CHAPTER III

ISOLATION AND STUDY OF THE TRITERPENOIDAL SAPONIN:

OLEAN-12-ENE-28-OIC-3-O-\(\beta\)-D-GLUCOPYRANOSYL (1→4)-

O-\(\alpha\)-L-XYLOPYRANOSIDE FROM *GARDENIA LATIFOLIA* (AIT.) STEMS.
*Gardenia latifolia* (Ait.) \(^1,^3\) belongs to natural order Rubiaceae and is commonly known as papra in Hindi. It is found throughout the greater part of India, mostly in dry forests. It is a bushy crown and grey bark peeling off in flakes. It is sometime found as an epiphyte.

The wood is creamy to yellowish white in colour, with no distinct, heartwood, somewhat lustrous with smooth peel, hard, strong, heavy (sp.gr. e, 0.85 wt. 54 lb/cuft.) fine and even textured. The calorific value of wood is 4,661 cal., 8.390 Btu. The timber is used as a substitute of boxwood.

Because of non-availability of good information about the research on this plant in literature its stem was taken up by the authoress for phytochemical studies.

In this present investigation a saponin has been isolated from the stem of *Gardenia latifolia* (Ait.) and analysed by the use of both classical degradation methods and modern spectroscopic techniques and has been found to be, Olean-12-ene-28-oic-3-O-β-D-glucopyranosyl (1→4)-0-α-L-xylopyranoside.

The air dried powdered and defatted stem of *Gardenia latifolia* (Ait.) was extracted with 95% ethanol
and the extract concentrated under reduced pressure to get a brown syrupy mass which was treated with methanol and methanol soluble part concentrated under reduced pressure, then the saponin was precipitated from it by the addition of excess of solvent ether and separated by decantating off the mixture of solvents. The saponin was purified by dissolving it in methanol and reprecipitating by the addition of solvent ether repeatedly.

The precipitate was dissolved in methanol and its homogeneity was tested by TLC examination when single spot was observed.

It was decolourised by passing it over animal charcoal when a light yellow coloured crystals were obtained. Its homogeneity was ascertained by paper chromatography on Whatmann No. 1 filter paper using the following solvent systems:

(a) n-butanol:acetic acid:water (4:1:5) upper layer.
(b) n-butanol:pyridine:water (5:4:2). The compound gave positive tests for saponin.

The saponin analysed for molecular formula $C_{41}H_{66}O_{12}$, m.p. 218°C, $[\alpha]_D^{25} = -16.8^\circ$ (in pyridine), $M^+$ = 750.
The saponin was insoluble in solvent ether and sparingly soluble in acetone and methanol. It was also soluble in pyridine giving a light yellow viscous solution.

It gave positive haemolytic, honey comb, foam test and other characteristic tests of saponin ⁴.

1. Pale yellow solution, conc. H₂SO₄, changing to red on addition of conc. HNO₃.

2. With Liebermann-reagent cherry red colour obtained.

3. Pink colour with acetic anhydride and pyridine.

**PRESENCE OF PENTACYCLIC TRITERPENOID**

It gave violet colour with 2,6 ditertiary butyl-p-cresol in ethanol ⁵ thereby confirming it to be a penta-cyclic triterpenoid.

**IR SPECTRUM OF THE SAPONIN**

The significant peaks obtained in the IR spectrum of the saponin and the structural assignments ⁶,⁷ made with the help of available literature are given in the Table I.
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Peaks Cm(^{-1})</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3575</td>
<td>-OH group</td>
</tr>
<tr>
<td>2.</td>
<td>2932, 2850</td>
<td>CH(_2)-CH(_2) stretching vibration</td>
</tr>
<tr>
<td>3.</td>
<td>2964</td>
<td>-CH asymmetrical stretching of CH(_3).</td>
</tr>
<tr>
<td>4.</td>
<td>2865</td>
<td>-CH symmetrical stretching of CH(_3).</td>
</tr>
<tr>
<td>5.</td>
<td>1725</td>
<td>C=O stretching</td>
</tr>
<tr>
<td>6.</td>
<td>1640</td>
<td>C=C stretching</td>
</tr>
<tr>
<td>7.</td>
<td>1455</td>
<td>C-H bending</td>
</tr>
<tr>
<td>8.</td>
<td>1385, 1380</td>
<td>CH(_3) gem dimethyl</td>
</tr>
<tr>
<td>9.</td>
<td>1355</td>
<td>-OH bending</td>
</tr>
<tr>
<td>10.</td>
<td>1380, 1375</td>
<td>Triterpene type nucleus</td>
</tr>
<tr>
<td>11.</td>
<td>1247</td>
<td>Vinylvidine type double bond</td>
</tr>
<tr>
<td>12.</td>
<td>820</td>
<td>C-H bending</td>
</tr>
</tbody>
</table>

**Presence of Hydroxyl Groups**

Peaks in the IR spectrum at 3575 Cm\(^{-1}\) indicated the presence of -OH group(s) in it which was estimated by the acetylation of the saponin by Wiesenberger's 8 method.
as described by Belcher and Godbert (26%) thereby indicating the presence of six hydroxyl group in it.

