CHAPTER IV

STUDIES ON TRITERPENOIDAL SAPONINS
FROM THE STEMS OF THYMUS SERPYLLUM
(LINN.)
Thymus serpyllum (Linn.)\textsuperscript{1,2} is known as Banajwain in Hindi and belongs to natural order Labiatae. It is one of the most effective ground coverers, forming dense mats.

The plant is found in the Himalayas from Kashmir to Nepal and is also reported to be grown in the gardens of Western India.

A detailed description of the plant and its important medicinal uses have been given in Chapter II of the thesis.

\textbf{STUDY OF 95\% ETHANOLIC EXTRACT:}

The stems of \textit{Thymus serpyllum} (Linn.), natural order Labiatae were air dried, powdered, defatted and extracted with 95\% ethanol and the extract filtered while hot. On concentration under reduced pressure it gave a brown syrupy mass and poured with continuous stirring in distilled water to get the water soluble and water insoluble fractions.

The water insoluble part was successively extracted with benzene, chloroform, acetone, methanol and ethanol from which the solvents were removed under reduced pressure.

The benzene, chloroform and acetone soluble part resulted in very small amount of residue which were insufficient for any substantive study.
The ethanol soluble part is described in part II of Chapter IV of the thesis.
PART I:

ISOLATION AND STUDY OF (Ursolic acid-3-O-B-D-glucopyranosyl(1\,\,4)-O-\,\,L-rhamnopyranoside FROM THE STEMS OF THYMUS SERPYLLUM (Linn.)
STUDY OF THE METHANOL SOLUBLE PART:

ISOLATION OF THE SAPONIN:

The methanol soluble part on concentration under reduced pressure yielded a brown viscous mass which on addition of excess of solvent ether gave a precipitate from which the solvent was removed by decantation. The precipitate was again dissolved in methanol, and precipitated with ether. The process was repeated several times till the precipitate was completely free from impurities. Finally the precipitate on thin layer chromatographic examination over silica gel G using solvent system n-butanol : acetic acid : water (4:1:5) showed only one spot which confirmed its homogeneity.

The crude saponin was crystallised from chloroform: Methanol (1:1) in light yellow coloured crystals, which responded to all the tests for saponin.

It was insoluble in water and ether but soluble in methanol and ethanol. It dissolved in pyridine giving a yellow solution.

STUDY OF THE SAPONIN:

The saponin analysed for molecular formula \( C_{42}H_{68}O_{12} \). 

\[ D = 83.5^\circ \] (in pyridine) \( K^+ = 764 \) and m.p.\( 269^\circ C \).
It was insoluble in solvent ether, sparingly soluble in acetone and readily soluble in pyridine, giving a bright yellow viscous mass. It gave positive honey comb test and responded to other characteristic colour reactions of saponin\textsuperscript{3-5}.

On reaction with acetic anhydride and concentrated sulphuric acid it gave pink colour and in concentrated sulphuric acid a pale yellow solution turning red on addition of concentrated nitric acid, and with Dibemmann reagent (KNO\textsubscript{2} in conc. H\textsubscript{2}SO\textsubscript{4}) an intense cherry red colouration, thereby confirming it to be a triterpenoidal saponin\textsuperscript{3-5}.

**I.R. SPECTRUM OF THE SAPONIN (Fig. 1)**

In the I.R. spectrum of the saponin, the significant peaks obtained and the structural assignment made with the help of the available literature\textsuperscript{6,7} is given in Table I.

**TABLE - I**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Wave length cm\textsuperscript{-1}</th>
<th>Structural unit inferred</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>3590, 1050, 1110</td>
<td>-OH group</td>
</tr>
<tr>
<td>2</td>
<td>3025, 3030</td>
<td>CH = CH</td>
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<th>S.No.</th>
<th>Wave length cm(^{-1})</th>
<th>Structural unit inferred</th>
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<tbody>
<tr>
<td>3</td>
<td>2900, 2850</td>
<td>CH(_3)-OH(_2)</td>
</tr>
<tr>
<td>4</td>
<td>2960</td>
<td>CH(_3) stretching vibration of CH(_3)</td>
</tr>
<tr>
<td>5</td>
<td>2840</td>
<td>CH(_3) group</td>
</tr>
<tr>
<td>6</td>
<td>1666-1650</td>
<td>CH(_2)-OH</td>
</tr>
<tr>
<td>7</td>
<td>1420, 1105</td>
<td>OH bending and O-O stretching vibration</td>
</tr>
<tr>
<td>8</td>
<td>1390</td>
<td>Stretching of secondary OH group</td>
</tr>
<tr>
<td>9</td>
<td>1392, 1381, 1379</td>
<td>OH bending vibration of CH(_3)</td>
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<tr>
<td>10</td>
<td>1247</td>
<td>Triterpene type nucleus</td>
</tr>
<tr>
<td>11</td>
<td>800</td>
<td>Vinylidine type = bond</td>
</tr>
</tbody>
</table>

**PRESENCE OF OH GROUP(S):**

Peaks in the I.R. spectrum at \(\text{ABr max} 3590, 1050\) cm\(^{-1}\) indicated the presence of OH group(s) which were estimated by acetylation with acetic anhydride and pyridine when it formed an acetyl derivative; molecular formula \(C_{48}H_{82}O_{18}\), m.p. 261-2\(^{\circ}\)C acetyl % = 14.90 thereby indicating the presence of six acetyl groups.
IR SPECTRUM OF THE SAPONIN

FIG. (I)
**Presence of Double Bond(s):**

On reaction with tetranitromethane\(^{25}\) the saponin gave a positive test indicating the presence of olefinic bond. It did not decolourise bromine water thereby confirming the presence of double bond inside the ring. Peaks in the I.R. spectrum at \(\text{KBr} \text{ max } 3025-3030 \text{ cm}^{-1}\) showed the presence of olefinic bond\(^{6,7}\) in the compound. On catalytic hydrogenation over Raney nickel it yielded a dihydro-derivative, molecular formula \(C_{42}H_{70}O_{12}\), m.p. 259°C, \(M^+ = 766\), further confirming the presence of only one double bond in the compound.

**Presence of Methyl Group(s):**

In the I.R. spectrum of the saponin other peaks at \(\text{KBr max } 2840 \text{ cm}^{-1}\) showed the presence of methyl group(s). Which when estimated by Zeisel's method (14.00%) indicated the presence of seven methyl groups in it.

The position of various methyl groups and the structure of saponin was established by the hydrolysis of the saponin and elucidating the structure of the sapogenin and study of various sugar moieties separately.
HYDROLYSIS OF THE SAPОGENIN:

On hydrolysis with 2N H₂SO₄ the saponin with 2N H₂SO₄ gave sapogenin and sugar moieties. They were separated by filtration and the structure of the saponin was established by studying the sapogenin and sugar moieties.

STRUCTURAL STUDY OF THE SAPОGENIN:

The sapogenin analysed for molecular formula C₃₀H₄₈O₃, M⁺ = 456,  Δ₂⁰ = +52 cmH₂O m.p. 292°C responded to the positive colour reactions characteristic of triterpene 8-14.

On reaction with 2,6-ditertiary butyl para cresol in ethanol it gave violet colour which support it to be of pentacyclic nature 14.

U.V. 15 SPECTRUM OF THE SAPОGENIN:

In the U.V. spectrum it did not show any absorption beyond 208 nm in methanol.

