
1'	4.22, d (7.8)		100.64 (CH)
2'	2.89		73.35 (CH)

3'	3.12, dt (8.7, 4.8)		76.75 (CH)
4'	3.01, dt (9.0, 5.1)		69.98 (CH)
5'	3.07, ddd (9.6, 7.9, 3.8)		76.65 (CH)
6'	3.64, ddd (11.7, 5.7, 2.0)	3.4	60.97 (CH_2)
OHs			
2',3',4'	4.90-4.85		
6'	4.42, t (5.8)		

*Connectivity of H-C established by HSQC. Deduced from DEPT experiments. [a] position of proton, carbon or group. s = **Singlet**. d = Doublet. t = Triplet. m = Multiplet. [m]Multiplet unless other thing mentioned

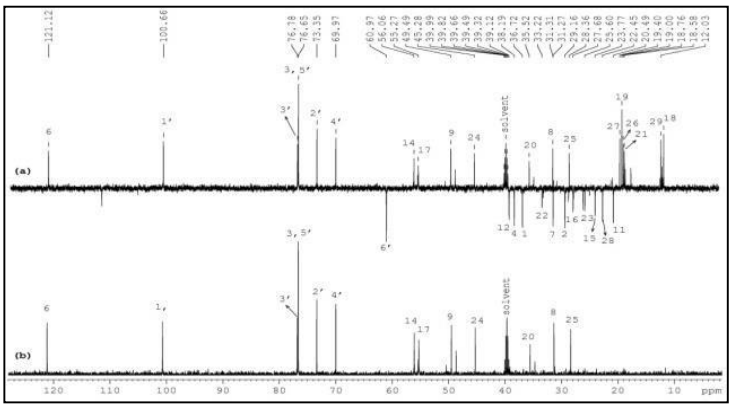
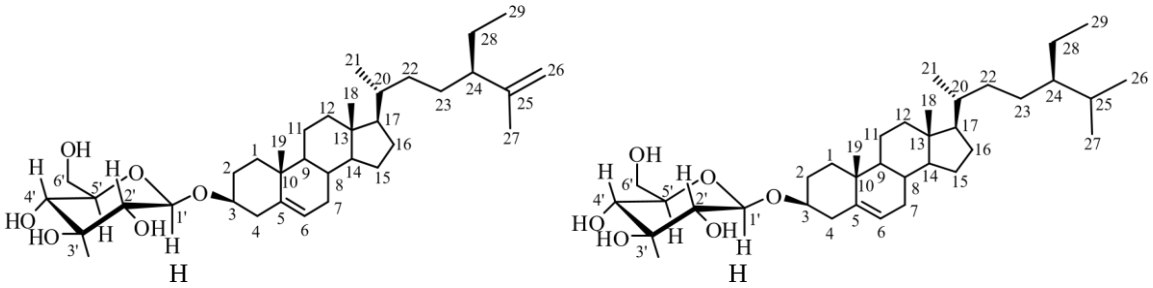


Figure 3: (a) DEPT 135 and (b) DEPT 90 NMR spectra of Compound 1 (DMSO-d_6, 125 MHz)

