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Isolation and Characterization of β-Sitosterol-3-O-β-D-glucoside from the Extract of the Flowers of *Viola odorata*

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Authors' contributions

This work was carried out in collaboration between the both authors. Both authors designed the study and protocol. Author TP performed the literature search and did the experimental part and analyses of the study. Both authors read and approved the final draft.

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ABSTRACT

Isolation from the ethanol extract of the flowers of *Viola odorata* resulted in the isolation of β-Sitosterol-β-D-glucoside. This compound has not been previously isolated or reported from the flowers of this variety. The isolated β-Sitosterol-3-O-β-D-glucosidewas characterized on the basis of spectroscopic techniques viz. infrared and nuclear magnetic resonance spectroscopy. A tetra acetyl derivative of β-Sitosterol-3-O-β-D-glucoside was synthesized and characterized. On hydrolysis of β-Sitosterol-β-D-glucoside, β-sitosterol was obtained and characterized.

Keywords: Aglycone; glycoside; phytosterol; sitosterol; Viola odorata.

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1. INTRODUCTION

Viola odorata is a species of genus Viola, which is native of Asia and Europe [1]. It is also known as wood violet or garden violet. It is a herbaceous flowering perennial plant, which as a whole is used for various therapeutic purposes and perfumery [2]. The flowers of this plant have a peculiar scent and are blue in color [3]. Sterols, predominantly sitosterol, stigmasterol and campesterol are present in practically all the plant species. Their synthetic derivatives are known as statins, which are used as cholesterol lowering drugs [4]. Sterols are the first choice of potential natural preventive dietary products [5]. Stigmasterol and β-sitosterol are two phytosterols, structurally similar to cholesterol and are well spread in plants, fungi and animals [6]. These are secondary metabolites and are used as health promoting constituents of natural foods [6]. The sterol glucoside contains the hydrophilic glucose moiety which inhibits the entry of cholesterol into the esterification of cholesterol [7-8]. European foods and safety authority recommends consuming about 1.5 to 2-4 g/day of phytosterol in order to reduce blood pressure [8]. In addition, the US Food and Drug Administration has approved the role of foods containing phytosterol esters, and a low saturated fat and cholesterol diet in reducing the risk of heart attack [9-10]. β-sitosterol-β-D-glucoside has been proposed as a useful candidate for the development of new drugs to treat endotoxemia and inflammation accompanied by the overproduction of nitric oxide. This compound reduces nitric oxide production from lipopolysaccharide-induced RAW 264.7 cells. In addition, it strongly inhibits the interleukin 6 activities of stimulated macrophages [11-12].

2. MATERIALS AND METHODS

The flowers of *Viola odorata* were collected from Udhampur district, Jammu and Kashmir, India. The plant was identified and the violet flowers were allowed to dry in open air under direct sunlight. The dried flowers were then used as such for further investigation. The herbarium specimen was prepared and deposited in University School of Medicine and Para-medical Health Sciences, Delhi. All the solvents used were of analytical reagent grade. Thin layer chromatography (TLC) was performed on silica gel GF_{254}. All evaporations were carried out under reduced pressure.

2.1 Spectroscopic Characterization

Different spectroscopic methods were used to elucidate the structure of compounds. The infrared spectra were recorded on Shimadzu -1S affinity. The ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded on Bruker R-32 (400-MHz) in a deuterated solvent CD_3OD and $CDCl_3$ with tetra methyl silane (TMS) as internal standard. Structural elucidation was carried out using 1D, ^{1}H, ^{13}C, and various 2D experiments.

2.2 Extraction and Isolation

The dried flower (500 g) was exhaustively extracted with petroleum ether, dry ether and dry ethanol successively [13]. The ethanol extract was concentrated on a rota-evaporator and this extract was dissolved in dry methanol. Further, it was kept at 4ºC for 72hours. A light brown solid separated which was filtered and collected. A dry mass was obtained which was given washing with dry ether and then it was vacuum dried. The solid 'A' so obtained was then applied to vacuum liquid chromatography on a silica column using hexane (60º-80ºC), diethyl ether and ethyl alcohol in the ratio of 10:3:1 [14]. The elutes were collected in a number of test tubes and monitored using TLC [15]. The elutes of similar retention factor (R_f) were then combined and evaporated. One of the fractions was used for further characterization.

The isolated fraction was washed with n-heptane to give compound 'A'. The TLC was done over silica gel (GF_{254}) using hexane-ether (30:70) as developing solvent. The R_f was found to be 0.50. The compound was observed as a yellow colored single spot upon exposure to iodine chamber, and as a violet colored spot upon spraying with sulphuric acid reagent followed by heating with hot air gun [16] m.pt 285ºC (corrected). A yield of 15mg was obtained.

