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Oligofurostanosides-Furostanol Saponins from *Agave vera-cruz* Mill.

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ABSTRACT: Ethanolic extract of fresh leaves of *Agave vera-cruz* Mill. have been isolated for two new oligofurostanosides-furostanol saponins. These on purification and chromatographic separation, followed by various chemical and spectral studies have been assigned structures 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]-26-O-β-D-glucopyranosyl-22α-methoxy- (25R)-5α-furostan-3β, 26-diol (Veracruzoside-C) and 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]-26-O-β-D-glucopyranosyl- (25R)-5α-furostan-3β, 22α, 26-triol (Veracruzoside-D).

Keywords: *Agave vera-cruz,* furostanol saponin, oligofurostanosides, Veracruzoside-C& D

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, hemolytic, anticancer, biofuel, beverages etc. properties [1-7]. Recently, steroidal saponins [3, 4, 7-17] have been isolated and characterised from various *Agave* species. *Agave vera-cruz* (Amaryllidaceae) commonly called 'Kuwarbuti' is a succulent, short stem, evergreen perennial plant, having erect sword-shaped fleshy spiny tipped leaves with small spines on the edges. The plant is widely used in hedging, fencing, rope making etc. In continuation of studies on the leaves of this plant for saponin contents [15-17], two new oligofurostanosides have been isolated, purified and characterised from the mixture of saponins by various chemical and spectral studies.

II. EXPERIMENTAL

The leaves of *Agave vera-cruz* Mill. were collected from village Hatwar, District Bilaspur (Himachal Pradesh), India. Extraction was carried out in open vessel at atmospheric pressure. Column Chromatography (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 (Thin Layer Chromatography: 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. Paper Chromatography (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)

A. Extraction and Isolation

The chipped fresh leaves (3kg) of *Agave vera-cruz* were extracted with petroleum ether (4 × 6 hrs.), EtAc (3 × 7 hrs.) and finally with EtOH (5 × 8 hrs.). The ethanol extract was concentrated under vacuum and extracted with n-BuOH, which was dried under vacuum 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 inseparable mixture for two new oligofurostanosides, named as: Veracruzoside-C **(1)** & Veracruzoside-D **(2).**

Veracruzoside-C (1) & Veracruzoside-D (2). Inseparable mixture **1 & 2** (2.5gm.) could not be separated by CC, showed no spiroketal absorbance in the IR spectrum. On two dimensional TLC their mixture showed three diagonal spots which gave intense red colour on visualisation with Ehrlich Reagent. The Inseparable mixture 1 & 2, however could be easily converted into Veracruzoside-C **(1)** and Veracruzoside-D **(2)** as follow:

Veracruzoside-C (1). Mixture **1&2** (100 mg) was refluxed with dry MeOH (50 ml) for 6 hrs. on a water bath to yield **1**. mp 189-94 °C, $[\alpha]_D^{20}$ - 52° (MeOH), R_f 0.68 (Solvent- A).

Veracruzoside-D (2). Mixture **1&2** (100 mg) was refluxed with aqueous acetone (50 ml, 1:1) for 8 hrs. on a water bath to yield **2**. mp 178-83 °C, $[\alpha]_D^{20}$ -53° (Py), R_f 0.51 (Solvent- A).

Acidic Hydrolysis. Acidic hydrolysis of **1& 2** mixture (100 mg) with 8-10% H_2SO_4 (50 ml) was carried out by refluxing for 4 hrs. on a steam bath. After usual work up, an aglycone was 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 concentrated under vacuum. 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 & 2 (50 mg) was taken up in distilled water (25 ml) and β-glucosidase (10 mg) was added to it along with toluene (3 drops) to cover the aqueous layer. The reaction mixture was kept at room temperature for 72 hrs. The PC (Solvent–D) showed the presence of a D-glucose (R_f 0.18) and TLC a prosaponin, Veracruzonin-A (R_f 0.88, Solvent-A).

Kiliani Hydrolysis. Mixture **1&2** (50 gm) was kept with Kiliani mixture (25 ml, AcOH: H_2O: 35% HCl, 35:55:10) at room temperature and analysed after regular intervals. The reaction mixture after 3 hrs. on PC (Solvent-D) showed one spot corresponding to D-glucose (R_f 0.18), whereas its TLC(Solvent-A) showed the presence of Veracruzonin-B (R_f 0.82, Co-TLC). PC analysis after 12 hrs. showed the presence of two more spots corresponding to D-xylose (R_f 0.28) and L-rhamnose (R_f 0.37) but the intensity of D-glucose was almost double. 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 three and four times respectively with respect to 3hrs. spot. The spots remained unchanged after 84 hrs. and even upon heating.

