International Journal of Theoretical & Applied Sciences, 12(2): 30-33(2020)

ISSN No. (Print): 0975-1718
ISSN No. (Online): 2249-3247

A New Oligospirostanoside-Spirostanol Saponin from *Agave vera-cruz* Mill. Leaves

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(Received 17 August, 2020, accepted 28 October, 2020)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: The ethanolic extract of fresh leaves of *Agave vera-cruz* Mill. on purification and chromatographic separation has been found to contain a complex mixture of steroidal saponins, out of which one new oligospirostanoside -a spirostanol saponin has been isolated and assigned the structure as: 3-O-[{ β-D-glucopyranosyl (1→6)}{ β-D-xylopyranosyl (1→4) }{ α-L-rhamnopyranosyl (1→2)}-β-D-glucopyranosyl (1→4)-β-D-glucopyranosyl] (25R)-5α-spirostan, 3β, ol (Veracruzonin-B) by chemical (permethylation, methanolysis followed by hydrolysis, Kiliani hydrolysis etc.) and spectral studies(EIMS,FAB-MS, ^{13}C-NMR, IR).

Keywords: *Agave vera-cruz,* oligospirostanoside, spirostanol saponin, Veracruzonin-B.

I. INTRODUCTION

The genus *Agave* is very widely distributed in sub-tropical and tropical regions of the world and reported to have diuretic, antiseptic, antisyphilitic, antifungal, anti-inflammatory, haemolytic, anticancer etc. properties [1-7]. Recently, steroidal saponins [3, 4, 7-15] have been isolated and characterised from various *Agave* species. *Agave vera-cruz* (Amaryllidaceae) commonly called 'Kuwarbuti' has been reported previously for the presence of, saponins [15], furostans [16] and sapogenins [17]. In continuation of studies on the leaves of this plant for saponins [15], an attempt was made to isolate and characterise a new oligospirostanoside [18] from the mixture of steroidal saponins.

II. EXPERIMENTAL

The leaves of *Agave vera-cruz* Mill. were collected from village Hatwar, Distt. Bilaspur (HP), India. Extraction was carried out in open vessel at atmospheric pressure. CC was carried out over silica gel (60-120 mesh, BDH) with $CHCl_3$: MeOH solvent system in the order of increasing polarity. Homogeneity of the fractions was tested by TLC (silica gel-G, BDH with binder) and spots were visualised by 8-10% H_2SO_4 and Ehrlich Reagent followed by heating. Melting points were determined in open capillaries in an electro thermal melting point apparatus. PC (descending) was carried out on Whatman Filter Paper No. 41 and spots were visualised by 'aniline hydrogen phthalate' reagent followed by heating. IR, EIMS, FAB-MS and ^{13}C-NMR spectra were recorded on Perkin Elmer, Jeol D-300, Jeol SX-102/DA-6000 (6KV, 10 mA, Acc. Volt. 10 KV) and Bruker WM-400 (400 MHz) respectively. The solvent systems used were:

A. $CHCl_3$: MeOH: H_2O (60: 50: 10)
B. C_6H_6: EtAc (8 : 2)
C. C_6H_6 : Pet. ether (1 : 1)
D. n-BuOH : AcOH : H_2O (4 : 1 : 5)
E. C_6H_6: MeOH (9:1)
F.n-BuOH : EtOH: H_2O (5 : 1 : 4)

Extraction and Isolation

The chipped fresh leaves (3kg) of Agave vera-cruz were extracted with pet. ether (4 × 6 hrs.), Et Ac (3 × 7 hrs.) and finally with EtOH (5 × 8 hrs.). The ethanol extract was conc. under vac. and extracted with n-BuOH, dried under vac. and dissolved in minimum quantity of MeOH. This was then precipitated drop-wise-drop in large volumes of acetone with constant shaking. The resulting residue was purified and separated by CC to get an Oligospirostanoside, Veracruzonin- B (**1**).

Veracruzonin- B (1)

1 was crystallised from MeOH; mp 200-8º,$[\alpha]_D^{20}$ -56 º (MeOH), R_f 0.82 (Solvent-A, 2.2g). It was positive to Liebermann- Burchard test and negative to Ehrlich Reagent test. Its IR spectrum showed well defined spiroketal absorption bands (902>920 cm^{-1}, 25R). FAB-MS showed molecular ion peak at1181 $[M+H]^+$ and ^{13}C-NMR data as in Table 1.