A portion of the isolated, washed and dried fraction (8 mg) was then treated with acetic anhydride along with 1µl of sulphuric acid and kept at room temperature for 2 days [17-18]. The reaction mixture was poured in ice cold water, stirred and extracted with ethyl acetate. Ethyl acetate was washed repeatedly with water and dried over anhydrous sodium sulphate. The resulting dry layer was evaporated in vacuum to give a tetra acetate derivative. The solution was centrifuged and solid 'B' as acetyl derivative was

isolated and vacuum dried. A yield of 10 mg was obtained.

A small amount of solid ‘A’ glucoside was hydrolyzed [19-20] by adding 8mg of glucoside to a mixture of amyl alcohol (3 mL), dilute hydrochloric acid (4 mL) and methanol (25 mL). The resulting solution was refluxed for about 6hours and the refluxed solution was concentrated to about 10 mL. To the concentrated solution, ethyl acetate was added in order to extract the sitosterol. The extracted mass was washed with water and dried over anhydrous sodium sulphate. The resulting mass was evaporated under reduced pressure and dried to give a white powder of β-sitosterol (compound ‘C’). Recrystallization was done using methanol.

3. RESULTS AND DISCUSSION

β-Sitosterol-3-O-β-D-glucoside (A) from the flowers of *Viola odorata* was isolated and reported for the first time as a light yellow amorphous solid. It showed a positive liberman burchard test with the formation of violet ring indicating a steroid skeleton. The ultraviolet (UV) maximum value of compound A was observed at 256 nm [21]. The IR spectrum of compound A showed broad band in the region 3450cm^{-1}, thus indicating the presence of –OH of glucoside linkage [22]. The absorption observed at 2958-2850 cm^{-1} was assigned to asymmetric aliphatic –CH stretching of CH_3 and CH_2 groups [22]. A band observed at 1095 cm^{-1} was assigned to C-O-C linkage. The weak absorption observed at 1664cm^{-1} was assigned to (C=C) stretching. Besides these, another three bands at 1452, 1379 and 836 cm^{-1} were observed. These observed values are in good agreement with the reported literature [23].

^{1}H NMR spectrum of compound A showed a chemical shift in the range 0.87-0.97 ppm, thus suggesting the presence of methyl protons (12H, H-21,26,27,29). A singlet observed at 0.70ppm was assigned to methyl protons (H-18). The proton attached to olefinic linkage was observed at 5.35 ppm. The signals observed at 0.87 and 0.88 ppm were assigned to methyl protons of isopropenyl moiety. Methyl proton (H-21) were observed at 0.95 ppm with coupling constant J=6.5 Hz. The proton of glucose was observed at 2.89-4.27ppm as a multiplet. The proton of –CH group of glycoside was observed at 4.20ppm. H-3 proton was observed at 2.98 ppm. The hydroxyl protons of sugar moiety (Table 1) showed resonance at 3.56-3.60 ppm (J=4.5 Hz) and were assigned to C-2’, C-3’ C-4’ and C-6’ respectively [24]. All these hydroxyl signals disappeared on shaking with D_2O.

The ^{13}C NMR spectrum of compound A indicated 35 carbon signals, of which six were for the sugar moiety and 29 were attributed to the aglycone moiety. The carbon signals of the sugar moiety observed at 62.82 (C-6'), 70.04 (C-4'), 73.42 (C-2'), 76.72 (C-5'), 76.86 (C-3'), and 100.73 (C-1') ppm were well consistent with those of glucose moiety. The aglycone moiety signals were observed at 140.41 (C-5), 121.20 (C-6), 76.85 (C-3), 56.13 (C-14), 55.38 (C-17), 49.55 (C-9), 45.09 (C-24), 41.82 (C-13), 39.28 (C-4), 38.26 (C-12), 36.79 (C-1), 36.18 (C-10), 35.45 (C-20), 33.29 (C-22), 31.38 (C-7), 31.26 (C-8), 29.23 (C-2), 28.64 (C-25), 27.76 (C-16), 25.36 (C-23), 23.83 (C-15), 22.06 (C-28), 20.56 (C-11), 19.69 (C-26), 19.07 (C-19), 18.89 (C-27), 18.58 (C-21), 11.75 (C-29) and 11.64 (C-18) ppm [25]. These data confirmed that compound A is β-sitosterol-3-O-β-D-glucoside (Fig. 1).