An important peak at 1725 cm\(^{-1}\) in the IR spectrum of the saponin revealed the presence of \(-\text{COOH}\) group(s) in it, which was further confirmed by the formation of an insoluble salt with KOH.

The saponin was found to be a monocarboxylic compound since on methylation with diazomethane it formed a methyl ester, molecular formula \(\text{C}_{42}\text{H}_{68}\text{O}_{12}\), m.p. 198\(^{\circ}\), \(M^+ = 764\), therefore confirming the presence of only one \(-\text{COOH}\) group in the saponin.

A peak in the IR spectrum at 2964 cm\(^{-1}\) showed the presence of \(\text{-CH}_3\) (group(s). The number of \(\text{-CH}_3\) group(s) was estimated by Zeisel's method (13%) indicating the presence of seven methyl groups in it.

A peak in the IR spectrum at 1640 cm\(^{-1}\) showed the presence of olefinic double bond(s). It did not decolourise bromine water, but gave a yellow colour with tetranitromethane \(^{10}\), (Ruzica's reaction), thereby the presence of double bond inside the ring was confirmed.

On catalytic hydrogenation over Raney nickel it yielded a dihydroderivative molecular formula \(\text{C}_{41}\text{H}_{68}\text{O}_{12}\),
m.p. 210°C, $M^+ = 752$, which further confirmed the presence of only one double bond in the compound.

On the basis of the position of $-\text{COOH}$ group, $-\text{OH}$ groups, double bond and that of various methyl groups and by the hydrolysis of the saponin and studying the structure of sapogenin and sugar moieties, separately the structure of the saponin was established.

**HYDROLYSIS OF THE SAPONIN**

The saponin was hydrolysed with $2\text{NH}_2\text{SO}_4$ it yielded sapogenin and sugar moieties. The sapogenin which precipitated out during hydrolysis was separated by filtration.

**STUDY OF THE SAPOGENIN**

The sapogenin analysed for molecular formula $\text{C}_{30}\text{H}_{48}\text{O}_3$, m.p. 306°C and $M^+ = 456$ (by mass spectroscopy) 

$[\alpha]_D^{25} = +78.5^\circ$ (in $\text{CHCl}_3$). It was soluble in methanol, ethanol and responded to all characteristic colour reactions of penta-cyclic triterpene.

1. A red colour in Liebermann-Burchard reaction.
2. Cherry red colouration with Liebermann reagent.
3. A reddish violet colour in Tschugajew reaction.
4. A yellow colour changing to red in Salkowski reaction.  
5. A reddish violet colour in Brieskorn reaction.  
6. A pink colour changing to violet in Noller's reaction.  

**IR SPECTRUM OF THE SAPOGENIN**

The characteristic important peaks obtained in the IR spectrum (Fig. II) of the sapogenin and the structural assignments inferred with the help of available literature are given below in the Table II.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Position of absorption band $\text{cm}^{-1}$</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3425</td>
<td>-OH group(s)</td>
</tr>
<tr>
<td>2.</td>
<td>3020, 3030</td>
<td>$\text{CH}_2$-$\text{CH}$</td>
</tr>
<tr>
<td>3.</td>
<td>2900, 2850</td>
<td>$\text{CH}_3$-$\text{CH}_2$</td>
</tr>
<tr>
<td>4.</td>
<td>1710</td>
<td>-COOH group</td>
</tr>
<tr>
<td>5.</td>
<td>1665, 1654</td>
<td>$\text{C}=$-$\text{C}$ stretching</td>
</tr>
<tr>
<td>6.</td>
<td>1392, 1381, 1370</td>
<td>Triterpenic acid</td>
</tr>
<tr>
<td>7.</td>
<td>1274</td>
<td>Vinylidene type double bond.</td>
</tr>
<tr>
<td>8.</td>
<td>820, 800</td>
<td>-C=$=$-$\text{CH}_2$</td>
</tr>
</tbody>
</table>
PRESENCE OF COOH GROUP

A peak at 1710 cm\(^{-1}\) in the IR spectrum of sapogenin revealed the presence of -COOH group in it. On methylation with diazomethane it yielded a methyl ester, molecular formula C\(_{31}\)H\(_{50}\)O\(_{3}\), m.p. 197\(^{0}\)C, \(M^+ = 470\), therefore showed the presence of only one -COOH group in it.

