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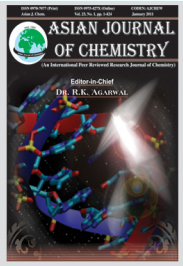

Digitoxigenin-3-O-β-D-Glucopyranoside from The Roots of *Sapium sebiferum*

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The plant *Sapium sebiferum* is commonly known as Vilayati shisham in Hindi and belongs to natural order *Euphorbiaceae*. The plant is widely cultivated in India. The ethyl acetate soluble fraction of the concentrated ethyl alcohol soluble part of its roots when subjected to column chromatography yielded a yellow coloured compound, m.f. $C_{29}H_{44}O_9$, m.p. 200-201 ºC $[\alpha]_D$ -19.3º, M^+ = 536, which on various chemical degradations, colour reactions and spectral analysis was identified as digitoxigenin-3-O-β-D-glucopyranoside AC-4.

Key Words: Chemical degradation, Rectified spirit, Digitoxigenin-3-O-β-D-Glucopyranoside.

INTRODUCTION

The plant *Sapium sebiferum*[1-4] is commonly known as Vilayati shisham in Hindi and belongs to natural order *Euphorbiaceae*. The plant is widely cultivated in India. The acrid juice is powerful Visciant. The roots of *Sapium sebiferum* were procured from Dehradun and authenticated by the faculty of botany. The roots were air-dried, powdered and defatted with petroleum ether and the defatted and powered roots were extracted with rectified spirit. The rectified spirit extract was filtered while hot and concentrated under reduced pressure to get a brown viscous mass. This brown viscous mass was successively extracted with petroleum ether, benzene, chloroform, ethyl acetate and with methanol.

The ethyl acetate soluble fraction of the concentrated ethyl alcohol soluble part of its roots when subjected to column chromatography yielded a yellow coloured compound, m.f. $C_{29}H_{44}O_9$, m.p. 200-201 ºC, $[\alpha]_D$ -19.3º, M^+ = 536, which on various chemical degradations, colour reactions and spectral analysis was identified as digitoxigenin-3-O-β-D-glucopyranoside (AC-4).

EXPERIMENTAL

Isolation of the cardiac glycoside AC-4

Extraction of the roots: About 5 kg of chipped roots were completely dried and powdered and then defatted with petroleum ether in a soxhlet extractor till completely defatted. The defatted powdered roots were then taken out and extracted with rectified spirit in a 5 L round bottomed flask for several days and extract was filtered while hot and concentrated to get brown viscous mass. This brown viscous mass was subsequently subjected to extraction with various solvents of increasing polarity. On examination, the ethyl acetate soluble part of the extract of roots, was found to respond to positive test of cardenolides (kedde's, keller and Legal's test)[5-7]. Therefore, it was concentrated under reduced pressure to a dark yellow viscous mass, which was subjected to TLC examination using chloroform:methanol:water (2:1:1) and kedde's reagent (2 % 3,5-dinitrobenzoic acid) followed by KOH in menthol as visualizing agent, when it showed a single spot, thereby confirming its homogenous character.

This fraction was therefore purified over silica gel column (60-120 mesh) and eluted with chloroform:methanol (3:4) The fractions were collected and removal of the solvent yielded a colourless solid in a yield of 0.032 % (AC-4).

TLC of the glycoside AC-4: The cardiac glycoside (AC-4) was dissolved in 10 mL of methanol and used for the TLC purpose. TLC examination was carried out on (20 cm × 20 cm) glass plate using silica gel G as stationery phase and chloroform: methanol:water (80:18:2) as mobile phase. When the solvent reached about 0.75 height of the plate it was removed and dried at room temperature and sparyed with kedde's reagent and heated in the electric oven at 100 ºC for 2 min when only one spot was noticed (R_f = 0.78).

The elutes which were of same R_f value were collected and combined and removal of the solvent yielded a brown coloured compound (3.01 g) which on crystallization from methanol, yielded a yellow crystalline compound, m.p. 200-201 ºC, $[\alpha]_D$-19.3, m.f. $C_{29}H_{44}O_9$, M^+ 536, UV λ_{max} 216, 270 nm, IR λ_{max} 3595, 2917, 1786-1750, 1630, 1446, 1380, 1340, 1060 and 1025 and found (%) C 64.90, H 8.60, calcd. (%) C 64.92, H 8.20 for $C_{29}H_{44}O_9$.