I.R. SPECTRUM OF THE SAPОGENIN (Fig. II)

In the I.R. spectrum of the sapogenin, the significant peaks obtained and the structural assignment made with the help of the available literature 16, 17 are given in Table II.
### TABLE - II

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Peaks cm(^{-1})</th>
<th>Assignment</th>
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<td>1</td>
<td>3590</td>
<td>-OH group</td>
</tr>
<tr>
<td>2</td>
<td>3035</td>
<td>(\text{RC} = \text{CH}) stretching vibration</td>
</tr>
<tr>
<td>3</td>
<td>2915, 2860</td>
<td>(\text{CH}_3 - \text{CH}_2)</td>
</tr>
<tr>
<td>4</td>
<td>1685</td>
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<tr>
<td>5</td>
<td>1670</td>
<td>(-\text{OH}_2)</td>
</tr>
<tr>
<td>6</td>
<td>1392, 1381, 1370</td>
<td>Triterpenic acid</td>
</tr>
<tr>
<td>7</td>
<td>1247</td>
<td>Vinylidine type = bond</td>
</tr>
<tr>
<td>8</td>
<td>820</td>
<td>(-\text{OH} = \text{CH}_2) stretching vibration</td>
</tr>
</tbody>
</table>

### PRESENCE OF OH GROUP(S) :

In the I.R. spectrum of the sapogenin, the absorption band at \(\text{KBr max}\ 3590\ \text{cm}\^{-1}\) indicated the presence of OH group(s) in it. Acetylation of the sapogenin with acetic anhydride and pyridine formed an acetyl derivative \(\text{C}_3\text{H}_5\text{O}_4\\text{O}^+\) \(M^+ = 448\), m.p. 270\(^\circ\)C. The percentage of acetyl group 8.80% in the acetylated product was determined by the method of Weisenberger\(^{18}\) as described by Belcher and Godbert\(^{19}\).
IR SPECTRUM OF THE SAPOGENIN

FIG (II)
The formation of acetyl derivative was further supported by the appearance of the acetyl absorption at 1480 cm\(^{-1}\) in the I.R. spectrum and disappearance of the hydroxyl absorption in the I.R. spectrum of the acetylated sapogenin. The signal for the methylene proton at 4.54 in \(^1\)H NMR further confirmed the presence of the hydroxyl group at C\(_3\).

**POSITION OF OH GROUP(S):**

On oxidation with chromic acid\(^{20}\) the methyl ester of the sapogenin formed an oxidation product which responded to positive Zimmermann test\(^{21}\), for the presence of C-3 keto group. This indicated the presence of one secondary hydroxy group at C\(_5\).

Conclusively the above facts indicated the genin to be pentacyclic triterpene of \(-\)amyrin series with one carboxyl group.

**PRESENCE OF COOH GROUP:**

In the I.R. spectrum of the sapogenin the absorption band at KBr max 1685 cm\(^{-1}\) indicated the presence of COOH group in it. Therefore on methylation it formed a methyl ester \(C_{31}H_{50}O_3\), m.p. 197-200\(^\circ\). The molecular weight 470 of the methyl ester confirmed the presence of only one COOH group in it.
POSITION OF COOH GROUP:

The methanolic KOH solution could not saponify the methyl ester of the sapogenin thus indicating that the COOH group was hindered and therefore must be attached at C$_{17}$.

The presence of COOH group at C$_{17}$$^{22,23}$ was further confirmed by signal in NMR at 3.62 of its methyl ester.

POSITION OF DOUBLE BOND:

The sapogenin decolourised bromine water, indicating the presence of unsaturation in it. On reaction with tetranitromethane$^{24}$ the sapogenin gave positive colour, indicating the presence of an olefinic double bond in one of the ring. Also a peak in I.R. spectrum at 1247 cm$^{-1}$ confirmed the presence of vinylidene type double bond$^{25,26}$.

POSITION OF THE DOUBLE BOND:

U.V. absorption of the sapogenin showed high terminal absorption typical of 12-13 double bond present in the triterpene of -amyrin series$^{27}$. The fact was further confirmed by $^1$HNMR spectrum of the monoacetyl derivative of the sapogenin at $= 5.31$ (vinyllic proton) (Fig. IV).

On the basis of the above facts the structure of the sapogenin was established as Ursolic acid.
This structure was further confirmed by its mass and NMR spectra.

The important signals obtained in the $^1$H NMR spectrum of the monoacetate derivatives of the sapogenin and the structural assignment made with the help of available literature$^{29,30}$ are given in the Table III and further confirmed the structure (I) assigned to it.
TABLE - III

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Value</th>
<th>Pattern</th>
<th>J. Value</th>
<th>No. of Protons</th>
<th>Structural assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.66</td>
<td>s</td>
<td>-</td>
<td>6</td>
<td>$2 \times \text{CH}_3$</td>
</tr>
<tr>
<td>2</td>
<td>0.67</td>
<td>s</td>
<td>-</td>
<td>6</td>
<td>$2 \times \text{CH}_3$</td>
</tr>
<tr>
<td>3</td>
<td>0.52</td>
<td>s</td>
<td>-</td>
<td>6</td>
<td>CH$_3$ group</td>
</tr>
<tr>
<td>4</td>
<td>0.59</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>CH$_3$ group</td>
</tr>
<tr>
<td>5</td>
<td>1.01</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>CH$_3$ group</td>
</tr>
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<td>s</td>
<td>-</td>
<td>3</td>
<td>CH$_3$ COCH$_3$</td>
</tr>
<tr>
<td>7</td>
<td>1.20-2.00</td>
<td>m</td>
<td>-</td>
<td>20</td>
<td>Polymethlene envelop (CH$_2$ and CH)</td>
</tr>
<tr>
<td>8</td>
<td>2.03</td>
<td>d,d</td>
<td>4 &amp; 12</td>
<td>2</td>
<td>C$_{11}$ H</td>
</tr>
<tr>
<td>9</td>
<td>4.54</td>
<td>d,d</td>
<td>5 &amp; 9</td>
<td>1</td>
<td>Methenic proton at $\text{O}_3$</td>
</tr>
<tr>
<td>10</td>
<td>5.31</td>
<td>d,d</td>
<td>4 &amp; 8</td>
<td>1</td>
<td>C$_{12}^-$H vinyllic proton</td>
</tr>
<tr>
<td>11</td>
<td>2.50</td>
<td>Triplet</td>
<td>4</td>
<td>1</td>
<td>C$_{16}^-$H</td>
</tr>
</tbody>
</table>

The important fragments obtained in the electron impact mass spectrum of the sapogenin is given below which further supported the structure (I) assigned to it.
'$^1$H NMR SPECTRUM OF THE ACETYLATED SAPOGENIN
60 MHz, SOLVENT CDCl$_3$ + CF$_3$COOH.'
M* = 456 (5%), and m/e = 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 accord with the structure assigned to it.

**STUDY OF THE SAPONIN HYDROLYSATE**

After the hydrolysis of the saponin, the aqueous hydrolysate obtained was neutralized with \( \text{BaCO}_3 \) and filtered. The filtrate was concentrated to a golden yellow mass, which reduced Fehling's solution and produced brown colour with aniline hydrogen phthalate, thereby confirming its carbohydrate nature. On paper chromatography by Whatman No. 1 filter paper it revealed the presence of \( \text{L-rhamnose} \) and \( \text{D-glucose} \) (Co-PC and Co-TLC).

**QUANTITATIVE ESTIMATION OF SUGARS**

On hydrolysis of the saponin (100 mg) on hydrolysis with 2N sulphuric acid for four hours yielded sapogenin (65 mg) and carbohydrate moieties (35 mg) by difference. The sugars when estimated by the procedure of Mishra and Rao\(^\text{31}\), showed that the two sugars (glucose, rhamnose) were present in the saponin in equimolecular ratio (1:1).
Scheme I

m/e = 456

m/e = 248

m/e = 233

m/e = 207

m/e = 203

m/e = 133

m/e = 189

m/e = 175
It was thus concluded from the percentage of sapogenin and the amount of carbohydrate moieties that one molecule of the saponin was made up of one molecule of Urosolic acid and one molecule each of D-glucose and L-rhamnose.

**POSITION OF ATTACHMENT OF SUGAR(S) TO THE SAPOGENIN**

It was concluded on looking to the structure of Urosolic acid that either all the sugars were attached to the -OH group in a sequence or to the COOH group in a sequence or some may be attached to the -OH group and other to the -COOH group. The position of attachment was finally established at C₃ as both sapogenin and saponin gave the test for free-COOH group. This is also supported by the fact that the saponin was not hydrolyzable with 5N NH₄OH (a specific reagent for the hydrolysis of sugar esters), thus concluding that all the sugar units were linked as a bisside unit to the -OH group at C₃.