POSITION OF -COOH GROUP

The methyl ester of the sapogenin could not be saponified with methanolic KOH solution. Of course when saponified with diethylene glycolic KOH it regenerated the sapogenin molecular formula C\(_{36}\)H\(_{48}\)O\(_{3}\), m.p. 306\(^{0}\) (by Co-PC, Co-TLC and m.m.p.).

From the above studies it was concluded that the -COOH group was hindered, thereby it should be attached at C\(_{17}\). The presence of -COOH group at C\(_{17}\) was also confirmed by the signal at \(\delta = 3.62\) in the \(^1\)HNMR spectrum of the methyl ester of the sapogenin.

PRESENCE OF HYDROXYL GROUP

An absorption band at 3425 cm\(^{-1}\) in the IR spectrum of the sapogenin indicated the presence of -OH group(s) in it. The number of -OH group(s) were estimated
when the sapogenin was acetylated, with \( \text{Ac}_2\text{O}/\text{pyridine} \) to get an acetylated product molecular formula \( \text{C}_{32}\text{H}_{50}\text{O}_4 \), m.p. 270\( ^\circ \)C, \( M^+ = 498 \). The percentage of acetyl group (8.25\%) was estimated by the procedure of Weisenberger as described by Belcher and Godbert when it confirmed the presence of one \(-\text{OH} \) group in it.

The presence of only one acetoxylic group was also confirmed by the signal at \( \delta = 2.04 \) in the \(^1\text{H} \text{NMR} \) of the acetylated sapogenin.

**POSITION OF HYDROXYL GROUP**

The methyl ester of the sapogenin molecular formula \( \text{C}_{31}\text{H}_{50}\text{O}_3 \), m.p. 199\( ^\circ \)C, \( M^+ = 470 \), on oxidation with chromic acid \(^{17} \) formed an oxidation product which responded to positive Zimmermann Test \(^{18} \) for 3 Keto group, therefore confirming the presence of one secondary hydroxyl group in the sapogenin at \( \text{C}_3 \).

**PRESENCE OF OLEFINIC DOUBLE BOND**

The sapogenin did not decolourise bromine water but produced yellow colour with tetranitromethane (TNM) thus indicated the presence of an olefinic double bond in one of the ring. The presence of a double bond in one of the rings was also confirmed by the IR spectrum of the
sapogenin in which a peak at 1654 Cm\(^{-1}\) was observed.

The sapogenin on catalytic hydrogenation, in presence of Raney nickel yielded a dihydroderivative molecular formula \(C_{31}H_{52}O_3\), \(M^+ = 472\), which clearly revealed the presence of only one olefinic double bond in the sapogenin.

**POSITION OF DOUBLE BOND**

The sapogenin showed UV absorption at \(\lambda_{max}^{MeClH} 207\) nm a signal of very high terminal absorption which is typical at \(\Delta^{12-13}\) double bond when present in the triterpene of a \(\alpha\) and \(\beta\)-amyrin series\(^{19}\).

The position of double bond at \(\Delta^{12-13}\) was further confirmed by \(^1\)HNMR spectrum of the monoacetyl derivative of the sapogenin at \(\delta = 5.30\) (vinyllic proton) (Fig. iii).

On the basis of above facts the tentative structure to the sapogenin was established as, Olean-12-ene-28-oic acid (I).
The structure (I) to the sapogenin was further confirmed by the $^1$HNMR spectrum of its monoacetyl derivative and mass spectral analysis.

$^1$HNMR SPECTRUM OF THE MONOACETYL DERIVATIVE OF SAPOGENIN

The significant signals (Fig. III) obtained in the $^1$HNMR spectrum of the monoacetyl derivative of the sapogenin and the structural assignments inferred with the help of available literature $^{20}$ are given in Table III and further confirmed structure (I) assigned to it.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Values</th>
<th>Pattern</th>
<th>J.Values</th>
<th>No. of protons</th>
<th>Structural assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.96</td>
<td>S</td>
<td>-</td>
<td>6</td>
<td>$2\times$CH$_3$(t.methyl)</td>
</tr>
<tr>
<td>2.</td>
<td>0.97</td>
<td>S</td>
<td>-</td>
<td>6</td>
<td>$2\times$CH$_3$(t.methyl)</td>
</tr>
<tr>
<td>3.</td>
<td>0.82</td>
<td>S</td>
<td>-</td>
<td>3</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>4.</td>
<td>0.88</td>
<td>S</td>
<td>-</td>
<td>3</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>5.</td>
<td>1.10</td>
<td>S</td>
<td>-</td>
<td>3</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>6.</td>
<td>2.08</td>
<td>S</td>
<td>-</td>
<td>3</td>
<td>C$_3$-OCOCH$_3$</td>
</tr>
<tr>
<td>7.</td>
<td>1.20-2.00</td>
<td>m</td>
<td>-</td>
<td>20</td>
<td>polymethylenic envelope(CH$_2$ and CH)</td>
</tr>
<tr>
<td>8.</td>
<td>2.04</td>
<td>d,d</td>
<td>4 and 12</td>
<td>2</td>
<td>C$_{11}$-H</td>
</tr>
<tr>
<td>9.</td>
<td>3.10</td>
<td>d,d</td>
<td>4 and 9</td>
<td>1</td>
<td>methenic proton at C$_3$</td>
</tr>
<tr>
<td>10.</td>
<td>5.30</td>
<td>d,d</td>
<td>4 and 8</td>
<td>1</td>
<td>C$_{12}$-H vinylic proton</td>
</tr>
<tr>
<td>11.</td>
<td>2.45</td>
<td>Triplet</td>
<td>4</td>
<td>1</td>
<td>C$_{18}$-H</td>
</tr>
</tbody>
</table>
FIG-11