Permethylation. Mixture **1 & 2** (300 mg) was permethylated by modified Hakomori's method (NaH, CH_3I, DMSO/N_2 atm.) to get permethylate (250 mg) which was purified by CC.(R_f 0.86 (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) added and hydrolysed. After usual work up, the aqueous neutralised hydrolysate on PC(Solvent-F) showed the presence of five methylated sugars as: 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. Mixture **1&2** (1 gm.) was refluxed on a steam bath with 5% aq. HCl-MeOH (50 ml, 1:1, 45 min.), neutralised with Ag_2CO_3 and filtered. The filtrate was dried under vacuum and chromatographed to obtain an aglycone-Tigogenin (mp, mmp, Co-TLC) along with six prosaponins PS_1 to PS_6. Each prosaponin was acid hydrolysed and usual work up showed only one aglycone-Tigogenin. The aqueous neutralised hydrolysates on PC (Solvent-D, with authentic samples) showed sugars as: D-glucose (R_f 0.18) in PS_1, PS_2 and PS_3; D-glucose (R_f 0.18), L-rhamnose (R_f 0.37) in PS_4; D-glucose (R_f 0.18), D-xylose (R_f 0.28) in PS_5 and D-glucose (R_f 0.18) in PS_6. 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_G 1.00); 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, 4, 6 tetra-O-methyl-D-glucose (R_G 1.00, 2 moles); PS_4 – 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_5– 2, 3, 6 tri-O-methyl-D-glucose (R_G 0.83, 2 moles) and 2, 3, 4 tri-O-methyl-D-xylose (R_G 0.94) and PS_6-2, 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

The concentrated ethanolic extract of the fresh leaves of *Agave vera-cruz* showed an inseparable mixture of two new oligofurostanosides, Veracruzoside**-C (1)** and Veracruzoside**-D (2)** by column chromatography.

This inseparable mixture of **1**&**2** showed no characteristic spiroketal absorption bands [18-21] in IR spectrum, however gave positive results with Liebermann–Burchard [22-23] and Ehrlich Reagent [18, 24] indicating its furostanolic nature. The inseparable mixture **1**&**2**, on refluxing with dry methanol converted into **Veracruzoside-C (1),** while on refluxing with aqueous acetone gave **Veracruzoside-D (2)**. Both these compounds showed all the characteristic tests of furostanosides [18-24].

Acid hydrolysis [25-27] of **1& 2** afforded an aglycone-Tigogenin (mp, mmp, Co-TLC, EIMS, IR, its acetate) and the aqueous neutralised hydrolysate contained D-glucose, D-xylose and L-rhamnose (R_f and Co-PC). It revealed that in **1& 2** aglycone part contains Tigogenin and glycone part contains D-glucose, D-xylose and L-rhamnose. Enzymatic hydrolysis [19, 28] of the mixture **1**&**2** with β-glucosidase liberated β-D-glucose and a prosaponin-Veracruzonin-A, negative to Ehrlich reagent test. This revealed that β-D-glucose is

liberated from C-26 of the oligofurostanoside result the closure of ring-F. The formation of corresponding oligospirostanoside (prosaponin) Veracruzonin-A resulted due to the liberation of one β-D-glucose molecule from the terminal position of sugar chain.

In order to find out the sequence of the sugars, **1 & 2** was subjected to Kiliani hydrolysis [29]. Examination of the reaction mixture with the passage of time on PC showed that D-glucose appeared first must be the sugar attached at C-26; since the resulted reaction mixture was negative to Ehrlich reagent test. D-glucose, D-xylose, L-rhamnose emerging out then, must be the terminal sugars of another sugar chain. Two glucose molecules emerging out later are the inner sugars through which D-glucose, D-xylose, L-rhamnose are linked to the aglycone – Tigogenin at C-3. The configurations of the sugars were deduced by Klyne's Rule [30] as well as from ^{13}C-NMR data [31-32].

Mixture **1 & 2** was permethylated by modified Hakomori's method [19, 33] to get a permethylate, which on methanolysis 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; 2,3,4-tri-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 one sugar chain linked through two molecules of D-glucose attached with C-3 of aglycone. The rest D-glucose is the sugar moiety of open ring-F attached at C-26 of aglycone– Tigogenin.