Identification of Compound 2 in mixture

Comp.2 was obtained from DCM extract as white powder. The spectroscopic data showed that **comp.2** is a mixture of two close sterols in the ratio of 1:1.2., with Comp.2 of higher quantity. The ^{1}H-NMR spectrum (Fig. 4, Table (2)) of **comp.2** displayed characteristic signals of **Comp.2** in addition to **comp.1** in pyridine. The spectrum showed integration of two sets of H-4a at δ_H 2.73 (dm, J = 13.3 Hz), H-4b at δ_H 2.48 (t, J = 12.2 Hz), H-3/5′ at δ_H 3.96-4.03, H-2a at δ_H 2.14, H-2′ at δ_H 4.06 (t, J = 8.2 Hz), H6′a at δ_H 4.56 (dd, J = 11.8, 2.2 Hz), H-6′b at δ_H 4.40, H-1′ at δ_H 5.04 (d, J = 7.8 Hz) and H-6 at δ_H 5.35 which indicated the presence of two structurally closed sterol glucosides. The spectrum also showed the presence of one set of geminal protons appeared as pair at δ_H 4.88 and δ_H 4.80, and methyl group resonated in the downfield region as a singlet at δ_H 1.62 belong to H-26 and H-27, respectively which pointed out to clerosterol-3-O-βD-glucoside. The coupling constant (d, J = 7.8 Hz) associated with the anomeric proton at δ_H 5.04 established β-configuration of the sugar moiety. ^{1}H-^{1}H COSY spectrum (Appendix 6) of **comp.2** disclosed most of ^{1}H-^{1}H vicinal and geminal correlations. It showed cross peaks of vicinal H-3 with H-2 and H-4, H-6 with H-7, H-1′ with H-2′, and of H-5′ with H-4′ and H-6′. Also showed cross peaks of geminal H-2, H-4, H-7 and H-6′. These evidences established the identity of the clerosterol-3-O-β-D-glucoside. The ^{13}CNMR spectrum (Fig. 5) of **comp.2** exhibited signals at δ_C 147.99, 141.20, 122.22 and 112.30 which indicated the presence of two double bonds. The signals at δ_C 141.20 and 122.22 are characteristic peaks of carbons with double bond (C-5 and C-6) in steroids [11]. Similarly, the signals at δ_C 147.99 and 112.30 are distinctive peaks of the double bond at C-25 and C-26 of clerosterol-3-O-β-D-glucoside. DEPT-135 and 90 (Fig. 6) confirmed the identities of these carbons. In ^{13}C-NMR spectrum of **comp.2**, sitosterol-3O-β-D-glucoside and clerosterol-3-O-β-D-glucoside shared carbons C-5 and C-6, and the signals of the sugar moieties were found to be common for both sitosterol-3-O-β-Dglucoside and clerosterol-3-O-β-D-glucoside. However, C-25 and C-26 of both the compounds resonated at different chemical shifts. The double bond at C-25 and C-26 of the clerosterol-3-O-β-D-glucoside resonated in the downfield region at δ_C 147.99 and 112.30, respectively, while that of the sitosterol-3-O-β-D-glucoside appeared at δ_C 29.54 and 19.20, respectively. Similarly, two magnetically non-equivalent pairs of C-24 and C-27 (δ_C 50.51 and 18.15 for cler.) and (δ_C 46.55 and 20.07 for sito.) were observed. The full assignment of carbon chemical shifts of **comp.2** is outlined in Table (2). ^{1}H-^{13}C HSQC spectrum (Appendix 7) of **comp.2** showed the attachment of the sugar protons of sito. and cler. to their respective carbons including H-1′ (δ_H 5.04) with C-1′ (δ_C 102.82) and H-6′ (δ_H 4.56 and 4.40) with C-6′ (δ_C 63.06). It also showed that H-26 (δ_H 4.88 and 4.80) of cler. are attached to C-26 (δ_C 112.30) and H-27 of cler. (δ_H 1.62) to C-27 (δ_C 18.15). ^{1}H-^{13}C HMBC spectrum (Appendix 8) of **comp.2** showed long range connectivities

of H-26 of cler. (δ_H 88 and 4.80) with C-24 and C-27 at δ_C 50.04 and 18.15, respectively. HMBC spectrum also showed cross peaks of H-27 of cler. With C25 of cler. at δ_C 147.99. These correlations confirmed clerosterol-3-O-*β*-D-glucoside skeleton. This is the second report of the compound in *Lamiaceae* family [12].



Clerosterol-3-O-*β*-D-glucoside **Sitosterol-3-O-*β*-D-glucoside**

Figure 7: The isolated compounds

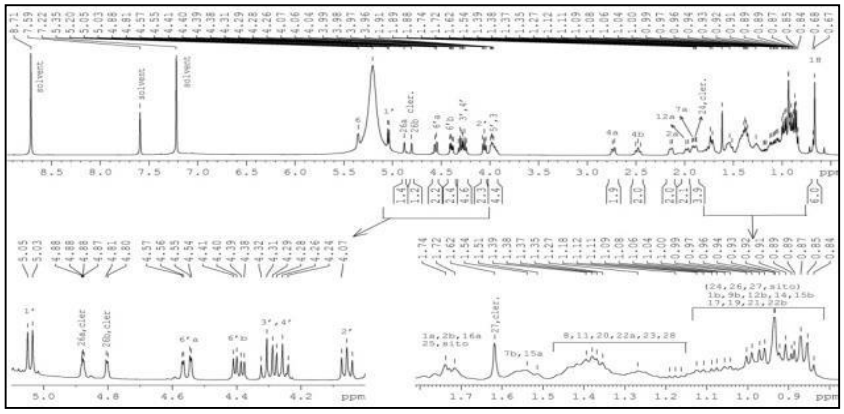


Figure 4: ^{1}H NMR spectrum of Compound 2 (pyridine-d_5, 500 MHz)