1H NMR SPECTRUM OF ACETYLATED SAPOGENIN
MASS SPECTRUM \textsuperscript{21} OF SAPOGENIN

The important fragments obtained in the electron impact mass spectrum of the sapogenin is given which further confirmed the structure (I) assigned to it $M^+ = 456$ and m/e at 248, 233, 207, 203, 190, 189, 175 and 133.

The different species obtained during fragmentation are shown in Scheme (I) and were found to be in complete agreement with the structure assigned to it.

STUDY OF THE SAPONIN HYDROLYSATE

The aqueous hydrolysate obtained by the hydrolysis of the saponin was neutralized with BaCO\textsubscript{3} and BaSO\textsubscript{4} filtered off. The filtrate reduced Fehling's solution and with aniline hydrogen phthalate gave brown colour. It was then concentrated to a golden yellow mass and the concentrated hydrolysate on paper chromatography over Whatman No. 1 filter paper showed the presence of D-glucose and L-xylose.

QUANTITATIVE ESTIMATION OF SUGARS

The quantitative hydrolysis of the sapogenin (120 mg.) by refluxing with 7\% H\textsubscript{2}SO\textsubscript{4} for five hours yielded 80 mg. of Olean-12-ene-28-oic acid indicating that the sapogenin is 60\% and carbohydrate moieties 40\% (by difference).
Scheme I

(a) \( m/e = 248 \)

(b) \( m/e = 207 \)

\( m/e = 190 \)

\( m/e = 189 \)

\( m/e = 175 \)
Estimation of two sugars present in the sapogenin by the procedure of Mishra and Mohan Rao revealed that the sugars were present in equimolecular ratio (1:1).

Therefore, from the percentage of sapogenin and the amount of carbohydrate moiety and the molecular weight of the sapogenin, it was concluded that one molecule of saponin must be made up of one molecule of oleanolic acid and one molecule of D-glucose and L-xylose.

POSITION OF ATTACHMENT OF THE SUGARS TO THE GENIN

The saponin on hydrolysis with 2N H\textsubscript{2}SO\textsubscript{4} yielded a sapogenin which on oxidation with CrO\textsubscript{3} yielded a keto compound which responded to positive Zimmermann test for the presence of \( \text{C}=\text{O} \) group at C\textsubscript{3} whereas the saponin on oxidation with CrO\textsubscript{3} yielded the compound which did not respond to positive Zimmermann test thereby concluding that C\textsubscript{3}-OH group was free in the sapogenin but was not free in the saponin and hence must be involved in the glycosidic linkage with the sugars.

Thus from the above facts a tentative structure to the saponin was assigned as (II).
SEQUENCE OF SUGAR RESIDUES IN THE SAPOGENIN

The sequence of sugar moieties was determined by partial hydrolysis of the saponin. On partial hydrolysis of the saponin, with Kiliani mixture for one day at room temperature then it was observed that it released first D-glucose followed by L-xylose thereby concluding that D-glucose was the terminal sugar and L-xylose was involved in glycosidic linkage.

The sequence was further confirmed by the isolation of mixture of two prosapogenins P0₁ and P0₂ by column chromatography over silica gel G. after hydrolysis. The
hydrolysate on paper chromatography showed the presence of D-glucose and L-xylose.

STUDY OF PROSAPOGENIN PO

The prosapogenin PO on hydrolysis with 7% H₂SO₄ yielded oleanolic acid and L-xylose. The genin content on quantitative estimation was found to be 75.70%, thereby concluding that prosapogenin PO is formed from one molecule of oleanolic acid and L-xylose, was therefore assigned the structure (III).
The nature of the linkage \( ^{\text{PO}_{1}} \) was established by the permethylation and hydrolysis of the permethylated \((\text{PO}_{1})\) prosapogenin.