Also that, on hydrolysis with 2N sulphuric acid the saponin yielded a sapogenin, the methyl ester of which on CrO₃ gave a positive Zimmermann's test and thus showed the presence of one secondary hydroxyl group at position C₃, but the saponin gave a negative test, thus concluded
that the sugar must be attached at $C_3$. Thus a tentative structure to the saponin assigned as II.

\[
\text{II}
\]

**SEQUENCE OF SUGAR(S) IN SAPONIN**

The sequence of sugar residues was determined by partial hydrolysis with Kiliani mixture of the saponin.

The saponin on partial hydrolysis$^{33}$ with Kiliani mixture for one day at room temperature yielded a mixture of two prosapogenins designated as $\text{P}_1$ and $\text{P}_2$ which were separated by a column of silica gel using chloroform.
methanol (1:2) as an eluant. The hydrolysate on paper chromatography showed the presence of D-glucose and L-rhamnose (Co-PC and Co-TLC).

**STUDY OF THE PROSAPOGENIN H51:**

The prosapogenin H51 analysed for molecular formula C35H56O7; M^+ = 588, m.p. 266°C and on hydrolysis with 7% sulphuric acid yielded Ursolic acid and L-rhamnose. The sapogenin content on quantitative estimation was found to be 72.00% thereby confirming that prosapogenin H51 was formed by one molecule of Ursolic acid and one molecule of L-rhamnose.

**PERMETHYLATION AND HYDROLYSIS OF H51**

H51 on permethylation^{34}, followed by hydrolysis showed the presence of 2,3,4-tri-O-methyl rhamnose in the hydrolysate (identified by paper chromatography with authentic sample). The formation of 2,3,4-tri-O-methyl-L-rhamnose indicated that the O1 of the L-rhamnose was involved in the formation of glycosidic linkage and also suggested that L-rhamnose was present in the pyranose form.
On the basis of above facts the prosapogenin $R_1$ may be assigned as III below:

![Chemical Structure](image)

**STUDY OF THE PROSAPOGENIN $R_2$:**

The prosapogenin $R_2$ analysed for molecular formula $C_{38}H_{60}O_{15}$, $m.p.$ and on hydrolysis with 7% sulphuric acid yielded Ursolic acid, D-glucose and L-rhamnose. The sapogenin content was found to be 60.00% of the saponin, confirming that prosapogenin $R_2$ was formed by one molecule of Ursolic acid and one molecule each of D-glucose and L-rhamnose.
PERMETHYLATION AND HYDROLYSIS OF RS₂:

RS₂ on permethylation followed by hydrolysis showed the presence of 2,3,di-O-methyl-L-rhamnose and 2,3,4,6-tetra-O-methyl-D-glucose indicating that C₄ of L-rhamnose was involved in the formation of the glycosidic linkage.

ENZYMATIC HYDROLYSIS OF THE SAPONIN:

The saponin on enzymatic hydrolysis with emulsin gave D-glucose, thereby indicating that L-rhamnose was attached to D-glucose through B-linkage and that L-rhamnose was attached to the sapogenin through L-linkage since it could not be hydrolysed by enzyme emulsin³⁵.

On the basis of above facts the sapogenin could be assigned the structure as Ursolic acid-3-O-β-D-glucopyranosyl (1→4)-O-L-rhamnopyranoside and represented as IV.
Finally the above proposed structure was confirmed by its $^1$H NMR and mass spectral studies.

$^1$H NMR SPECTRUM OF THE HEXA ACETYL DERIVATIVE OF SAPONIN:

$^1$H NMR spectrum of the hexaacetyl derivative of saponin was found to be in complete conformity with the above structure. The significant signals obtained in $^1$H NMR spectrum of the saponin and structural units inferred with the help of available literature is given below in Table IV.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Value</th>
<th>Pattern</th>
<th>$J$ value (Hz)</th>
<th>No. of protons</th>
<th>Structural assignment</th>
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<td>1</td>
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<td>6</td>
<td>$2 \times \text{CH}_3$ tertiary methyl</td>
</tr>
<tr>
<td>2</td>
<td>0.67</td>
<td>s</td>
<td>-</td>
<td>6</td>
<td>$2 \times \text{CH}_3$ tertiary methyl</td>
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<tr>
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<td>$\text{CH}_3$ group</td>
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<tr>
<td>4</td>
<td>0.59</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>$\text{CH}_3$ group</td>
</tr>
<tr>
<td>5</td>
<td>1.01</td>
<td>s</td>
<td>-</td>
<td>3</td>
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<tr>
<td>6</td>
<td>1.20-2.00</td>
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<td>Polymethylene envelop ($\text{CH}_2$ &amp; CH3)</td>
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<tr>
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<td>d,d</td>
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<td>C11-H</td>
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<td>Methenic proton at C3</td>
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<td>Protons of rhamnosyl unit</td>
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<td>6''-OAc</td>
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**MASS SPECTRUM OF THE SAPONIN**

The important fragmentation pattern obtained in the electronic impact mass spectrum of the sapolin is given which further supported the structure (IV) assigned to it.
HNMR SPECTRUM OF THE ACETYLATED SAPONIN FIG-IV
$M^+ = 764$ and other signals at m/e = 601, 455, 248, 233, 207,
203, 190, 189, 175, 133.

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

3.0 Kg of the stems of *Thymus serpyllum* (obtained from Himalayan range drug field, Simla) and identified by the Botany Department of this University, were air dried, powdered and defatted. The defatted stems were extracted with rectified spirit (3.50 lit.) in a reflux condenser till complete extraction. The rectified spirit extract (3.0 lit.) was filtered while hot and the extract concentrated to a brown syrupy mass.

The brown syrupy mass was poured with continuous stirring in distilled water and the water insoluble part was treated with methanol and the methanol soluble part was concentrated under reduced pressure and excess of solvent ether added in it to precipitate saponin(s) which was separated by decantation. Again the residue was dissolved in methanol and the process of precipitation of saponin(s) repeated till complete precipitation.

The precipitate responded to characteristic colour with Antimony trichloride, Thionyl chloride and Phosphomolybdic acid. The precipitate was again dissolved in methanol and subjected to thin layer chromatography which was found to be homogeneous.
THIN LAYER CHROMATOGRAPHY:

The brown syrupy mass (5.0 mg) was suspended in ethanol and its spot was applied with the help of a fine capillary (1.0 cm) above the bottom of the plate. The solvent system used was chloroform : methanol (50:50) and 10% sulphuric acid was used as spraying reagent when single spot (Rf = 0.64) was obtained.

STUDY OF THE COMPOUND:

The saponin crystallised in light yellow crystals, molecular formula $C_{42}H_{68}O_{12}$, $M^* = 764$, and m.p. 269°C. It was insoluble in solvent ether and sparingly soluble in acetone and readily soluble in pyridine giving pale yellow viscous mass.

ELEMENTAL ANALYSIS OF THE SAPONIN:

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 65.93%</td>
<td>C = 65.96%</td>
</tr>
<tr>
<td>H = 8.7%</td>
<td>H = 8.9%</td>
</tr>
</tbody>
</table>

Molecular formula $C_{42}H_{68}O_{12}$

Molecular weight = 764

It gave positive honey comb test and haemolytic test which were carried out as follows:-
FOAM TEST:

The test was performed as described on page.

HARMOlytic TEST:

The test was done in the same way as described on page.

HYDROlysis OF THE SAPoGENIN:

The saponin (500 mg) was taken in a quick fit ground joint B-24 flask (250 ml) and 0.02N of 7% sulphuric acid (10.0 ml) was added in it and a reflux condenser attached to it. The conical flask was heated for three hours on a water bath and then poured into ice cold water (200 ml) when a yellow crystalline sapogenin separated out which was dissolved in least quantity of chloroform and the solution kept overnight when a yellow crystalline sapogenin m.p. 292°C was obtained. The aqueous layer was separated by filtration and neutralised with barium carbonate and barium sulphate filtered off. The filtrate was concentrated to a golden yellow mass and subjected to column chromatography for the identification of the sugars.