Table 2: ^{1}H and ^{13}C NMR (500/125 MHz) Assignments* of Compound 2 in mixture in pyridine-d5

	^{1}H		^{13}C
Pos.[a]	δppm, multiplicity (J in Hz)		δ_{ppm} (type)[†]
	Ha	Hb	
1	1.72	0.98	37.76 (CH_2)
2	2.14	1.75	30.52 (CH_2)
3	4.03-3.96		78.45 (CH)
4	2.73, dm (13.3)	2.48, t (12.2)	39.61 (CH_2)
5			141.20 (C)
6	5.35, d (3.6)		122.22 (CH)
7	1.90	1.54	32.47 (CH_2)
8	1.37		32.34 (CH)
9	0.90		50.62 (CH)
10			37.20 (C)
11	1.42	1.86	21.57 (CH_2)
12	1.98	1.10	40.25 (CH_2)
13			42.77 (C)
14	0.94		57.12 (CH)
15	1.54	1.03	24.78 (CH_2)
16	1.82	1.26	28.75 (CH_2)
17	1.12-1.02		56.54 (CH)
18	0.68		12.61 (CH_3)
19	0.93		19.72 (CH_3)
20	1.40-1.39		36.04 (CH)
21	0.86		19.36 (CH_3)
22	1.37	1.04	34.44 (CH_2)
23	1.37	1.27	29.93 (CH_2)
24	1.9		50.04 (CH)

*Connectivity of H-C established by HSQC. [†]deduced from DEPT experiments. [a] position of proton or carbon. s = Singlet. d = Doublet. t = Triplet. m = Multiplet.

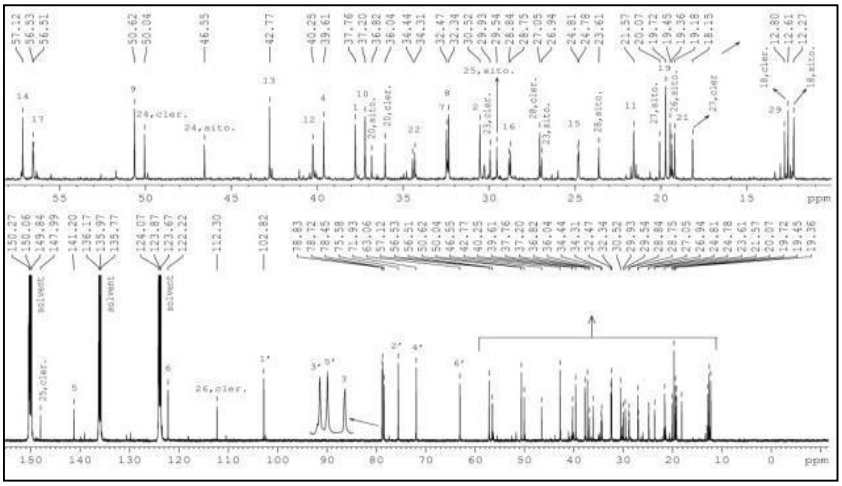


Figure 5: ^{13}C NMR spectrum of Compound 2 (pyridine-d_5, 125 MHz)

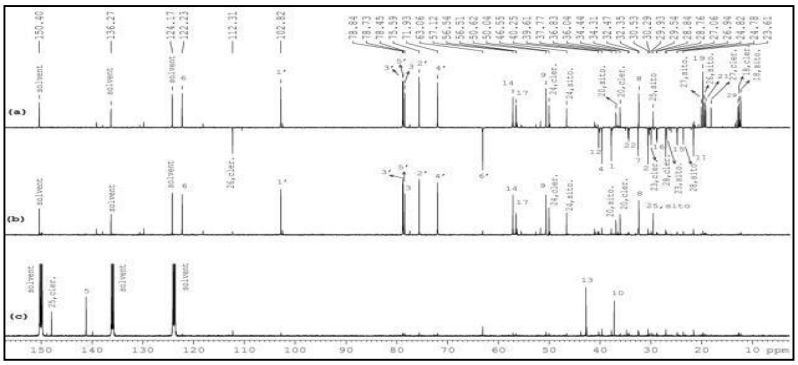


Figure 6: (a) DEPT 135, (b) DEPT 90 and (c) DEPT Q NMR spectra of Compound 2 (pyridine, 125 MHz)

CONCLUSION

Two sterol glucosides; sitosterol-3-O-*β*-D-glucoside and clerosterol-3-O-*β*-Dglucoside were isolated from DCM ext. of the aerial parts of *Teucrium barbeyanum* plant, and full characterization of the two compounds was achieved using EI-mass spectrometry and spectroscopic techniques including IR and, one and two dimension NMR. The rarely compound, clerosterol-3-O-*β*-D-glucoside was challengingly identified in mixture. This is the second report of this compound which is so far restrictedly found in *Lamiaceae* family.