The chemical shift at 19.69 and 18.89 ppm were assigned to two methyl group. The signals observed at downfield 11.64, 19.07, and 18.58 ppm were assigned to angular methyl group moiety linked at C-18, C-19 and C-21. The signals at 140.41, 36.18 and 41.82ppm were assigned to the quaternary carbon at point C-5, C-10 and C-13. The signals observed at 31.26, 49.55 and 56.13ppm were assigned to protons at C-8, C-9 and C-14, respectively. The upfield chemical shift at 36.79, 29.23, 39.28, 31.38, 49.55, 20.56, 38.26, 56.13, 23.83, 27.76 and 55.38ppm were assigned to cyclohexyl and cylopentyl of A, B, C and D rings. The carbon of cyclic linkage part joint to the side chain was observed at 35.45ppm. Anomeric carbon 100.73 (C-1’) and that of proton (H-1’) at 4.20 with 76.85 ppm, respectively, confirmed the glucose moiety at 3 position. The ^{1}H and ^{13}C NMR values for all the protons and carbons were assigned on the basis of Heteronuclear Multiple Bond Correlation (HMBC) spectroscopy (COSY) and nuclear overhauser effect spectroscopy (NOESY) which are given in Table 1.

The IR spectrum of compound ‘B’ (tetra acetyl derivative) showed strong absorptions due to –CH_2 at 2870- 2830 cm^{-1}; absorptions at 1476-1144 cm^{-1}, 1380 cm^{-1}, and 1370 cm^{-1} were attributed to methyl and methylene group vibrations of sterol molecule. Broad absorption in the region 1125-1010 cm^{-1} was characteristic of

sugar. The strong peak observed at 1736 cm^{-1} was assigned to υ(C=O) of acetate of glucose [20].

^{1}HNMR spectrum of compound 'B' in $CDCl_3$ showed a downfield shift for the glucose moiety protons. Further, methyl protons of acetoxy group were observed at 2.0, 2.1, 2.1and 2.2 ppm, attributed to 2',3',4 and 6' attached to O- of acetyl group. The ^{1}HNMR spectra of aglycone moiety showed signals at 0.68 and 1.01ppm. Three methyl doublets appeared at 0.80, 0.82 and 0.98 ppm and a methyl triplet appeared at 0.86 ppm. The olefinic proton was observed at 5.33 ppm. All other protons were observed at their usual positions (Table 1).

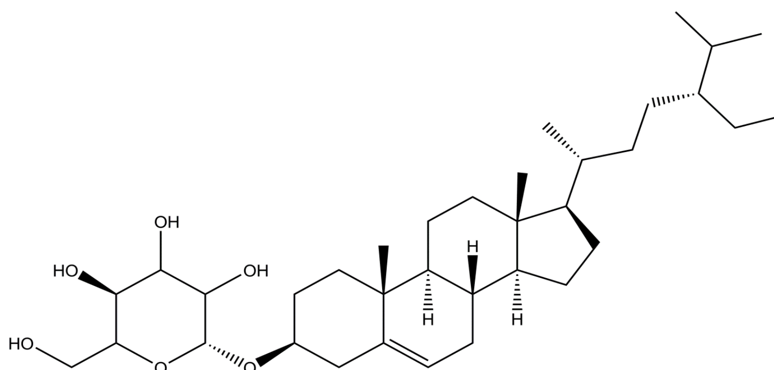

Fig. 1. Structure of β-sitosterol-3-O-β-D-glucoside

Table 1. ^{1}H and ^{13}C NMR chemical shift values of β-Sitosterol-3-O-β-D-glucoside (A) and tetra acetate derivative (B)

A)

HeteroCOSY		HMBC	^{1}H x ^{1}H	
δC	**δH**		**COSY-45º**	**NOESY**
1 (CH_2) 36.79	1.0 m	H-2α, H-4α. H-6	H-2α, H2β	-
	1.40 m	-	-	-
2 (CH_2) 29.23	1.58 m	H-4	H-1α, H-3α	
	1.26 m	-	H-1β, H-1α	-
3 (CH) 76.85	2.98 m	H-1β, H-4α, H-1	H-2α, H-2β	H-1
4 (CH_2) 39.28	2.26 (dt, J = 4.69, 8.10)	H-2α, H-6	H-3α	H-6
-	1.98 (ddd, 1.98, 12.94, 12.94)	-	H-3α	-
5 (qC) 140.41		H-1β, H-4α, H		
6 (CH) 121.20	5.35 (t, J = 3.6)	H-4α	H-4β, H-7α, H-7β	H-4
7 (CH_2) 31.38	1.73 (ddd, J = 2.5, 7.0, 16.0)	-	H-6	H-14α
-	1.95 (ddd, J = 16.0, 2.5, 7.0)	-	H-6	-
8 (CH) 31.26	1.36 m	H-6, H-14α	H-9α	H-4α
9 (CH) 49.55	0.85 m	H-7β, H-14α, H-19	H-8β	-
10 (qC)36.18	-	H-1β, H-19	-	-
11(CH_2)20.56	1.42 m	-	-	-
	1.42 m	-	-	-
12(CH_2)38.26	1.52 (dd, J = 4.3, 12.37)	H-18	-	-
-	1.20 m	-	-	-
13(qC)41.82	-	H-14α, H-15β, H-18	-	-
14(CH) 56.13	0.95 m	H-7β, H-15β	H-15α	H-7α