Acidic Hydrolysis

Acidic hydrolysis of **1** (100 mg) with 8-10% H_2SO_4(50 ml) was carried out by refluxing for 4 hrs. on a steam bath. The usual work up afforded an aglycone, crystallized as colourless needles from MeOH; mp 202-205°, $[\alpha]_D^{20}$ -65.5° ($CHCl_3$) [Tigogenin, Lit. mp 205-208°, $[\alpha]_D^{20}$ -67°($CHCl_3$)], R_f 0.70 (Solvent- B).IR$_{\nu max}^{KBr}$ cm^{-1} 3500-3400 (OH),984, 920, 902, 860 (902 > 920, 25R). EIMS -m/z 416$[M]^+$, 398,357, 347, 344, 302, 287, 273, 139 (base peak) and 115. Its acetate was prepared in cold in usual manner and crystallized as colourless needles from MeOH; mp 203-6°, $[\alpha]_D^{20}$ – 72°($CHCl_3$) [Tigogenin acetate, Lit. mp 206-8°, $[\alpha]_D^{20}$ – 74°($CHCl_3$)], R_f 0.55 (Solvent – C).The aq. hydrolysate was neutralised with $BaCO_3$, filtered and conc. under vac. PC studies (Solvent-D) revealed the presence of D-glucose (R_f 0.18), D-xylose (R_f 0.28) and L- rhamnose (R_f 0.37)

Enzymatic Hydrolysis

1 (50 mg) was taken up in distilled water (25 ml) and β-glucosidase (10 mg) was added along with toluene(3 drops) to cover the aq. layer. The reaction mixture was kept at room temp. for 72 hrs. The PC (Solvent-D) and TLC (Solvent-A) showed the presence of D-glucose (R_f 0.18) and a prosaponin, Veracruzonin-A [15] (R_f 0.82, Co- TLC) respectively.

Kiliani Hydrolysis

1 (50 gm) was kept with Kiliani mixture 25 ml, (AcOH :H_2O : 35% HCl, 35 : 55 :10) at room temp. PC (Solvent -D) after 12 hrs. showed the presence of three spots corresponding to D-glucose (R_f 0.18), D-xylose (R_f 0.28) and L-rhamnose (R_f 0.37). The probe samples after 36 hrs. and 60 hrs. on PC though showed the same spots but the intensity of D-glucose's (R_f 0.18) was almost double and triple respectively. There was no change on PC after 84 hrs. and even upon heating.

Permethylation

1 (250 mg) was permethylated by modified Hakomori's method (NaH, CH_3I, DMSO/N_2 atm.) to get permethylate (200 mg) which was purified by CC.{R_f 0.91 (Solvent -E)}

Methanolysis followed by hydrolysis

The above permethylate (200 mg) was refluxed with dry MeOH -1N HCl (50 ml) for 4 hrs. on a steam bath, MeOH evaporated, H_2O (25 ml) was added and hydrolysed. After usual work up, the aq. neutralised hydrolysate on PC (Solvent-F) showed the presence of 2, 3, 6 tri-O-methyl-D-glucose(R_G 0.83); 3 mono-O-methyl-D-glucose(R_G 0.26); 2, 3, 4 tri-O-methyl-D-xylose (R_G 0.94);2,3,4 tri-O-methyl-L-rhamnose (R_G 1.01) and 2, 3,4, 6 tetra-O-methyl-D-glucose (R_G 1.00).