**PERMETHYLA\_TION AND HYDROLYSIS OF \( ^{\text{PO}_{1}} \)**

Permethylation of \( ^{\text{PO}_{1}} \) followed by hydrolysis showed the presence of 2:3:4 tri-\( \text{O}-\)methyl-\( \text{L}-\)xylose in the hydrolysate (identified by the paper chromatography with an authentic sample). The formation of 2,3,4 tri-\( \text{O}-\)methyl-\( \text{L}-\)xylose indicated that \( \text{C}_{1} \) of the \( \text{L}-\)xylose was involved in the formation of glycosidic linkage and suggested it to be in pyranose form.

**STUDY OF PROSAPOGENIN \( ^{\text{PO}_{2}} \)**

The prosapogenin \( ^{\text{PO}_{2}} \) on hydrolysis with 7% \( \text{H}_{2}\text{SO}_{4} \) yielded olean-12-ene-28-oic acid and \( \text{D}-\)glucose and \( \text{L}-\)xylose. On quantitative estimation the genin content was found to be 58.60% further confirming the prosapogenin \( ^{\text{PS}_{2}} \) to be made up of one molecule of sapogenin and one molecule of \( \text{D}-\)glucose and \( \text{L}-\)xylose each.
PERMETHYLATION AND HYDROLYSIS OF PROSAPOGENIN PO₂

On complete permethylation PO₂ by Kuhn procedure followed by hydrolysis the prosapogenin PO₂ yielded 2:3 di-O-methyl-L-xylose and 2,3,4,6 tetra-O-methyl-D-glucose. This indicated that PO₂ must be linked with D-glucose which is also present in the pyranoside form.

Thus based on the above facts, the saponin could be assigned the structure as a clean-12-ene-28-oic-3-O-β-D-glucopyranosyl (1→4)-O-α-L-xylopyranoside and represented as (V).
The structure of the saponin was further confirmed by its $^1$HNMR and mass spectral studies.

ENZYMATIC HYDROLYSIS OF SAPONIN

The saponin on enzymatic hydrolysis with enzyme emulsion (specific for β-linkage) gave D-glucose confirming that D-glucose was attached to L-xylose by β-linkage and L-xylose was attached to sapogenin by α-linkage.

In the light of the above facts that saponin was assigned the structure as, Olean-12-ene-28-oic-3-O-β-D-glucopyranosyl (1→4)-O-α-L-xylopyranoside.

$^1$HNMR SPECTRUM OF THE HEXA ACETYL DERIVATIVE OF THE SAPONIN

The important signals (Fig. 14) observed $^1$HNMR spectrum of the hexa acetyl derivative of the saponin and the structural assignments made with the help of available literature 24,25 are given in Table IV.
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Value (δ)</th>
<th>Pattern</th>
<th>J. Value (Hz.)</th>
<th>No. of Protons</th>
<th>Structural assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.95</td>
<td>S</td>
<td>-</td>
<td>6</td>
<td>2xCH₃ tertiary methyl</td>
</tr>
<tr>
<td>2.</td>
<td>0.97</td>
<td>S</td>
<td>-</td>
<td>6</td>
<td>2xCH₃ tertiary methyl</td>
</tr>
<tr>
<td>3.</td>
<td>0.82</td>
<td>S</td>
<td>-</td>
<td>3</td>
<td>CH₃</td>
</tr>
<tr>
<td>4.</td>
<td>0.89</td>
<td>S</td>
<td>-</td>
<td>3</td>
<td>CH₃</td>
</tr>
<tr>
<td>5.</td>
<td>1.10</td>
<td>S</td>
<td>-</td>
<td>3</td>
<td>CH₃</td>
</tr>
<tr>
<td>6.</td>
<td>1.26-2.00</td>
<td>m</td>
<td>-</td>
<td>20</td>
<td>Polymethyl-ene envelop(CH₂ &amp; CH)</td>
</tr>
<tr>
<td>7.</td>
<td>2.03</td>
<td>d,d</td>
<td>4 and 11.2</td>
<td>2</td>
<td>C₁₁-H</td>
</tr>
<tr>
<td>8.</td>
<td>3.06</td>
<td>d,d</td>
<td>5 and 5.9</td>
<td>1</td>
<td>Methenonic proton at C₃H</td>
</tr>
<tr>
<td>9.</td>
<td>5.31</td>
<td>d,d</td>
<td>4 and 8</td>
<td>1</td>
<td>C₁₂-H Vinlylic proton</td>
</tr>
<tr>
<td>10.</td>
<td>2.50</td>
<td>triplet</td>
<td>6 and 8</td>
<td>1</td>
<td>C₁₈-H</td>
</tr>
<tr>
<td>11.</td>
<td>4.29</td>
<td>d</td>
<td>7.5</td>
<td>1</td>
<td>C₁⁻H anomic proton</td>
</tr>
<tr>
<td>12.</td>
<td>5.47</td>
<td>d</td>
<td>7.8</td>
<td>1</td>
<td>C₁⁻IH anomic proton</td>
</tr>
<tr>
<td>13.</td>
<td>3.40-4.18</td>
<td>m</td>
<td>-</td>
<td>9</td>
<td>Sugar protons</td>
</tr>
<tr>
<td>14.</td>
<td>2.06</td>
<td>S</td>
<td>-</td>
<td>3</td>
<td>C₂-OCOCH₃</td>
</tr>
<tr>
<td>15.</td>
<td>2.08</td>
<td>S</td>
<td>-</td>
<td>3</td>
<td>C'₃-OCOCH₃</td>
</tr>
<tr>
<td>16.</td>
<td>2.00</td>
<td>S</td>
<td>-</td>
<td>3</td>
<td>C₂'-OCOCH₃</td>
</tr>
<tr>
<td>17.</td>
<td>2.15</td>
<td>S</td>
<td>-</td>
<td>3</td>
<td>C''₃-OCOCH₃</td>
</tr>
<tr>
<td>18.</td>
<td>2.18</td>
<td>S</td>
<td>-</td>
<td>3</td>
<td>C''₄-OCOCH₃</td>
</tr>
<tr>
<td>19.</td>
<td>2.22</td>
<td>S</td>
<td>-</td>
<td>3</td>
<td>C₆-OCOCH₃</td>
</tr>
</tbody>
</table>
The important fragmentation pattern obtained in the electron impact mass spectrum of the saponin were \( M^+ = 750 \) and other signals at m/e 587, 455, 248, 233, 207, 203, 190, 189, 175, 133 which further supported the structure (V).
SCHEME - II
EXPERIMENTAL