STUDY OF THE SAPoGENIN:

The sapogenin analysed for molecular formula $C_{30}H_{48}O_3$, $M^+ = 456$ and m.p. 292°C.
ELEMENTAL ANALYSIS OF THE SAPOGENIN:

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 78.55%</td>
<td>C = 78.94%</td>
</tr>
<tr>
<td>H = 10.50%</td>
<td>H = 10.52%</td>
</tr>
</tbody>
</table>

Molecular formula = C_{30}H_{48}O_{3}

Molecular weight = 456

On reaction with acetic anhydride and concentrated sulphuric acid it gave pink colour and responded to the following colour reactions.

SALKOWASKI REACTION:

The test was performed as described on page.

LIEBERMANN BURCHARD REACTION:

It was done in the same way as described on page.

TSCHUGAJEW REACTIONS:

The test was done in similar manner as described on page.

METHYL ESTER OF THE SAPOGENIN:

The sapogenin (100 mg) was taken in solvent ether and treated with ethereal solution of diazomethane with constant
cooling till a permanent yellow colour was produced acetic acid was added to destroy the excess of diisomethane and ethereal solution was washed with aqueous sodium bicarbonate and dried over anhydrous sodium sulphate. The solvent was distilled off under reduced pressure to get a white residue which was dissolved in small amount of methanol and chromatographed over a column of neutral alumina and eluted with acetic acid: methanol (1:1) thereby yielding its methyl ester, molecular formula $C_{31}H_{50}O_3$ which crystallised from methanol, m.p. 197°C.

ELEMENTAL ANALYSIS OF METHYL ESTER OF THE SAPOGENIN :

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 79.13%</td>
<td>C = 79.14%</td>
</tr>
<tr>
<td>H = 10.5%</td>
<td>H = 10.6%</td>
</tr>
</tbody>
</table>

Molecular formula = $C_{31}H_{50}O_3$

Molecular weight = 470

CHROMIC ACID OXIDATION OF THE METHYL ESTER :

The methyl ester (60 mg) was suspended in 90% acetic acid (5 ml) in a conical flask (100 ml) and solution of $CrO_3$ (200 mg) in acetic acid (10 ml) was added to it with constant cooling till a permanent orange brown colour was produced.
A crystalline product m.p. 169°C separated out which responded to positive Zimmermann colour for C-3 keto-group.

**Zimmermann Test:**

Oxidised product (20 mg) was dissolved in 2N KOH (10 ml) in absolute alcohol and kept for about 10 minutes when a violet colour was developed.

**Saponification of the Methyl Ester:**

The methyl ester (50 mg) was taken in ethylene glycol (20 ml) in B-14 ground joint round bottomed flask (250 ml) attached with reflux condenser and 2N KOH (50 ml) in absolute alcohol was added in it and contents were heated at 160°C on a sand bath for about 3 hours. Thereafter the contents were cooled and poured into cold water the constant stirring. The contents were extracted with solvent ether and on concentration yielded a gelatin mass which crystallised with chloroform : methanol (1:1) m.p. 294°C.

**Study of the Sugars in the Saponin Hydrolysate:**

The aqueous hydrolysate obtained by the separation of sapogenin after hydrolysis of the saponin was neutralised and barium sulphate filtered off. The filtrate on concentration yielded a golden yellow mass which was subjected
to paper chromatography using different solvent systems and aniline hydrogen phthalate as spraying reagent, when the presence of L-rhamnose and D-glucose was confirmed.

**TABLE**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Solvent system</th>
<th>Rf value Unknown</th>
<th>Rf value Known</th>
<th>Sugar identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n-butanol:Acetic acid:water (4:1:5)</td>
<td>0.29</td>
<td>0.30</td>
<td>D-glucose</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.36</td>
<td>0.37</td>
<td>L-rhamnose</td>
</tr>
<tr>
<td>2</td>
<td>n-butanol and 1% NH₄OH</td>
<td>0.08</td>
<td>0.08</td>
<td>D-glucose</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.22</td>
<td>0.21</td>
<td>L-rhamnose</td>
</tr>
<tr>
<td>3</td>
<td>n-butanol:ethanol:water (45:5:49) and 1% NH₄OH</td>
<td>0.10</td>
<td>0.11</td>
<td>D-glucose</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.26</td>
<td>0.27</td>
<td>L-rhamnose</td>
</tr>
</tbody>
</table>

**PARTIAL HYDROLYSIS OF THE SAPONIN WITH KILIANI MIXTURE**

The saponin (100 mg) was suspended in kiliani mixture (75 ml, hydrochloric acid : acetic acid: water, 15:35:50) in a conical flask (150 ml) and the reaction mixture kept at room temperature for the whole night. The contents of the flask extracted with n-butanol and subjected to TLC examination when two spots were noticed, thereby showing the presence of two compounds. The butanol extract was subjected to column chromatography over column of silica gel. The column when eluted with methanol yielded two compounds which were assigned as Rₐ₁ and Rₐ₂.
STUDY OF THE PROAGLYCONE RS₁;

HYDROLYSIS OF PROAGLYCONE RS₁:

The proaglycose RS₁ (40 mg) when hydrolysed with 7% sulphuric acid for four hours yielded a precipitate which was extracted with chloroform with the aid of a separating funnel and chloroform extract crystallised from a mixture of chloroform and methanol (1:1) as colourless crystals m.p. 295°C.

Paper chromatography of the aqueous hydrolysate revealed the presence of L-rhamnose.

PERMETHYLATION AND HYDROLYSIS OF RS₁:

RS₁ (40 mg) was taken in a B-14 ground joint conical flask (150 ml) and treated with methyl iodide (2.0 ml) and silver oxide (80 mg) in dimethyl formamide (4 ml) and a reflux condenser was attached to it. The flask was heated for 40 hours at room temperature on a water bath and cooled, the residue was taken up in chloroform (15 ml). After removal of the solvent a syrupy mass was obtained which when hydrolysed with kiliani mixture⁴⁶ yielded the sapogenin which was taken up in ether.
The aqueous hydrolysate was neutralised with barium carbonate and barium sulphate filtered off. The filtrate was concentrated to a golden yellow mass and subjected to paper chromatography over Whatman No. 1 filter paper, using butanol : ethanol : water (4:1:5) as developing reagent and aniline hydrogen phthalate as spraying reagent. The sugar was identified as L-rhamnose. The methylated sugar was identified as 2, 3, 4, trimethyl rhamnose.

HYDROLYSIS AND PERMETHYLATION OF \( R_2 \)

The same procedure was followed as described for \( R_1 \). Hydrolysis of \( R_2 \) yielded 2,3,4,tri-O-methyl rhamnose and 2,3,4,6 tetra-O-methyl-glucose (confirmed by Co-PC and Co-TLC).

ENZYMATIC HYDROLYSIS OF THE SAPONIN:

The saponin (50 mg) was suspended in an emulsion (25 to 30 ml) and kept at 50°C for 30 hours, in a round bottomed flask with a reflux condenser attached to it and refluxed for 30 hours. The hydrolysate was chromatographed over Whatman No. 1 filter paper using butanol : acetic acid : water (4:1:5) as solvent system and aniline hydrogen phthalate as spraying reagent, when only one spot was visualised corresponding to L-glucose, thereby indicating
that L-rhamnose was attached to D-glucose through B-linkage and also that L-rhamnose was attached to the sapogenin through -linkage, since it could not be hydrolysed by enzyme emulsin.

PERIODATE OXIDATION OF THE SAPONIN:

The saponin (40 mg) was taken in (250 ml) ground joint conical flask attached with a reflux condenser and the contents were treated with sodium metaperiodate (20 ml of 0.1N). The reaction mixture was allowed to stand at room temperature for two days and a blank was run with the same procedure simultaneously. The amount of the formic acid liberated and molecules of sodium metaperiodate consumed was estimated by the procedure of Jones et al.\textsuperscript{37}
PART - II:

ISOLATION AND STUDY OF (Oleanolic acid-3-O-\-L-rhamnopyranosyl(1 4)-O-B-D-glucopyranoside) FROM THE STEMS OF THYMUS SERPYLLUM (LINN.)
STUDY OF THE ETHANOL SOLUBLE PART:

The ethanol soluble part was obtained as described on page 144 of the thesis.