Hydrolysis of the cardiac glycoside AC-4: 750 mg of glycoside (AC-4) was dried at 100 ºC in vacuum dessicator and boiled under reflux with a mixture of alcohol (100 mL) and water (100 mL) containing 1 mL of sulphuric acid for 0.5 h. On removal of alcohol, the cardiogenin separated in well defined crystal and after drying at 80 ºC in vacuum weighed (400 mg) (AC-4a).

AC-4a is soluble in methanol and absolute alcohol, it has m.p. 253-550 ºC, $[\alpha]_D$ +19.1º in ($CHCl_3$), m.f. $C_{23}H_{34}O_4$, M^+ = 374, UV λ_{max} 218, 276 nm, IR λ_{max} 3590, 2900, 1786-1750, 1620, 1448, 1336, 1168, 1555 and 1060, 1025 and found (%) C 73.69, H 8.94, calcd. (%) C 73.79, H 9.09.

Preperation of acetyl derivative of the cardiogenin: The cardiogenin AC-4a (50 mg) was taken in a flask and 2 mL of acetic anhydride and 1.5 mL of pyridine were added to it. The mixture was refluxed and cooled when the crystalline acetylated product was obtained which was separated by filtration and crystallized from methanol m.p. 219-220 ºC, m.f. $C_{25}H_{36}O_5$, M^+ = 416, ^1H NMR ($CDCl_3$ 100 MHz), δ 0.82, 0.90, 4.95, 4.51, 2.56, 2.78, 5.90, 4.40, 3.42-4.30, 3.38, 2.14, 1.25, -2.00. Found (%) C 72.21, H 8.65, calcd. (%) C 72.11, H 8.65.

Preperation of anhydrous cardiogenin: A solution of cardiogenin (100 mg) in 50 % alcohol containing hydrochloric acid (0.5 mL) was boiled under reflux for 2 h and the contents diluted with 10 mL of water and alcohol removed by distillation under reduced pressure. After standing for some time the semi-crystalline deposit (100 mg) was separated and crystallized first from aqueous acetone and then from ethyl acetate, from which it separated in brilliant prisms, m.p. 202 ºC, $[\alpha]_D$ -13.3º, m.f. $C_{23}H_{32}O_3$. M^+ = 356, found (%) C 77.42, H 8.94 %, calcd. (%) C 77.52, H 8.98.

Preperation of dihydro cardiogenin: 100 mg of cardiogenin was dissolved in 10 mL of 80 % alcohol and shaked with pd-black and hydrogen until no more gas was observed. Reduction proceeded slowly for 2 h and was completed after about 12 h after the catalyst had been removed, the solution was concentrated until solid separated out. The solid (80 mg) after crystallization from ethyl acetate melted at 110-13 ºC and had $[\alpha]_D$+10 (MeOH). m.f. $C_{23}H_{36}O_4$, M^+ = 376, found (%) C 73.40, H 9.95, calcd. (%) C 73.40, H 9.57.

Preperation of isocardiogenin (AC-4a): The cardiogenin (100 mg) was dissolved in 10 % solution of KOH in MeOH (2 mL) and kept for 0.5 h. The solution was then diluted with water (2 mL) and alcohol (2 mL) and acidified with 10 % HCl (3 mL). After standing for 0.5 h, the solution was further diluted and then concentrated under reduced pressure.The solid, which separated, was filtered and purified by crystallization, m.p. 275 ºC, m.f. $C_{23}H_{34}O_4$, M^+ = 374, found (%) C 73.94, H 9.16, calcd. (%) C 73.79, H 9.09.

Identification of sugar after hydrolysis of cardiac glycoside (AC-4a): The aqueous layer obtained after the hydrolysis of the glycoside (AC-4a) was neutralized by the addition of $BaCO_3$ and $BaSO_4$ filtered off. The filtrate was subjected to paper chromatography for identification of sugar and aniline hydrogen phthalate was used as spraying reagent, when only one spot was noticed and the sugar identified as D-glucose (by Co-PC and Co-TLC).