HeteroCOSY		HMBC	^1H x ^1H	
δC	δH		COSY-45º	NOESY
15(CH_2)23.83	1.05 m	-	H-14α, H-16β	-
-	1.57 m	-	-	-
16(CH_2)27.76	1.25 m	-	H-15α	-
	1.85 m	-	-	-
17(CH) 55.38	1.20 m	H-14α, H-21, H-18	-	-
18(CH_3)11.64	0.70 s	H-11	-	-
19(CH_3)19.07	0.94 s	-	-	-
20(CH) 35.45	1.40 m	H-16α, H-21, H-23	-	-
21(CH_3)18.58	0.95(d, J = 6.5)	-	-	-
22(CH_2)33.29	1.20 m	H-21	-	-
23(CH_2)25.36	1.25 m	H-28	H-24	-
24(CH) 45.09	0.94 m	H-28, H-29	H-23, H-25, H-28	
25(CH) 28.64	1.68 m	H-23, H-28, H-27, H-26	H-24	-
26(CH_3)19.69	0.87 (d, J = 7.0)	-	-	-
27(CH_3)18.89	0.88 (d, J = 7.0)	-	-	-
28(CH_2)22.06	1.30 m	H-29	H-24	-
29(CH_3)11.75	0.97 (t, J = 7.5)	-	-	-
1´100.73	4.20 (d, J = 7.9)	H-3, H-2´	H-2´	H-3
2´73.42	2.89 (dt, J = 4.5, 8.0)	H-1´, H-4´	H-1´, H-3´	-
3´76.86	3.27 (dt, 8.0, J = 4.5, 8.0)	H-1´, H-2´, H-4´, H-5´, H-6´	H-2´, H-4´	H-5´
4´70.04	3.00 (dt, , J = 4.5, 8.0)	H-3´, H-6´	H-3´, H-5´	-
5´76.72	3.06 m(dt, , J = 4.5, 8.0)	-	H-4´, H-6´	H-3´
6´(CH_2) 62.82	4.55 (dd, J = 2.5, 11.77)	-	H-5´	-
-	4.40 (dd, J = 5.2, 11.77)	-	H-5´	-
2'(OH)	3.56 (d,J=4.5)	-	-	-
3'(OH)	3.56 (d,J=4.5)	-	-	-
4'(OH)	3.40 (d,J=4.5)	-	-	-
5'(OH)	3.60 (t,J=4.5)	-	-	-

Assignments were made on the basis of COSY, HMQC and HMBC coorelation. Coupling constant are given in Hz.

B)

HeteroCOSY		HMBC	^1H x ^1H	
δC	δH		COSY-45º	NOESY
1 (CH_2) 37.03	1.00 m	H-6	H-2α	-
-	1.40 m	-	H-2β	-
2 (CH_2) 29.46	1.28 m	H-4α	H-1α, H-3	-
-	1.58 m	-	H-1β	-
3 (CH) 81.6	3.48 (dd, J = 5.16, 10.95)	H-2α, H-4α, H-4β	H-2α, H-4α, H-4β	-
4 (CH_2) 39.0	2.21 m	-	H-3, H-6	-
-	2.15 m	-	H-3, H-6	-
5 (qC) 140.45	-	-	-	-
6 (CH) 122.20	5.33 (d, J = 5.3)	H-4α, H-4β, H-7α	H-4α, H-4β, H-7α, H-7β	-
7 (CH_2) 31.10	1.60 m	-	H-6	-