ISOLATION OF THE SAPONIN:

The ethanol soluble part, on concentration under reduced pressure gave a brown mass, which when poured into large amount of solvent ether, gave a precipitate which was separated by filtration. The precipitate was again dissolved in ethanol and precipitated with ether and this process repeated several times till the precipitate was completely free from impurities. On thin layer chromatographic examination over silica gel G using solvent system n-butanol : acetic acid : water (3:2:5), it showed only the spot which confirmed its homogeneity.

It crystallised from chloroform : ethanol in pale yellow needles, m.p. 276-78°C and responded to all the tests for saponin3-5.

It was insoluble in water and solvent ether and soluble in methanol and ethanol. It dissolved in pyridine giving a yellow solution.
STUDY OF THE SAPONIN

The saponin analysed for molecular formula \( C_{42}H_{68}O_{12} \),
\[
\frac{25}{D} = 95.3 \quad \text{(in pyridine)} \quad M^+ = 764 \quad \text{and m.p. 276-78°C.}
\]
It was insoluble in solvent ether but soluble in pyridine, forming a bright yellow viscous mass. It gave positive
honey comb test and respond to all the characteristic colour
reactions of saponin\(^3-5\).

With acetic anhydride and concentrated sulphuric
acid, it gave a pink colour and in concentrated sulphuric
acid it gave a pale yellow solution, becoming red on
addition of concentrated nitric acid, and with Liebermann
reagent (\( \text{KNO}_2 \) in conc. sulphuric acid) gave intense cherry
red colouration, thereby confirming it to be a triter-
penoidal saponin\(^3-5\).

I.R. SPECTRUM OF THE SAPONIN (Fig. I):

The significant peaks obtained in the I.R.
spectrum and the structural assignment made with the help
of available literature\(^6,7\) are given in the Table I.
TABLE - I

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Peaks cm⁻¹</th>
<th>Structural unit inferred</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3578</td>
<td>-OH group</td>
</tr>
<tr>
<td>2</td>
<td>2929, 2854</td>
<td>CH₃-CH₂ stretching vibration</td>
</tr>
<tr>
<td>3</td>
<td>2960</td>
<td>-C-H asymmetrical stretching of CH₃</td>
</tr>
<tr>
<td>4</td>
<td>2864</td>
<td>C-H symmetrical stretching of CH₃</td>
</tr>
<tr>
<td>5</td>
<td>1722</td>
<td>C=O stretching</td>
</tr>
<tr>
<td>6</td>
<td>1644</td>
<td>C=C stretching</td>
</tr>
<tr>
<td>7</td>
<td>1452</td>
<td>C-H bending</td>
</tr>
<tr>
<td>8</td>
<td>1384, 1378</td>
<td>CH₃ gem. dimethyl</td>
</tr>
<tr>
<td>9</td>
<td>1348, 1254</td>
<td>-OH bending</td>
</tr>
<tr>
<td>10</td>
<td>1388, 1374</td>
<td>Triterpenic type nucleus</td>
</tr>
<tr>
<td>11</td>
<td>1248</td>
<td>Vinylidene type double bond</td>
</tr>
<tr>
<td>12</td>
<td>818</td>
<td>C-H bending</td>
</tr>
</tbody>
</table>

PRESENCE OF HYDROXYL GROUP(S) :

Peaks in the I.R. spectrum at KBr max 3578, indicated the presence of -OH group(s) in it, which when estimated by acetylation with acetic anhydride and pyridine gave an acetyl derivative, molecular formula C₅₄H₈₀O₁₈.m.p. 212°13°C, m. = 1016, acetyl group % = 27.91, thereby indicating the presence of six hydroxyl groups.
Presence of COOH group(s):

A peak at $\text{KBr}_{\text{max}} 1722 \text{ cm}^{-1}$ in the I.R. spectrum of the saponin showed the presence of $-\text{COOH}$ group(s) in it, which was further confirmed since it formed an insoluble salt with KOH.

One saponin was found to be a mono carboxylic compound since on acetylation with diacetyl methane it formed a methyl ester, molecular formula $\text{C}_{43}\text{H}_{70}\text{O}_{12}$, m.p. 223-24°C, $M^+ = 778$, thereby confirming the presence of only one $-\text{COOH}$ group in it.

Presence of methyl group(s):

In the I.R. spectrum, of the saponin, other peak at $\text{KBr}_{\text{max}} 2960 \text{ cm}^{-1}$ showed the presence of $-\text{CH}_3$ group(s) which when estimated by Zeisel’s method (1941) indicated the presence of seven methyl groups in it.

Presence of double bond(s):

A peak in the I.R. spectrum at $\text{KBr}_{\text{max}} 1248 \text{ cm}^{-1}$ indicated the presence of olefinic double bond(s)$^{6,7}$. It did not decolourise bromine water but gave a yellow colour with tetranitromethane$^{24}$, thereby confirming the presence of double bonds inside the ring.
On catalytic hydrogenation over Raney nickel the saponin yielded a dihydroderivative molecular formula $C_{42}H_{70}O_{12}$, m.p. 266$^\circ$C, $M^+$ = 766, further confirming the presence of only one double bond in the compound.

The position of various methyl groups and the structure of saponin was established by the hydrolysis of the saponin and elucidating the structure of the sapogenin and a study of various sugar moieties separately.

The structural studies of the saponin was made by its hydrolysis and separately studying the sapogenin and sugar hydrolysate.

**HYDROLYSIS OF THE SAPONIN**

The saponin on hydrolysis with 2N sulphuric acid gave sapogenin and sugar moieties. They were separated by filtration and the structure of the saponin was established by elucidating separately the structure of the sapogenin and the sugar moieties.

**STRUCTURAL STUDY OF THE SAPOGENIN**

The sapogenin analysed for molecular formula $C_{30}H_{48}O_{3}$, $M^+$ = 456, $\delta = 76.5^\circ$ (CHCl$_3$), m.p. 306$^\circ$C, and
responded to the colour reactions characteristic of triterpenes\textsuperscript{8-14}.

Its pentacyclic nature was supported because it gave violet colour with 2,6-ditertiary butyl para cresol in ethanol\textsuperscript{15}.

\textbf{15} \\
\textit{U.V. SPECTRUM OF THE SAPOGENIN:}

The sapogenin showed high terminal U.V. absorption and did not absorb above 208 nm in methanol (Fig. II).

\textbf{I.R. SPECTRUM OF THE SAPOGENIN (Fig. III):}

The different peaks obtained in I.R. spectrum of the sapogenin and the structural assignment made with the help of available literature\textsuperscript{16,17} are recorded in the table II.

\textbf{TABLE - II}

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Peaks cm\textsuperscript{-1}</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3578</td>
<td>-OH grouping</td>
</tr>
<tr>
<td>2</td>
<td>1640</td>
<td>-C=C stretching vibration</td>
</tr>
<tr>
<td>3</td>
<td>2924, 2864</td>
<td>(\text{CH}_3)-(\text{CH}_2) stretching vibration</td>
</tr>
<tr>
<td>4</td>
<td>1711</td>
<td>-COOH group</td>
</tr>
<tr>
<td>5</td>
<td>1390, 1380, 1369</td>
<td>Triterpenic acid</td>
</tr>
<tr>
<td>6</td>
<td>1246</td>
<td>Vinylidene type double bond</td>
</tr>
<tr>
<td>7</td>
<td>818</td>
<td>-CH=CH\textsubscript{2} stretching vibration</td>
</tr>
</tbody>
</table>
FIG. NO. XI
U-V SPECTRUM OF THE "SAPOGENIN"

(% TRANSMTTANCE)

WAVE LENGTH (MILLI MICRON)
IR SPECTRUM OF THE SAPOGENIN

FIG. (III)
**Presence of \(-\text{COOH}\) group:**

The sapogenin formed on insoluble salt with potassium hydroxide and a methyl ester, m.p. 197°, \(C_{31}H_{50}O_{3}\), \(M^+ = 470\) with diacethylene, indicating the presence of only one \(-\text{COOH}\) group in it. It was further supported by a peak in I.R. spectrum at 1711 cm\(^{-1}\) (ester carbonyl).