ISOLATION OF THE SAPONIN

About (2 Kg.) air dried, powdered and defatted stem of *Gardenia latifolia* (Ait.) was taken up for present study.

The precipitate (5.0 gm.) was treated with methanol (100 ml.) and methanol soluble part separated by filtration. It was concentrated under reduced pressure and excess of ether (200 ml.) added when a precipitate appeared which was separated by decantating off the mixture of solvents. It was purified by dissolving it again in methanol and precipitating with excess of solvent ether and the process repeated five times.

The crude saponin responded to positive Liebermann Burchard test and gave characteristic colour with antimony trichloride, thionylchloride and phosphomolybidic acid. It gave positive results with foam test and haemolytic test which were carried as follows:

(I) **FOAM TEST**

5 mg. of the saponin was taken in a boiling tube and 10 ml. of distilled water was added to it and shaken well.
One inch thick soapy layer was formed which remained as such for a long time.

(II) **HAEMOLYTIC TEST**

Gelatine (5 gms.) was suspended in 100 ml. (0.9%) aqueous sodium chloride in a boiling tube and allowed to stand for half an hour. 5.0 ml. blood was taken in a (50 ml.) conical flask and stirred with a small quantity of the above gelatine suspension. Blood was poured on a glass plate and spread uniformly and then a drop of the above extract was applied using a micro pipette. After about an hour red blood gelatine film became transparent at the place of application of the extract thereby confirming the presence of saponin in the extract.

**THIN LAYER CHROMATOGRAPHY OF THE SAPONIN**

The crude saponin was dissolved in ethanol and subjected to the preparatory thin layer chromatography. The glass plates of (10 x 10 cm.) were used and a thin film of silica gel G. was applied with the help of applicator. The plates were activated at 110°C for an hour in an electric oven.

Spot of small quantity of the saponin (in ethanol) was applied with the help of a fine capillary at one cm. above from the base of the TLC plates. The solvent system
used was chloroform:methanol:water in the ratio of (11:7:2) while 10% sulphuric acid was the spraying reagent. Only single spot was noticed thereby confirming the homogeneity of the saponin. The saponin (4.5 gm.) was decolourised by passing it over a column of animal charcoal and crystallised from a mixture of chloroform:methanol (1:1) in light yellow crystals. m.p. 218°C, \([\alpha]_D^{25} = -16.8\) (in pyridine).