Hydrolysis of the glycoside with enzyme: 30 mg the cardiac glycoside was suspended in an almond emulsion (30 mL) in a conical flask and kept at 40 ºC for 30 h, the hydrosylate was subjected to paper chromatography over Whatmann No. 1 filter paper with authentic sample and aniline hydrogen phthalate was used as spraying reagent. Only one spot appearing with R_f value corresponding of D-glucose (confirmed by authentic sample), thus indicating the D-glucose was attached to the cardiogenin by β-linkage.

RESULTS AND DISCUSSION

The compound (AC-4)was obtained as colourless solid in a yield of 0.032 %. The compound gave positive test of glycoside and responded to characteristic colour reaction with (i) Kedder's (ii) killiani reagent and (iii) Legal's reagent, which are specific for cardiac glycoside.

Cardiac glycoside AC-4: Characteristic band at 1446 cm^{-1} in the IR spectrum of the cardiac glycoside showed the presence of CH_3 group (s) in it, which were estimated by the zeisel method (5.32 %) which showed the presence of two methyl group in AC-4. Other characteristic band was observed at 3595 cm^{-1} which indicate the presence of -OH groups(s) in the glycoside AC-4. The number of OH groups were estimated by acetylation with Ac_2O/pyridine to get an acetylated product. The acetyl group in the acetylated product was estimated by the method of Wiesenberger[8] as described by Belcher and Godbert[9], thereby showing the presence of only one OH group in the cardiac glycoside AC-4.

Another band at 1750 cm^{-1} in the IR spectrum of the cardiac glyocide AC-4 showed the presence of -C=O group in the lactone ring of AC-4[10].

The structure of the cardiac glycoside AC-4 was established by its hydrolysis and subsequent identification of the cardiogenin AC-4a and sugar moity(ies). Therefore, the cardiac glycoside (AC-4), when hydrolyzed with 2 N sulphuric acid yielded a cardiogenin (AC-4a) and sugar (s), which were studied separately.

Cardiogenin (AC-4a): (AC-4a) analyzed for m.f. $C_{23}H_{34}O_4$, M^+ = 374 (by mass spectroscopy) m.p. 253-55 ºC and $[\alpha]_D$ 19.1º (in $CHCl_3$). It responded to characteristic colour reactions of steroids and yielded Diels' hydrocarbon on the dehydrogenation with selenium powder.

Presence of -CH_3 group(s): Important band at 1448 and 2900 cm^{-1} in the IR spectrum of the cardiogenin AC-4a showed the presence of -CH_3 group(s) which when estimate by Zeisel method indicated the presence of two -CH_3 group (2.10 %) in the (AC-4a).

Presence of the lactone group in AC-4a: Characteristic band at 1786-1750 cm^{-1} in the IR spectrum of the cardiogenin AC-4a indicated the presence of a lactone group which was estimated by hydrolysis and titration when it showed the presence of one lactone ring in AC-4a only[11].

Position of lactone ring in AC-4a: The position of lactone ring was established at C-17, because the cardiogenin mono acetate (AC-4a_1) on oxidation with $KMnO_4$ in acetone and subsequent hydrolysis yielded a compound, m.f. $C_{20}H_{32}O_4$, m.p. 235-37 ºC, which was identified as 3 β-14 β dihydroxy etianic acid[12] by comparison with authentic sample.

Presence and position of double bond in AC-4a: The cardiogenin formed a dihydro cardiogenin (AC-4a_2) m.f. $C_{23}H_{32}O_4$, m.p. 110-13 ºC on catalytic hydrogenation[13], thereby

confirming the presence of only one double bond, which was further supported by its IR spectrum peak at 1620 cm^{-1}. The position of the double bond was fixed in the lactone ring, since it gave negative TNM test thereby ruling out the possibility of the double bond being present in the steroidal nucleus AC-4a. Also the production of deep red colour by the cardiogenin in pyridine with alkaline sodium nitropruside shows the presence of α : β unsaturated lactone ring in it, thus confirming[13] its position at C20-C22.