**Position of the \(-\text{COOH}\) group:**

The saponification of the methyl ester of the sapogenin with 10% methanolic KOH, resulted in its partial hydrolysis, but on saponification with diethylene glycolic KOH it regenerated the sapogenin m.p. 306°, indicating that \(-\text{COOH}\) group in the sapogenin was hindered and so must be attached at \(O_{17}\). The presence of \(-\text{COOH}\) group at \(O_{17}\) was further confirmed by a signal in \(^1\text{H}NMR\) at \(\delta = 3.62\) of its methyl ester\(^{22,23}\).

**Presence of Double Bond(s):**

The sapogenin decolourised bromine water, indicating the presence of unsaturation in it. With THM (Fusiicka reaction)\(^{24}\) it gave positive colour. Also a peak in the I.R. spectrum at 1246 cm\(^{-1}\) confirmed the presence of vinylidene type double bond\(^{26,27}\) in it.
In catalytic hydrogenation, in presence of Raney nickel, the sapogenin yielded a dihydro-derivative, molecular formula $C_{31}H_{52}O_2$, $M^+ = 456$, which clearly showed the presence of only one olefinic double bond in the sapogenin.

The sapogenin showed high terminal U.V. absorption typical of presence of double bond at $C_{12}-C_{13}$ in the triterpene of $B$-amyrin series$^{27}$. This fact was further supported by $^1$HNMR spectrum of the monoacetyl derivative of the sapogenin $= 5.31$ (vinyllic proton) (Fig. IV).

**Presence of $\text{OH}$ group(s):**

In the I.R. spectrum of the sapogenin, the peak at 3578 cm$^{-1}$ indicated the presence of hydroxyl group(s) in it. On acetylation of the sapogenin with acetic anhydride and pyridine it formed an acetyl derivative, crystallising from ethanol $C_{32}H_{50}O_4$, m.p. 266$^0$, $\delta = +65^0$ (CHCl$_3$). The percentage of the acetyl group 10.9 in the acetylated product was determined by the method of Wiesenberger$^{10}$ as described by Belcher and Godbert$^{15}$.

The sapogenin itself did not show any peak in the I.R. spectrum for acetyl group, so it must have formed by
acetylation of hydroxyl group. The formation of monoacetyl compound indicated the presence of only one hydroxyl group in the sapogenin.

POSITION OF OH GROUP:

On oxidation with chromic acid the methyl ester of the sapogenin gave an oxidation product which responded to positive Zimmermann rest indicating the presence of a keto group in position C-3 thereby indicating the sapogenin to have a secondary hydroxyl at C3 which is in conformity with the fact that most of the naturally occurring terpenic acids contain -OH group at C3.

The above facts concluded that the sapogenin is pentacyclic triterpene of B-amyrin series with one -COOH group.

The spectral data and a survey of the literature of the sapogenin confirmed the genin and its methyl ester as Olean-12-one-28 oic acid and methyl oleanolate, respectively. The identity of the compound was further confirmed by superimposable I.R. spectra. Thus the sapogenin and its methyl ester have been assigned the structure as (I) and (II) respectively.
$^1$H NMR SPECTRUM OF THE ACETYLATED SAPOGENIN

The important signals (Fig. IV) obtained in the $^1$H NMR spectrum of the monoacetyl derivative of the sapogenin and the structural assignment made with the help of the available literature$^{28,29}$ are given in the Table III and further confirmed structure (I) assigned to it.
TABLE - III

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Value</th>
<th>Pattern</th>
<th>J value Hz</th>
<th>No. of protons</th>
<th>Structural assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.95</td>
<td>s</td>
<td>-</td>
<td>6</td>
<td>2 x CH₃</td>
</tr>
<tr>
<td>2</td>
<td>0.97</td>
<td>s</td>
<td>-</td>
<td>6</td>
<td>2 x CH₃</td>
</tr>
<tr>
<td>3</td>
<td>0.82</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>CH₃ group</td>
</tr>
<tr>
<td>4</td>
<td>0.89</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>CH₃ group</td>
</tr>
<tr>
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<td>1.10</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>CH₃ group</td>
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<tr>
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<td>2.02</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>CH₃OCOCH₃</td>
</tr>
<tr>
<td>7</td>
<td>1.26-2.00</td>
<td>m</td>
<td>-</td>
<td>20</td>
<td>Polymethelene envelop (CH₂ and OH)</td>
</tr>
<tr>
<td>8</td>
<td>2.03</td>
<td>d,d</td>
<td>4 &amp; 12</td>
<td>2</td>
<td>C₁₁-H</td>
</tr>
<tr>
<td>9</td>
<td>4.54</td>
<td>d,d</td>
<td>5 &amp; 9</td>
<td>1</td>
<td>Methene proton</td>
</tr>
<tr>
<td>10</td>
<td>5.31</td>
<td>d,d</td>
<td>4 &amp; 8</td>
<td>1</td>
<td>C₁₂-H vinylcic proton</td>
</tr>
<tr>
<td>11</td>
<td>2.50</td>
<td>triplet</td>
<td>4</td>
<td>1</td>
<td>C₁₈-H</td>
</tr>
</tbody>
</table>

MASS SPECTRUM of the SAPOGENIN:

The various fragments in the mass spectrum of the sapogenin were at M⁺ = 456 and m/e = 441, 411, 410, 395, 300, 248, 207, 203, 189 (100%), 175 and 133 and its methyl ester M⁺ = 470 and m/e = 455, 411, 410, 262, 249, 207, 203, 189 and 133 and further supported the identity of sapogenin as: oleic-12-ene-28 oic acid (Scheme I)
$^1$H NMR SPECTRUM OF THE ACETYLATED SAPONENIN
60MHz, SOLVENT CDCl$_3$ + CCl$_4$. 
STUDY OF THE SAPONIN HYDROLYSATE:

The aqueous hydrolysate obtained after the separation of the sapogenin was neutralised with barium carbonate and barium sulphate filtered off. The filtrate was concentrated to a golden yellow syrup, which reduced Fehling's solution, Tollen's reagent and produced colour with aniline hydrogen phthalate, thereby confirming its carbohydrate nature. On paper chromatography over Whatman No.1 filter paper it revealed the presence of D-glucose and L-rhamnose (Co-PC and Co-TLC with authentic sample).

QUANTITATIVE ESTIMATION OF SUGARS:

The saponin (100 mg) on hydrolysis with 2N sulphuric acid for four hours gaveoleanolic acid (60 mg) and carbohydrate moieties (40 mg) by difference). The sugars present in the saponin were estimated by the procedure of Mishra and Mohan Rao\(^{31}\) revealed that the two sugars were present in equimolecular ratio (1:1).

It was therefore concluded from the percentage of the sapogenin and the amount of carbohydrate moieties that one molecule of the saponin was made up of one molecule of oleanolic acid and one molecule each of L-rhamnose and D-glucose.
POSITION OF ATTACHMENT OF SUGAR(S) TO THE SAPOGENIN:

Looking to the structure of oleanolic acid it was concluded that either all the sugars were attached to the -OH group in a sequence or to the -COOH group in a sequence or some may be attached to the -OH group and other to the -COOH group. Finally the position of attachment was established at C₃ as both the saponin and the sapogenin gave the test for free -COOH group. This is supported by the fact that the saponin was not hydrolysed with 5N NH₄OH (a specific reagent for the sugar esters hydrolysis). Thus concluding that all the sugar units were linked as a bioside unit to the -OH group at C₃.