ACKNOWLEDGMENT

Authors would like to thank Zahri Othman for help in NMR measurements.

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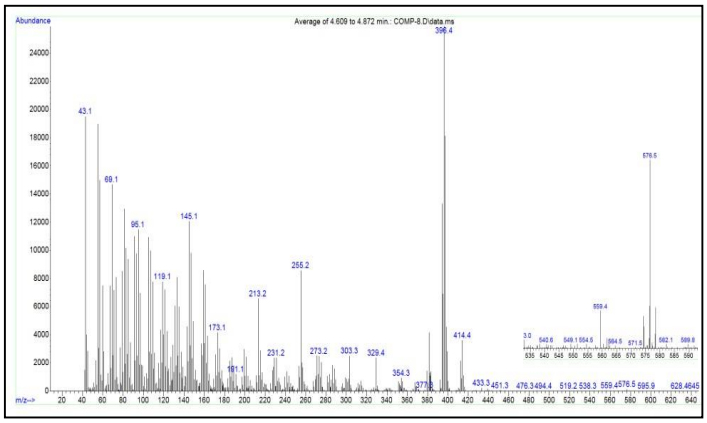
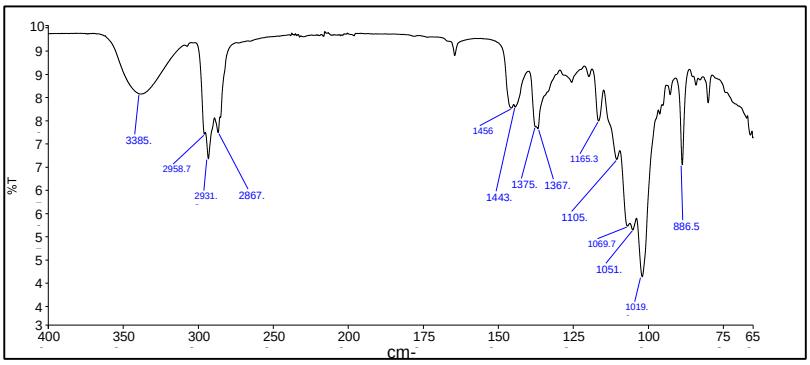
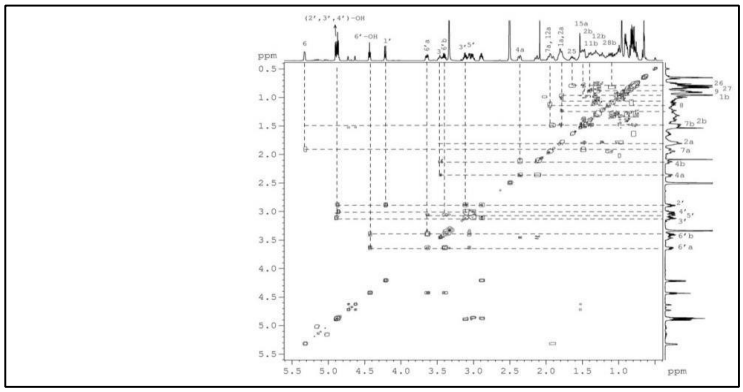
APPENDIXES



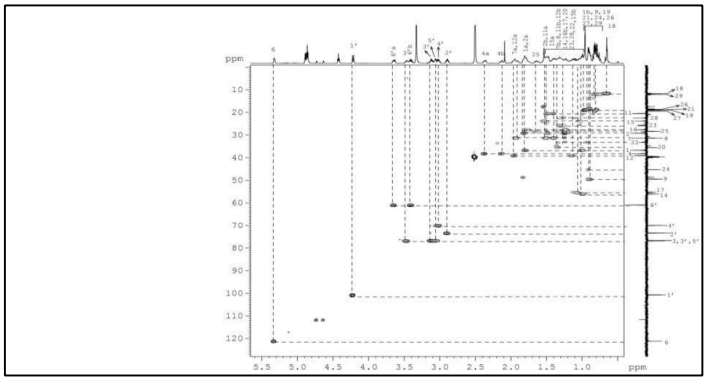
Appendix 1: EI Mass Spectrum of compound 1



