CHAPTER II

ISOLATION AND CHARACTERISATION OF A NOVEL FLAVONE GLYCOSIDE: 5,6-DIHYDROXY, 7,8,3'-TRIMETHOXY FLAVONE-4'-O-β-D-XYLOFURANOSYL (1→4)-O-β-D-GLUCOPYRANOSIDE FROM THE SEEDS OF TRICHOSANTHES ANGUINA LINN.

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**Trichosanthes anguina** Linn\(^1,2\) belongs to family Cucurbitaceae which is commonly known as Chachinda in hindi. It is an annual climber and cultivated throughout the hotter parts of India and China. Its fruits varies from 0.3 - 0.9 m in length.

The Ayurvedic system of medicine describes that the plant **T.anguina** is used as tonic, to cure cough, biliousness and is purgative and anthelmintic. Its seeds are used as antidiarrhoeal. It is also used in the treatment of syphilis\(^3\).

A number of bio-active constituents\(^4-6\) have been reported from this plant.

Since no systematic photochemical investigations have been done on this plant, therefore it was thought worthwhile to investigate it further phytochemically because of its medicinal importance.

This chapter deals with the isolation of a novel flavone diglycoside from the seeds of **Trichosanthes anguina** Linn.

**ISOLATION OF THE FLAVONE GLYCOSIDE TA**

The seeds of **Trichosanthes anguina** Linn. were procured from M/s United Chemicals and Allied
Products, Calcutta and authenticated by Department of Botany of this University.

Air-dried and powdered seeds of *T. anguina* were extracted exhaustively with hot 95% ethanol. The total extract was concentrated under reduced pressure to yield a pale yellow syrupy mass, which was dissolved in distilled water and the water soluble part was concentrated to viscous mass and successively extracted with the solvents of increasing basicity i.e.; petroleum ether, benzene, chloroform, ethyl acetate, acetone and methanol.

The petroleum ether, benzene, chloroform and methanol soluble fractions were insufficient to provide for any substantive investigations. The detailed description of acetone soluble fraction has been described in chapter IV of the thesis.

The study of ethyl acetate soluble fraction has been described in this chapter.

**STUDY OF THE ETHYL ACETATE SOLUBLE PART**

The ethyl acetate soluble fraction was concentrated under reduced pressure to yellow syrupy mass, which gave two spots on TLC examination using EtOAc-AcOH-H₂O (9:3:1) and I₂ vapours as visualising agent, which were separated by column chromatography
over si-gel G and eluted with chloroform-Methanol in various proportions.

**STUDY OF THE ELUATES FROM CHLOROFORM METHANOL (6:2)**

Eluates from CHCl₃-MeOH (6:2) were found to have same Rf value and so, were combined together. On evaporation of the solvent, a yellow coloured amorphous powder was (0.065%) obtained, which was found to be homogenous on TLC examination. It analysed for molecular formula C₂₉H₃₄O₁₇, m.p. 336°C and [M]⁺ 654 (EIMS).

It was soluble in ethanol, and crystallised from solvent ether. It gave positive Molisch test for glycoside and characteristic colour reactions for flavonoids⁷,⁸.

Study of eluates from chloroform-methanol (6:4) has been described in chapter III on page no. 61 of the thesis.

**UV SPECTRUM OF THE FLAVONE GLYCOSIDE TA**

The wave lengths of maximum absorption with various shift reagents were recorded below:

\[ \lambda_{\text{MeOH}}^{\text{max}} = 250, 287, 362 \text{ nm}; \]

\[ \lambda_{\text{NaOMe}}^{\text{max}} = 260, 272, 391 \text{ nm}; \]
\[ \text{AlCl}_3 \text{ max } 245, 260, 380 \text{ nm}; \]

\[ \text{AlCl}_3 + \text{HCl max } 245, 260, 380 \text{ nm}; \]

\[ \text{NaOAc max } 299, 309, 342, 395 \text{ nm}; \]

\[ \text{NaOAc} + \text{H}_3\text{BO}_3 \text{ max } 297, 340, 380 \text{ nm} \]

**IR SPECTRUM OF THE GLYCOSIDE TA**

The significant peaks observed in the IR spectrum (Fig. 1) and the structural units inferred with the help of available literature⁹,¹⁰ are given in the Table-I.