Also that, the saponin with 2N sulphuric acid gave a sapogenin the methyl ester of which gave a positive Zimmermann's test showing the presence of one secondary hydroxyl group at position C₃, while the methyl ester of the saponin gave a negative test for C₂ group thus concluding that the sugar must be attached at C₃. Thus a tentative structure to the saponin may be assigned as III.

\[ \text{III.} \]

\[ R = \text{Sugar moieties.} \]

\[ \text{(glucose, rhamnose)} \]
SEQUENCE OF THE SUGAR(S) IN THE SAPONIN:

The sequence of sugar residue in the saponin was determined by partial hydrolysis of the saponin.

Partial hydrolysis\(^3\) of the saponin with 1% sulphuric acid for 3 days at room temperature yielded a mixture of two prosapogenins designated as H\(_1\) and H\(_2\) which were separated by column chromatography, using chloroform: methanol (1:2) as an eluant. The hydrolysate on paper chromatography showed the presence of L-rhamnose and D-glucose (Co-PC and CO-TLC).

STUDY OF PROSAPOGENIN H\(_1\):

The prosapogenin H\(_1\) was analysed for molecular formula \(C_{36}H_{56}O_8\), m.p. 256\(^\circ\)C and on hydrolysis with 7% sulphuric acid gave, oleanolic acid and D-glucose. The sapogenin content on quantitative estimation was found to be 72.00% thereby confirming that prosapogenin H\(_1\) was formed by one molecule of oleanolic acid and one molecule of D-glucose.

PERMETHYLATION AND HYDROLYSIS OF H\(_1\):

H\(_1\) on permethylation\(^4\) followed by hydrolysis showed the presence of 2:3:4:6 tetra-O-methyl glucose in the
hydrolysate (identified by paper chromatography with authentic sample). The formation of 2:3:4:6, tetra-O-methyl-D-glucose indicated that the C₁ of the D-glucose was involved in the formation of glycoside linkage and also suggested that D-glucose was present in the pyranose form.

The prosapogenin P₁ on the basis of above facts was assigned the structure IV as below:
STUDY OF PROSAPONIN $\text{R}_2$:

The prosapogenin $\text{R}_2$ analysed for molecular formula $C_{42}H_{68}O_{12}$, $M^+ = 764$, m.p. $276^\circ C$ and on hydrolysis with 7% sulphuric acid yielded oleanolic acid, D-glucose and L-rhamnose. The sapogenin content was found to be 64.00%, thereby confirming that prosapogenin $\text{R}_2$ was formed by the one molecule of oleanolic acid, one molecule of L-rhamnose and one molecule of D-glucose. $\text{R}_2$ on permethylation.

PERMETHYLA TION AND HYDROLYSIS OF $\text{R}_2$:

$\text{R}_2$ on permethylation followed by hydrolysis yielded two methylated sugars (1) 2:3:6 tri-O-methyl-D-glucose and (2) 2:3:4 tri-O-methyl-L-rhamnose. This indicated that $C_4$ of D-glucose was involved in the formation of the glycoside linkage.

ENZYMATIC HYDROLYSIS OF THE SAPONIN:

The saponin on enzymatic hydrolysis with emulsin liberated the sapogenin (identified by m.m.p., Co-RE, Co-TLC) and sugar indicating that the linkage between the sapogenin and sugar was B-linkage.$^{35}$

Enzyme diastase was added to the hydrolysed products, when it was found that L-rhamnose and D-glucose were also
liberated. Thereby confirming the presence of L-linkage between L-rhamnose and D-glucose and B-linkage between the sapogenin and D-glucose.

The saponin on the basis of the above facts could be assigned the structure as: oleanolic acid-3-O- -L-rhamnopyranosyl (1--4)-O-B-D-glucopyranoside and is represented as below:

![Chemical Structure Image]

The above (V) structure was finally confirmed by its $^1$HNMR and mass spectral studies.
\textbf{\textit{\textsuperscript{1}H NMR} Spectrum of the Hexa Acetyl Derivative of Saponin}

\textit{\textsuperscript{1}H NMR} spectrum of the hexa acetyl derivative of saponin was found to be incomplete conformity with the above structure (IV). The significant peaks (Fig. V) obtained in \textit{\textsuperscript{1}H NMR} spectrum of the saponin and structure units inferred with the help of available literature are given below in the Table IV.

\textbf{Table IV}

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Value</th>
<th>Pattern</th>
<th>J value (Hz)</th>
<th>No. of protons</th>
<th>Structural assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.95</td>
<td>s</td>
<td>-</td>
<td>6</td>
<td>2 x CH\textsubscript{3} tertiary methyl</td>
</tr>
<tr>
<td>2</td>
<td>0.97</td>
<td>s</td>
<td>-</td>
<td>6</td>
<td>2 x CH\textsubscript{3} tertiary methyl</td>
</tr>
<tr>
<td>3</td>
<td>0.82</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>CH\textsubscript{3} group</td>
</tr>
<tr>
<td>4</td>
<td>0.89</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>CH\textsubscript{3} group</td>
</tr>
<tr>
<td>5</td>
<td>1.10</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>CH\textsubscript{3} group</td>
</tr>
<tr>
<td>6</td>
<td>1.26-2.00 m</td>
<td>-</td>
<td>20</td>
<td>polymethylenes envelop(CH\textsubscript{2} &amp; CH)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2.03</td>
<td>d,d</td>
<td>4 &amp; 12</td>
<td>2</td>
<td>C\textsubscript{11}-H</td>
</tr>
<tr>
<td>8</td>
<td>4.54</td>
<td>d,d</td>
<td>5 &amp; 9</td>
<td>1</td>
<td>Methenic proton at C\textsubscript{3}</td>
</tr>
<tr>
<td>9</td>
<td>5.31</td>
<td>d,d</td>
<td>4 &amp; 8</td>
<td>1</td>
<td>C\textsubscript{12}-H vinylic proton</td>
</tr>
<tr>
<td>10</td>
<td>2.50</td>
<td>triplet</td>
<td>4</td>
<td>1</td>
<td>C\textsubscript{18}-H</td>
</tr>
<tr>
<td>11</td>
<td>4.40</td>
<td>d</td>
<td>7.6</td>
<td>1</td>
<td>1' anomeric proton</td>
</tr>
</tbody>
</table>

\textit{Table IV: Contd..}
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Value</th>
<th>Pattern</th>
<th>J value (Hz)</th>
<th>No. of protons</th>
<th>Structural assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>5.47</td>
<td>d</td>
<td>2</td>
<td>1</td>
<td>1° anomic proton</td>
</tr>
<tr>
<td>13</td>
<td>0.78</td>
<td>d</td>
<td>6.0</td>
<td>3</td>
<td>6°-CH₃ of rhamnose</td>
</tr>
<tr>
<td>14</td>
<td>4.66-4.82</td>
<td>m</td>
<td>-</td>
<td>4</td>
<td>Protons of rhamnoseyl unit</td>
</tr>
<tr>
<td>15</td>
<td>5.48</td>
<td>m</td>
<td>-</td>
<td>6</td>
<td>Protons of glucose unit</td>
</tr>
<tr>
<td>16</td>
<td>2.04</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>2°-OAc</td>
</tr>
<tr>
<td>17</td>
<td>2.07</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>3°-OAc</td>
</tr>
<tr>
<td>18</td>
<td>3.94</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>6°-OAc</td>
</tr>
<tr>
<td>19</td>
<td>2.18</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>2°-OAc</td>
</tr>
<tr>
<td>20</td>
<td>2.28</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>3°-OAc</td>
</tr>
<tr>
<td>21</td>
<td>2.10</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>4°-OAc</td>
</tr>
</tbody>
</table>

**Mass spectrum**²² of the saponin:

The important fragmentation pattern obtained in the electronic impact mass spectrum of the saponin is given, which further supported the structure (IV) assigned to it. M⁺ = 764 and other signals at m/e = 617, 455, 248, 233, 207, 203, 190, 189, 175, 133.