Partial hydrolysis

1 (1 g) was refluxed on a steam bath with 5% aq. HCl-MeOH (50 ml, 1:1, 45 min.), neutralised (Ag_2CO_3) and filtered. The filtrate was dried under vac. And chromatographed to obtain an aglycone-Tigogenin (mp, mmp, Co-TLC) along with five prosaponins PS_1 to PS_5. Each prosaponin was acid hydrolysed and usual work up showed only one aglycone-Tigogenin. The aq. neutralised hydrolysates on PC (Solvent-D, with authentic samples) showed sugars as: PS_1 and PS_2-D-glucose (R_f 0.18); PS_3-D-glucose(R_f 0.18), L-rhamnose (R_f 0.37); PS_4 -D-glucose(R_f 0.18), D-xylose (R_f 0.28) and PS_5 –D-glucose only. Each prosaponin was subjected to permethylation and methanolysis followed by hydrolysis. After usual work up PC (Solvent-F) of the neutral hydrolysate showed different sugars viz: PS_1 – 2, 3, 4, 6 tetra-O-methyl-D-glucose (R_G1.00); PS_2 – 2, 3, 6 tri-O-methyl-D-glucose (R_G 0.83) and 2, 3, 4,6 tetra-O-methyl-D-glucose (R_G 1.00); PS_3 –2, 3, 6 tri-O-methyl-D-glucose (R_G 0.83); 3, 4, 6 tri-O-methyl-D-glucose(R_G 0.84) and 2, 3, 4, tri-O-methyl-L-rhamnose (R_G 1.01); PS_4– 2, 3, 6 tri-O-methyl-D-glucose (2 moles, R_G 0.83) and 2, 3, 4tri-O-methyl-D-xylose (R_G 0.94) and PS_5-2, 3, 6 tri-O-methyl-D-glucose (R_G 0.83); 2, 3, 4 tri-O-methyl-D-glucose (R_G 0.85) and 2, 3, 4, 6 tetra-O-methyl-D-glucose (R_G 1.00).

III. RESULTS AND DISCUSSION

Veracruzonin–B (**1**) was separated by CC and crystallised from MeOH. Its IR spectrum showed characteristic spiroketal absorption bands [19-22], was positive to Liebermann-Burchard test [23-24] but negative to Ehrlich Reagenttest [19, 25]. Acid hydrolysis [26-28] of **1** afforded an aglycone-Tigogenin (mp, mmp, Co-TLC, EIMS, IR, its acetate) and the aq. neutralised hydrolysate contained D-glucose, D-xylose and L-rhamnose (R_f and Co-PC). Enzymatic hydrolysis [20, 29] of **1** with β-glucosidase revealed the liberation of β-D-glucose on PC and a prosaponin, Veracruzonin-A [15]on TLC. These results indicated that there is one more D-glucose in **1** than that of Veracruzonin-A and it is the terminal sugar of the glycone moiety. This also became evident by its FAB-MS, showing molecular ion peak at 1181 $[M+H]^+$ as compared to Veracruzonin-A at 1019 $[M+H]^+$.

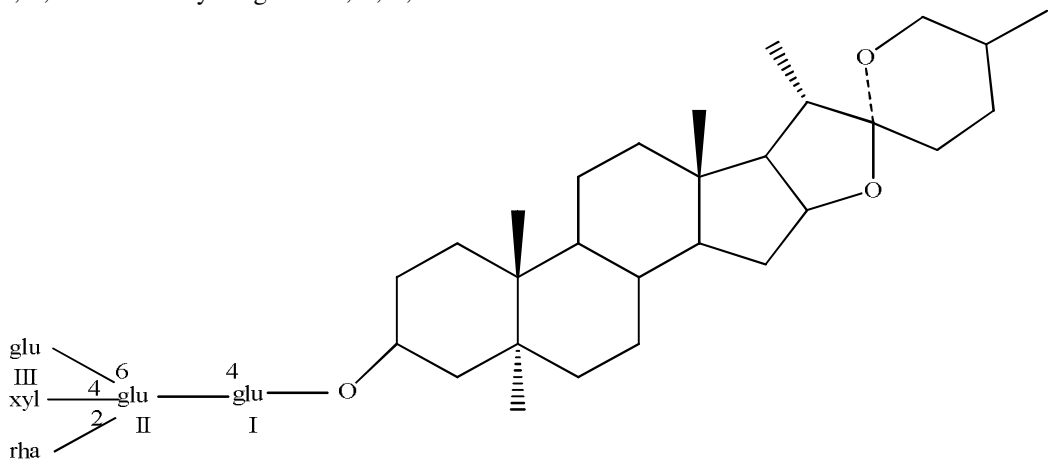
In order to find out the sequence of the sugars, **1** was subjected to Kiliani hydrolysis [30]. The reaction mixture on testing, with the passage of time on PC showed that D-glucose, D-xylose and L-rhamnose emerging out first must be the terminal sugars of the sugar chain. Two molecules of D- glucose emerging out later were the inner sugars through which the terminal sugars D-glucose, D-xylose and L-rhamnose were linked on one end and C-3 of aglycone at the other end as there is no other hydroxyl group available in the aglycone -Tigogenin for the linkage. The configurations of the sugars were deduced by Klyne's Rule [31] as well as from ^{13}C-NMR data [32-33].**1** was per methylated by modified Hakomori's method [20, 34] to get a permethylate, which on methanolys is followed by hydrolysis furnished five methylated sugars, identified by PC as: 2, 3, 6 tri-O-methyl-D-glucose; 3 mono-O-