**STUDY OF THE SAPONIN**

**ELEMENTAL ANALYSIS OF THE SAPONIN**

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C(<em>{41})H(</em>{66})O(_{12})</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 65.26%</td>
<td>C = 65.60%</td>
</tr>
<tr>
<td>H = 9.04%</td>
<td>H = 8.88%</td>
</tr>
<tr>
<td>Molecular weight = 750</td>
<td>Molecular weight = 750</td>
</tr>
</tbody>
</table>

(by mass spectroscopy)

Molecular formula C\(_{41}\)H\(_{66}\)O\(_{12}\)

**HYDROLYSIS OF THE SAPONIN**

60 mg. of the saponin was taken in a 250 ml. quick fit ground glass joint B-24 flask. 75 ml. of 7% \(\text{H}_2\text{SO}_4\) was added to the flask containing saponin and reflux condenser was attached to it. The conical flask was heated for three hours on a water bath and thereafter poured in a ice cold 150 ml. water taken in a 500 ml. beaker, when a crystalline
precipitate appeared which was separated by filtration.

The precipitate was dissolved in minimum amount of chloroform and the solution kept over night at room temperature when a yellow crystalline sapogenin was obtained having m.p. 306°C, \([\alpha]_D^{25} = + 78.5^\circ\) (in CHCl₃).

The aqueous layer was neutralised and worked up separately for identification of sugar(s).

**ELEMENTAL ANALYSIS OF THE SAPOGENIN**

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C₃₀H₄₈O₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 74.87%</td>
<td>C = 78.94%</td>
</tr>
<tr>
<td>H = 11.82%</td>
<td>H = 10.52%</td>
</tr>
</tbody>
</table>

Molecular weight = 456
(by mass spectroscopy)

Molecular formula C₃₀H₄₈O₃

**COLOUR REACTIONS OF THE SAPOGENIN**

The sapogenin responded to the following colour reactions.

1. **SALKOWSKI REACTION**

The solution of the compound in chloroform
produced yellow colour changing to red on addition of conc. $\text{H}_2\text{SO}_4$ to it.

2. **LIEBERMANN-BURCHARD REACTION**

A red violet colour was produced when a few drops of conc. $\text{H}_2\text{SO}_4$ were added to the solution of the compound dissolved in acetic anhydride.

3. **TSCHUGAJEW REACTION**

A red violet colour was developed when the solution of the compound in chloroform was boiled with acetyl chloride and zinc chloride.

4. **BRIESKORN REACTION**

A reddish violet colour was noticed when a small amount of compound was treated with 30% solution of chlorosulphuric acid in glacial acetic acid.

5. **NOLLER'S REACTION**

When a little of the compound was treated with Noller's reagent (prepared by adding 0.1% $\text{SnCl}_2$ in pure thienyl chloride) a pink colour changing to violet was observed.
METHYL ESTER OF THE SAPOGENIN

The sapogenin (100 mg.) was taken in a 250 ml. conical flask in ether and treated with ethereal solution of diazomethane with constant cooling under tap till a permanent yellow colour was obtained. The excess of diazomethane was destroyed by the addition of acetic acid and the ethereal solution was first washed with aqueous sodium bicarbonate and then with water and thereafter dried over anhydrous sodium sulphate. The solvent was removed under reduced pressure when a white residue was obtained. The residue was dissolved in minimum amount of methanol and chromatographed over a column of alumina. Elution with chloroform:methanol (1:1 v/v) yielded the methyl ester of sapogenin which was crystallised from chloroform m.p. 198°C.

ELEMENTAL ANALYSIS OF THE METHYL ESTER OF SAPOGENIN

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C_{31}H_{50}O_{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 82.03%</td>
<td>C = 79.14%</td>
</tr>
<tr>
<td>H = 9.13%</td>
<td>H = 10.63%</td>
</tr>
</tbody>
</table>

Molecular weight = 470 (by mass spectroscopy)

Molecular formula C_{31}H_{50}O_{3}
CHROMIC ACID OXIDATION OF THE METHYL ESTER

About 50 mg. of the methyl ester of the sapogenin was taken in 5 ml. of 90% acetic acid in a 100 ml. conical flask and fractions of solution of CrO₃ (500 mg.) in acetic acid (4 ml.) added to it with constant cooling under the tap water until a permanent orange brown colour was obtained.

The oxidised crystalline product m.p. 169°C was separated out which responded to positive Zimmermann test.

ZIMMERMANN COLOUR TEST

The oxidised product (10 mg.) was dissolved in 5 ml. of 2N KOH in absolute alcohol. The contents were distilled with 10 ml. of absolute alcohol after ten minutes a reddish violet colour developed.

SAPONIFICATION OF METHYL ESTER

50 mg. of the methyl ester of the sapogenin was dissolved in 20 ml. of ethylene glycol in a 250 ml. round bottom flask having a reflux condenser attached to it and then 40 ml. of 2N KOH was added. The contents were heated at 150⁰-160⁰C on a sand bath for three hours, cooled and poured into ice cold water (100 ml.) with constant stirring.
and extracted with ether. The ethereal extract on concentration yielded a gelatinous mass which was crystallized and melted at 308°C.