Presence of -OH groups: The cardiogening (AC-4a) on acetylation with acetic anhydride and pyridine gives a mono acetate (AC-4a_1) m.f. $C_{25}H_{36}O_5$, m.p. 219-20 ºC showing the presence of one primary or secondary OH group in it, *i.e.* AC-4a. The presence of OH group in it was further supported by the IR spectrum peak at 3595 cm^{-1} cardiogenin (AC-4a) now accounted for three oxygen atoms and according to its m.f. $C_{24}H_{34}O_4$ the nature and position of the fourth oxygen atom, remainted still to be accounted for in it, *i.e.* AC-4a, the nature of the fourth oxygen atom was shown to be as tertiary OH group, because the cardiogenin AC-4a when heated with dil. HCl, lost a molecule of water and yielded anhydro cardiogenin[14], m.f. $C_{23}H_{32}O_3$, m.p. 202 ºC which formed a monoacetate, m.f. $C_{25}H_{34}O_4$, m.p. 185 ºC. Therefore cardiogenin itself presumably contained two -OH group (one secondary or primary and the other tertiary).

Position of -OH groups in AC-4a

Presence of C-3 OH group: Chromium trioxide/pyridine oxidation of the cardiogenin AC-4a yielded a 'ketone' m.p. 200 ºC, m.f. $C_{23}H_{32}O_4$ which showed a positive Zimmermann test for C-3 keto group, thereby confirming the presence of one OH group at C-3 and further indicating its nature as secondary[15].

This is also in conforming with the fact most of the naturally occurring cardiogenin have a secondary -OH group at C-3.

Presence of C-14 OH group in AC-4a: The cardiogenin (AC-4a) on reaction with KOH formed an isocardiogenin[16], m.p. 275 ºC, m.f. $C_{23}H_{34}O_4$ and its formation may be explained by fixing the tertiary -OH group at C-14.

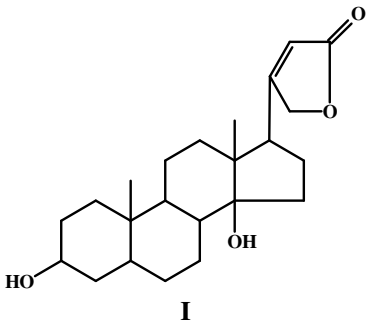
This fact of presence of -OH group at C-3 and C-14 was also supported by the formation of 3β,14 β dihydroxy etianic acid by the oxidation of cardiogenin mono acetate with $KMnO_4$ and subsequent hydrolysis.

Position of -CH_3 groups in AC-4a: Formation of 3β, 14 β dihydroxy etianic acid also supported the presence of two-CH_3 group at C-10 and C-13, respectively.

Based on above discussion the cardiogenion was assigned the structure as displayed compound (**I**) which explained all the reactions. ^{1}H NMR spectrum of the monoacetyl derivative of the cardiogenin further supported the structure of AC-4a.

Quantitative estimation of sugar: Quantitive estimate of sugar present in the glycoside was done by the procedure of Mishra and Rao[17], when it revealed that one mole of the glycoside was made up of one molecule of the cardiogenin and one molecule of D-glucose.

Position of attachement of the sugar to the cardiogenin AC-4a: The position of attachment of sugar residue to AC-4a cardiogenin, was shown to be at position C-3, as the glycoside



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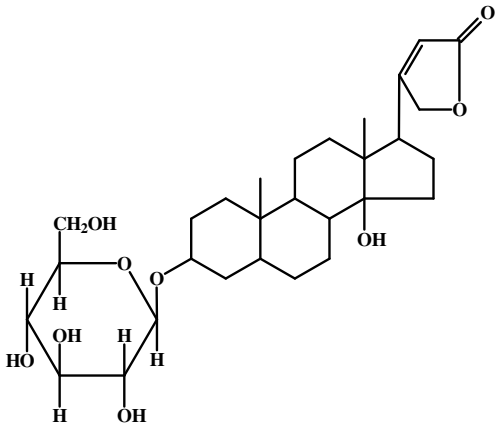

II

did not respond to Zimmermann test while cardiogenin did respond it, there by confirming the presence of -OH group at position C-3 in the cardiogenin and further confirmed its absence in the cardiogenin glycoside AC-4a.

Nature of the glycosidic linkage in AC-4: The cardiac glycoside AC-4 on enzymatic hydrolysis indicated the presence of β-linkage between the cardiogenin (AC-4a) and the sugar D-glucose.The structure of cardiogenin glycoside (AC-4) was thus established as; digitoxigenin-3-O-β-D-glucopyranoside (II) as shown below. The structure was further supported by ^{1}H NMR and mass spectral studies.

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