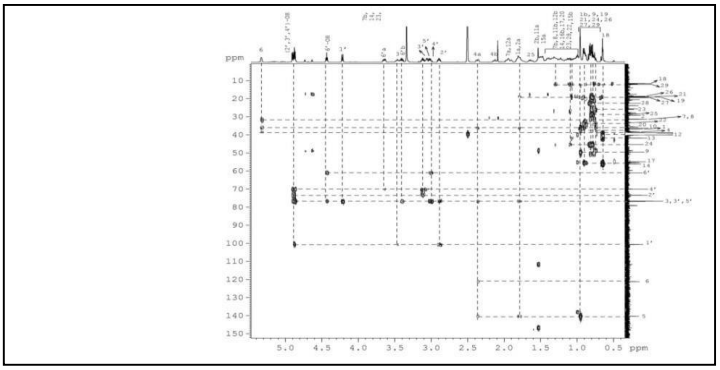
Appendix 2: IR spectrum of compound 1



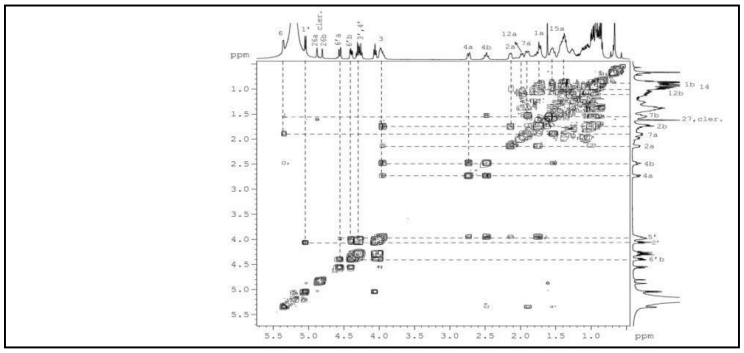
Appendix 3: ^{1}H-^{1}H COSY NMR spectrum of compound 1 (DMSO-*d*$_6$)



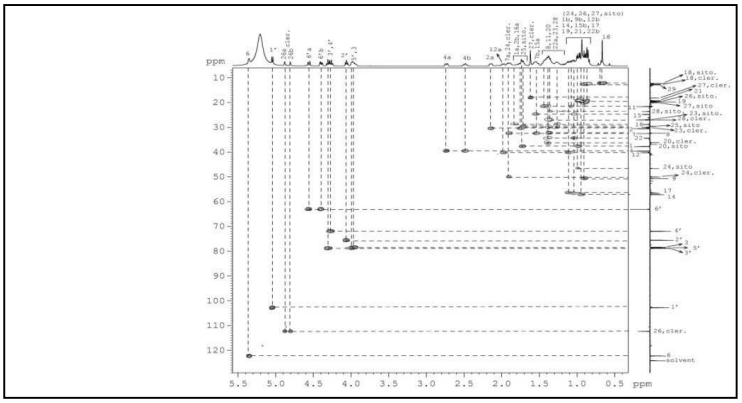
Appendix 4: ^{1}H-^{13}C HSQC NMR spectrum of compound 1 (DMSO-*d*$_6$)



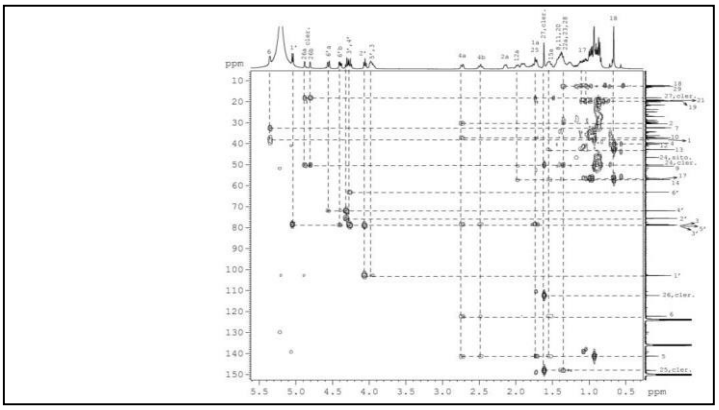
Appendix 5: ^{1}H-^{13}C HMBC NMR spectrum of compound 1 (DMSO-d_6)



Appendix 6 :^{1}H-^{1}H COSY NMR spectrum of compound 2 (pyridine)



Appendix 7: ^{1}H-^{13}C HSQC NMR spectrum of compound 2 (pyridine)



Appendix 8: ^{1}H-^{13}C HMBC NMR spectrum of compound 2 (pyridine)