The different species obtained during fragmentation are shown in Scheme (II) and were found to be in complete accord with the structure assigned to it.
$^1$H NMR SPECTRUM OF THE ACETYLATED SAPONIN FIG.V

60 MHz, SOLVENT CDCl$_3$ + CF$_3$COOH.
Scheme II
EXPERIMENTAL

The stems (3 kg) of Thymus serpyllum Linn., natural order Labiatae, obtained from Himalayan range Drug Field, Simla and authenticated by the Botany Department of this University were air dried powdered and defatted. The defatted stems were extracted with rectified spirit (3.75 lit.) for a week, and the extract (3.0 lit.) was filtered while hot and concentrated to a brown mass (0.650 gm).

The brown mass was then treated with distilled water and the water insoluble part treated with ethanol and the ethanol soluble part concentrated under reduced pressure and excess of solvent ether added when the saponins precipitated out which was separated by decantation. It was redissolved in ethanol and a reprecipitated with ether and the process was repeated several times till precipitation. Finally the precipitate was dissolved in ethanol and the crude saponin was obtained by distilling the solvent under reduced pressure.

The saponin responded to characteristic colour with Antimony trichloride, thionyl chloride and phosphomolybdic acid. The precipitate was again dissolved in ethanol and subjected to thin layer chromatography which was found to be homogeneous.
THIN LAYER CHROMATOGRAPHY:

TLC was done in the same way as described on page of the thesis (Rf. 0.74).

STUDY OF THE SAPONIN:

The saponin crystallised in pale yellow needles, molecular formula \( C_{42}H_{68}O_{12} \), \( M^+ = 764 \), and m.p. 221-22°C. It was insoluble in solvent ether but soluble in pyridine forming pale yellow viscous mass.

ELEMENTAL ANALYSIS OF THE SAPONIN:

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 65.97%</td>
<td>C = 65.96%</td>
</tr>
<tr>
<td>H = 8.8%</td>
<td>H = 8.9%</td>
</tr>
</tbody>
</table>

Molecular formula = \( C_{42}H_{68}O_{12} \)

Molecular weight = 764.

It gave positive honey comb test and haemolytic test which was carried out as follows:

FOAM TEST:

It was done in the same way as described on page of the thesis.
HAEMOLYTIC TEST:

The test was performed in the same way as described on page of the thesis.

HYDROLYSIS OF THE SAPONIN:

The hydrolysis was carried out in the same way as described on page of the thesis. A crystalline sapogenin m.p. 306°C was obtained.

STUDY OF THE SAPOGENIN:

The sapogenin analysed for molecular formula $C_{30}H_{48}O_3$. $M^+ = 456$ and m.p. 300°C.

ELEMENTAL ANALYSIS OF THE SAPOGENIN:

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 78.90%</td>
<td>C = 78.94%</td>
</tr>
<tr>
<td>H = 10.80%</td>
<td>H = 10.52%</td>
</tr>
</tbody>
</table>

Molecular formula = $C_{30}H_{48}O_3$

Molecular weight = 456.

On reaction with acetic anhydride and concentrated sulphuric acid it gave pink colour and responded to the following reactions.
SALOZABSKI REACTION:

It was performed as given on page

LIEBERMANN BURCHARD REACTION:

It was performed as given on page

TSCHUGASEW REACTION:

It was performed as given on page

NOLLER'S REACTION:

The compound when added to the Noller's reagent (0.01% SnCl₂ added in thioryl chloride) a pink colour changing to violet was observed.

METHYL ESTER OF THE SAPOGENIN:

It was prepared in the same way as described on page ... of the thesis.

The residue obtained was eluted with a mixture of CHCl₃: ethanol (1:1) when it yielded methyl ester of the sapogenin which was recrystallised from chloroform m.p. 197-98°C.

ELEMENITAL ANALYSIS OF THE METHYL ESTER OF THE SAPOGENIN:

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 79.10%</td>
<td>C = 79.14%</td>
</tr>
<tr>
<td>H = 10.2%</td>
<td>H = 10.6%</td>
</tr>
</tbody>
</table>

Molecular formula = C₃₁H₅₀O₃

Molecular weight = 470
CHROMIC ACID OXIDATION OF METHYL ESTER OF THE SAPOGENIN:

It was done in the same way as described on page of the thesis.

ZIMMERMANN COLOUR TEST:

Performed in the same way as described on page of the thesis.

SAPONIFICATION OF THE METHYL ESTER:

It was done in the same way as described on page of the thesis.

A gelatinous mass was obtained which crystallised from chloroform: ethanol (1:1) m.p. 302°C.

STUDY OF THE SUGARS IN THE SAPONIN HYDROLYSATE:

The aqueous hydrolysate obtained by the separation of sapogenin after hydrolysis of the saponin was neutralised with barium carbonate and barium sulphate filtered off. Concentration of the filtrate gave a golden yellow mass which was subjected to paper chromatography using different solvent systems and aniline hydrogen phthalate as spraying reagent when the presence of D-glucose and L-rhamnose was confirmed.
TABLE

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Solvent system</th>
<th>RF values</th>
<th>Sugar 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:</td>
<td>0.31</td>
<td>D-glucose</td>
</tr>
<tr>
<td></td>
<td>Water (4:1:5)</td>
<td>0.38</td>
<td>lr-rhamnose</td>
</tr>
<tr>
<td>2</td>
<td>n-butanol and 1%</td>
<td>0.09</td>
<td>D-glucose</td>
</tr>
<tr>
<td></td>
<td>NH₄OH</td>
<td>0.22</td>
<td>L-rhamnose</td>
</tr>
<tr>
<td>3</td>
<td>n-butanol:ethanol:</td>
<td>0.12</td>
<td>D-glucose</td>
</tr>
<tr>
<td></td>
<td>water (45:5:49)</td>
<td>0.28</td>
<td>L-rhamnose</td>
</tr>
<tr>
<td></td>
<td>and 1% NH₄OH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PARTIAL HYDROLYSIS OF THE SAPONIN WITH KILIANI MIXTURE:

The saponin (100 mg) was suspended in kiliaini mixture (75 ml), hydrochloric acid: acetic acid : water 15:35:50) with conical flask (150 ml). The reaction mixture was kept at room temperature for the whole night and then the contents was extracted with n-butanol and subjected to TLC examination when two spots were noticed, thereby showing the presence of two compounds. The butanol extract was subjected to column chromatography over column of silica gel and the column eluted with ethanol yielded two compounds which was assigned as $R_1$ and $R_2$.

STUDY OF THE PROSAPOGENIN $R_1$:

Hydrolysis of Prosapogenin $R_1$:

It was performed in the same way as described on page 171 of the thesis.
The chloroform extract when concentrated and crystallised from mixture of chloroform and ethanol (1:1) gave colourless crystals m.p. 295°C and identified as Oleanolic acid.

Paper chromatography of the aqueous hydrolysate showed the presence of D-glucose.

PERMETHYLATION AND HYDROLYSIS OF H51:

It was done in the similar way as described on page...

...The sugar was identified as D-glucose. The methylated sugar was identified as 2,3,4,6 tetra-methyl glucose.

STUDY OF PROSAPONIGIN H52:

PERMETHYLATION AND HYDROLYSIS OF H52:

The same procedure was followed as described on page when it yielded 2,3,4,6 tetra-O-methyl glucose (confirmed by Co-PC and Co-TLC).

ENZYMATIC HYDROLYSIS OF THE SAPONIN:

The saponin (5.0 mg) was suspended in a emulsion (28 ml) and kept at 50°C for 30 hours in a round bottom flask with a reflux condenser attached to it and refluxed for 30 hours. The hydrolysate was chromatographed over
Whatman No. 1 filter paper using butanol : acetic acid : water (4:1:5) as solvent system and aniline hydrogen phthalate as spraying reagent when only one spot was seen corresponding to L-rhamnose, thereby indicating that D-glucose was attached to L-rhamnose through linkage and that D-glucose was attached to the sapogenin through B-linkage since it was not be hydrolysable by the enzyme emulein.

PERIODATE OXIDATION OF THE SAPONIN :

The same procedure was followed as described on page.
REFERENCES