methyl-D-glucose; 2, 3, 4 tri-O-methyl-D-xylose; 2, 3, 4tri-O-methyl-L-rhamnose and 2, 3,4, 6 tetra-O-methyl-D-glucose. These results again revealed that D-glucose, D-xylose and L-rhamnose are the terminal sugars of sugar chain linked through two molecules of D-glucose attached with C-3 of aglycone.

In order to establish the exact linkages of the sugars with each other, **1** was subject to partial hydrolysis [35-37] to get five prosaponins PS_1 to PS_5. Acid hydrolysis of these prosaponins furnished the same aglycone - Tigogenin but different sugars viz: D-glucose in PS_1 and PS_2; D-glucose, L-rhamnose in PS_3; D-glucose, D-xylose in PS_4 and only D-glucose in PS_5. Each prosaponin on permethylation followed by methanolys is and hydrolysis gave the following methylated sugars:PS_1 – 2, 3, 4, 6 tetra-O-methyl-D-glucose ; PS_2 – 2, 3, 6 tri-O-methyl-D-glucose and 2, 3, 4, 6 tetra-O-methyl-D-glucose; PS_3 –2, 3, 6 tri-O-methyl- D-glucose ;3, 4, 6 tri-O-methyl-D-glucose and 2, 3, 4, tri-O-methyl-L-rhamnose ; PS_4 – 2, 3, 6 tri-O-methyl-D-glucose (2 moles) and 2, 3, 4 tri-O-methyl-D-xylose and PS_5-2, 3, 6 tri-O-methyl-D-glucose ; 2, 3, 4 tri-O-methyl-D-glucose and 2, 3, 4, 6 tetra-O-methyl-D-glucose. Hence, PS_1 = Tigogenin + glucose (at C-3); PS_2 = PS_1+ glucose (1→4); PS_3 = PS_2 + rhamnose (1→2); PS_4 = PS_2+ xylose (1→4) and PS_5 = PS_2 + glucose (1→6). These results established that D-glucose (I) is attached to C-3 of aglycone –Tigogenin on one end and to D-glucose (II) through (1→4) on another end, which in turn is linked to terminal sugars D-glucose (1→6), D-xylose (1→4) and L-rhamnose (1→2).

FAB-MS of **1** showed molecular on peak at 1181 $[M + H]^+$, indicating an aglycone of molecular weight 416 (Tigogenin), three molecules of hexoses (glucose), one molecules of pentose (xylose) and one molecule of methyl pentose (rhamnose). ^{13}C-NMR data (Table 1) further confirmed these results; hence the structure of Veracruzonin-B (**1**) was elucidated as:

3-O-[{ β-D-glucopyranosyl (1→6)}{ β-D-xylopyranosyl (1→4) }{ α-L-rhamnopyranosyl (1→2) }-β-D-glucopyranosyl (1→4)-β-D-glucopyranosyl](25R)-5α-spirostan, 3β, ol.



1

Table 1: ^{13}C-NMR chemical shifts of sugar moieties (D_2O).

Sugars	Carbon Nos. Chemical shifts (ppm)					
	1	**2**	**3**	**4**	**5**	**6**
Glucose (I)	103.4	72.8	81.0	70.1	81.2	61.3
Glucose(II)	105.5	72.6	81.7	69.5	81.6	61.1
Glucose(III)	103.5	73.2	75.2	71.1	75.2	61.4
Xylose	104.6	73.7	76.4	69.8	65.8	—
Rhamnose	102.2	71.8	72.4	73.8	69.0	18.4

ACKNOWLEDGEMENT

The author is highly thankful to the Director, CDRI, Lucknow for EIMS, IR, FAB-MS and ^{13}C-NMR spectra.

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