In order to establish the exact linkages of the sugars with each other, mixture **1&2** was subject to partial hydrolysis [34-36] to get six prosaponins PS_1 to PS_6. Acid hydrolysis of these prosaponins furnished the same aglycone -Tigogenin but different sugars viz: D-glucose in PS_1, PS_2 and PS_3; D-glucose, L-rhamnose in PS_4; D-glucose, D-xylose in PS_5 and D-glucosein PS_6. Each prosaponin on permethylation followed by methanolysis 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, 4, 6 tetra-O-methyl-D-glucose (2 moles); PS_4 –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_5 – 2, 3, 6 tri-O-methyl-D-glucose (2 moles) and 2, 3, 4 tri-O-methyl-D-xylose and PS_6-2, 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_1 + glucose (at C-26 and 22 α-OMe/-OH); PS_4 = PS_2 + rhamnose (1→2), PS_5 = PS_2 + xylose (1→4) and PS_6 = PS_2 + glucose (1→6). These results confirmed 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 (III) through (1→6), D-xylose (1→4) and L-rhamnose (1→2). The rest one D-glucose is attached at C-26 of open ring F of aglycone-Tigogenin.

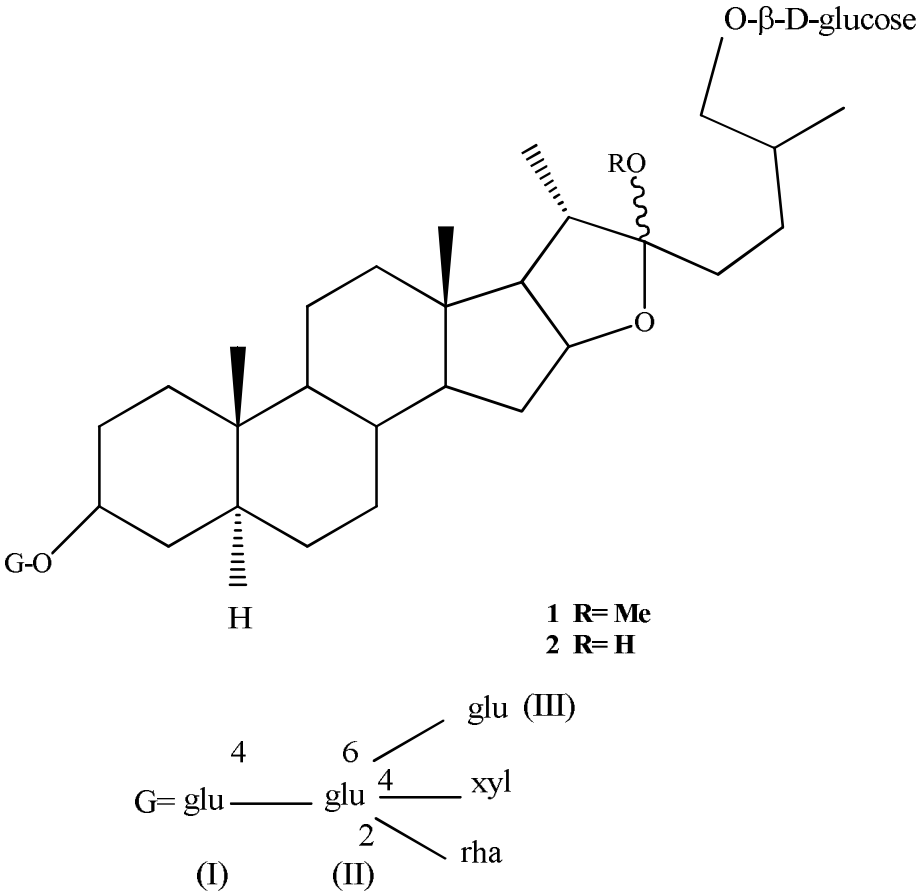
FAB-MS of mixture **1&2** showed molecular ion peak at 1381 $[M + Li]^+$, indicating an aglycone of molecular weight 416 (Tigogenin), four molecules of hexoses (glucose), one molecules of pentose (xylose) and one molecule of methyl pentose (rhamnose) along with open ring-F (22-OMe).

IV. CONCLUSION

FAB-MS, ^{13}C-NMR data (Table 1) and chemical studies confirmed all the above findings /results; hence the structures (Fig. 1) for **1 & 2** were 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]-26-O-β-D-glucopyranosyl-22α-methoxy- (25R)-5α-furostan-3β, 26-diol (**Veracruzoside-C**) and 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]-26-O-β-D-glucopyranosyl-(25R)-5α-furostan-3β, 22α, 26-triol (**Veracruzoside-D**).

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)	103.3	73	81.2	70.1	81.2	61.2
Glucose(II)	105.7	72.8	81.6	69.6	81.6	61.0
Glucose(III)	103.4	73.2	75.2	70.0	75.3	61.4
Xylose	104.6	73.4	76.5	69.8	65.9	--
Rhamnose	102.2	71.6	72.2	73.2	69.0	18.3
Glucose	103.6	73.5	75.4	70.1	75.4	61.4



glu = glucose, xyl = xylose, rha = rhamnose

Fig. 1.

Conflict of Interest. There is no conflict of interest.

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