HeteroCOSY		**HMBC**	**^1H x ^1H**	
δC	**δH**		**COSY-45⁰**	**NOESY**
-	1.95 m	-	H-6	-
8 (CH) 31.80	1.43 m	H-11α, H-15β	-	-
9 (CH) 50.26	0.88 (dd, J = 3.03, 6.66)	H-11α	-	-
10 (qC) 37.0	-	H-6	-	-
11(CH_2)20.80	1.48 m	-	-	-
	1.38 m		-	-
12(CH_2)39.98	2.03 m	H-18	-	-
-	1.19 m	-	-	-
13 (qC) 42.6	-	-	-	-
14(CH) 57.0	0.97 m	-	-	-
15(CH_2)24.40	1.24 m	-	-	-
-	1.58 m	-	-	-
16(CH_2)28.44	1.12 m	-	-	-
-	1.20 m	-	-	-
17(CH) 56.40	1.06 m	H-18, H-21	-	-
18(CH_3)11.90	0.86 s	-	-	-
19(CH_3)19.26	1.00 s	-	-	-
20(CH) 36.42	1.36 m	H-21	-	-
21(CH_3)18.78	0.98 (d, J = 6.5)	-	H-20	-
22(CH_2)34.00	1.54 m	-	-	-
-	1.38 m	-	-	-
23(CH_2)26.25	1.20 m	H-28	-	-
	0.72 m		-	-
24(CH) 46.0	0.96 m	H-23, H-26, H-28	-	-
25(CH) 29.23	1.65 m	H-23α	-	-
26(CH_3)19.09	0.80 (d, J = 6.5)	-	H-25	-
27(CH_3)20.80	0.82 (d, J = 6.5)	H-26	-	-
28(CH_2)23.10	1.30 m	H-29	-	-
29(CH_3)12.20	0.86 (t, J = 7.0)	-	-	-
2´(CH_3) 20.70	2.00 s	-	-	-
3´(CH_3) 21.0	2.1 s	-	-	-
4´(CH_3) 21.0	2.1 s	-	-	-
6´(CH_3) 20.63	2.2 s	-	-	-
6´(CH_2) 62.40	4.09 (dd, J = 2.5, 12.30)	-	H-5´	-
-	4.34 (dd, J = 4.8, 12.30)	-	H-5´	-
1´(CH) 104.20	4.65 (d, J = 8.0)	-	H-2´	H-3´
2´(CH) 71.59	4.98 (dd, J = 8.0,9.6)	-	H-1´, H-3´	H-4´
3´(CH) 72.99	5.54 (t ,J = 9.6)	-	H-2´, H-4´	H-1´
4´(CH) 68.66	5.35 (t, J = 9.6)	-	H-3´, H-5´	H-2´
5´(CH) 72.76	3.65 (ddd, J = 2.5, 4.8, 9.5)	-	H-4´, H-6´a, H-6´b	-
2´ (C=O) 170.10	-	H-2´, 2´ -$OCOCH_3$	-	-
3´(C=O) 170.20	-	H-3´, 3´ -$OCOCH_3$	-	-
4´(C=O) 170.10	-	H-4´, 4´ -$OCOCH_3$	-	-
6´(C=O) 170.20	-	H-6´, 6´ -$OCOCH_3$	-	-

^{13}CNMR spectrum showed 43 carbons out of which eight were from acetoxy group. The spectrum showed shifts from compound 'A' due to α, β effects of acetyl group. COSY 45º spectrum analysis showed vicinal couplings in the glucose moiety.

The ^{1}HNMR spectra of compound 'C' showed the presence of six methyl signals as two singlets at 0.68 and 1.01 ppm. Three methyl doublets appeared at 0.81, 0.83 and 0.93 ppm and a methyl triplet appeared at 0.84 ppm. The olefinic protons were observed at 5.36 ppm. The proton attached to hydroxyl group at C-3 appeared as triplet of doublet of doublet at 3.50 ppm. Vicinal coupling correlation was predicted using COSY45 and NOESY which are given in Table 1.

The ^{13}C NMR, COSY, HMQC and HMBC showed 29 carbon signals of methyl (six), methylene (11), quaternary carbon (three), and methine (nine). The structure of the isolated compound was confirmed as β-sitosterol and was consistent with the reported literature [26]. The spectral assignments were made on the basis of HMBC, COSY-45 and NOESY and are in comparison with the reported literature [27].

4. CONCLUSIONS

One phytosterol was isolated from the flowers of *Viola odorata*. The structure of isolated compound was identified as β-sitosterol-β-D-glucoside. This glucoside was acetylated to give acetate derivative and hydrolyzed to give β- sitosterol.

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CONSENT

Consent is not applicable

ETHICAL APPROVAL

Ethical approval is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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