IDENTIFICATION OF SUGARS IN THE SAPONIN HYDROLYSATE

The aqueous hydrolysate obtained by after the separation of the sapogenin was neutralized with barium carbonate and barium sulphate filtered off. The filtrate was concentrated to a brown mass and the sugars identified by paper chromatography using different solvent systems and aniline hydrogen phthalate as spraying reagent, whereupon the presence of D-glucose and L-xylose was noticed which was further confirmed by Co-PC and Co-TLC with authentic sugar samples.

The observations and results are recorded in the table given below:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Solvent systems</th>
<th>Values</th>
<th>Sugars identified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unknown</td>
<td>Known</td>
</tr>
<tr>
<td>1.</td>
<td>n-butanol:acetic acid:water (4:1:5)</td>
<td>0.19</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.26</td>
<td>0.28</td>
</tr>
<tr>
<td>2.</td>
<td>n-butanol:ethanol:water (45:5:49) add 1/4 NH₄OH</td>
<td>0.108</td>
<td>0.105</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.172</td>
<td>0.170</td>
</tr>
<tr>
<td>3.</td>
<td>n-butanol and 1/4 NH₄OH</td>
<td>0.068</td>
<td>0.070</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.122</td>
<td>0.125</td>
</tr>
</tbody>
</table>
PARTIAL HYDROLYSIS OF THE SAPONIN

The saponin was treated with 100 ml. of Kiliani mixture (hydrochloric acid:acetic acid:water, 15:35:50) in a 250 ml. round bottom flask and the reaction mixture kept at room temperature for five days.

The content of the flask were then extracted with n-butanol (50 ml.) and subjected to TLC examination when two spots were noticed showed the presence of two compounds. The butanol extract was then concentrated and chromatographed and over a column of silica gel, eluted with chloroform:methanol (1:1), yielded two compounds which were designated as PO₁ and PO₂ and were recrystallised from methanol.

HYDROLYSIS OF PO₁

The PO₁ was hydrolysed with 7% H₂SO₄ for four hours when a precipitate appeared which was extracted with chloroform in a separating funnel. The chloroform extract was concentrated and crystallised into colourless needles from a mixture of chloroform:methanol (1:1) m.p. 185-190°C (d), [α]D²⁵ = + 31° (in CHCl₃). It was identified as Lupeol-21-ene by m.m.p. and Co-PC.

The hydrolysate on paper chromatographic examination revealed the presence of L-xylose.
PERMETHYLATION AND HYDROLYSIS OF \( \text{PO}_1 \)

\( \text{PO}_1 \) (40 mg.) was taken in a B-14 ground joint conical flask (50 ml.) and treated with methyl iodide (10 ml.) and silver iodide (30 mg.) in dimethyl formamide (4 ml.) and a refluxed for 50 hours at room temperature. The contents of the flask were filtered and residue was taken up in chloroform (20 ml.). The chloroform extract was washed with water and chloroform was evaporated. It was hydrolysed with Kiliani mixture. The sapogenin precipitated out on addition of excess of water.

The aqueous part was neutralized with \( \text{BaCO}_3 \) and \( \text{BaSO}_4 \) and filtered off. The concentrated filtrate was chromatographed over Whatmann No. 1 filter paper using n-butanol:ethanol:water (4:1:5) as solvent system for methylated sugars and aniline hydrogen phthalate as spraying reagent. The methylated sugar was identified as 2:3:4 tri-O-methyl xylose by comparision with the authentic methylated sugar on paper chromatography.

HYDROLYSIS OF \( \text{PO}_2 \)

It was done in the same way as described for \( \text{PO}_1 \), when Lupeol-21-ene and D-glucose and L-xylose (confirmed by Co-PC and Co-TLC) were obtained.
PERMETHYLATION AND HYDROLYSIS OF PO₂

The prosapogenin PO₂ on complete permethylation followed by hydrolysis yielded 2:3:4:6 tetra-O-methyl-D-glucose and 2:3:4 tri-O-methyl-L-xylose (confirmed by Co-PC, Co-TLC and authentic sample of methylated sugars).

ENZYMATIC HYDROLYSIS OF THE SAPONIN

The saponin on hydrolysis with enzyme emulsion yielded D-glucose, confirming that glucose is attached to xylose by β-linkage. Further since xylose was not hydrolysable with enzyme emulsion (specific for β-linkage) so it could be concluded that L-xylose must be attached to sapogenin by α-linkage.

In the light of above facts structure to the saponin was assigned as; Olean-12-ene-28-oic-3-O-β-D-glucopyranosyl (1→4)-O-α-L-xylopyranoside.
REFERENCES


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