**TABLE – I**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Wave number cm⁻¹</th>
<th>Assignment</th>
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</thead>
<tbody>
<tr>
<td>1.</td>
<td>3345</td>
<td>-OH group(s)</td>
</tr>
<tr>
<td>2.</td>
<td>2872</td>
<td>-OCH₃ group(s)</td>
</tr>
<tr>
<td>3.</td>
<td>1660</td>
<td>&gt;C=O</td>
</tr>
<tr>
<td>4.</td>
<td>1110</td>
<td>C-O-C bending vibration</td>
</tr>
<tr>
<td>5.</td>
<td>2950</td>
<td>-C-H- stretching vibration</td>
</tr>
<tr>
<td>6.</td>
<td>1600, 1585</td>
<td>Aromatic ring system</td>
</tr>
<tr>
<td>7.</td>
<td>825</td>
<td>Two adjacent hydrogen atom</td>
</tr>
</tbody>
</table>
FIG. No.: 1

IR SPECTRUM OF THE FLAVONE GLYCOSIDE TA
PRESENCE OF -OH GROUP(S) IN TA

In the IR spectrum a peak at $\nu_{\text{max}}^{\text{KBr}}$ 3345 cm$^{-1}$ indicated the presence of free hydroxyl group(s) in the molecule. On acetylation ($\text{Ac}_2\text{O}$/pyridine), it formed an acetyl derivative, molecular formula $\text{C}_{45}\text{H}_{50}\text{O}_{25}$, m.p. 198°C, $[\text{M}]^+$ 990 (EI-MS). The acetyl percentage (34.74%) was determined by Wiesenberger method$^{11}$ as described by Belcher and Godbert$^{12}$, which suggested the presence of eight hydroxyl groups in the glycoside.

The appearance of IR signals of the acetyl derivative at $\nu_{\text{max}}^{\text{KBr}}$ 1710 cm$^{-1}$ and the disappearance of the IR signal at $\nu_{\text{max}}^{\text{KBr}}$ 3345 cm$^{-1}$ suggested the acetylation of all hydroxyl groups present in the glycoside.

PRESENCE OF -OCH$_3$ GROUP(S) IN THE GLYCOSIDE TA

A peak at $\nu_{\text{max}}^{\text{KBr}}$ 2872 cm$^{-1}$ in the IR spectrum of the glycoside showed the presence of methoxyl group(s) in it. Methoxyl groups estimation was carried out by Zeisel's method$^{13}$ (14.22%) which indicated the presence of three methoxyl groups in it.

ACID HYDROLYSIS OF THE GLYCOSIDE TA

On acid hydrolysis with 7% alcoholic $\text{H}_2\text{SO}_4$ yielded aglycone (TA-1) and sugar moiety (ies), which were studied separately.
STUDY OF THE AGLYCONE TA-1

The aglycone was crystallised from methanol to gave yellow coloured compound and was found to be homogenous on TLC examination using benzene-Methanol (6:3).

It analysed for molecular formula $C_{18}H_{16}O_8$, m.p. 235°C, $[M]^+\ 360$ (EIMS), and responded to all the characteristic colour reactions of flavonoids$^{14,15}$.

UV SPECTRUM OF THE AGLYCONE TA-1

The UV spectrum of the aglycone exhibited absorption maxima with various shift reagents, are as follows:

\[
\lambda_{\text{MeOH}}^\text{max}\ 249, 293, 344 \text{ nm;}
\]

\[
\lambda_{\text{NaOMe}}^\text{max}\ 260, 290, 390 \text{ nm;}
\]

\[
\lambda_{\text{AlCl}_3}^\text{max}\ 242, 262, 307, 381 \text{ nm;}
\]

\[
\lambda_{\text{AlCl}_3+\text{HCl}}^\text{max}\ 241, 260, 389 \text{ nm;}
\]

\[
\lambda_{\text{NaOAc}}^\text{max}\ 295, 309, 342, 393 \text{ nm;}
\]

\[
\lambda_{\text{NaOAc+H}_3\text{BO}_3}^\text{max}\ 297, 345, 376 \text{ nm;}
\]
IR SPECTRUM OF THE AGLYCONE TA-1

The prominent peaks observed in the IR spectrum (Fig. 2) and the structural units assigned with the help of available literature\textsuperscript{16,17} are recorded in Table-II.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Wave number cm(^{-1})</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3421</td>
<td>-OH group(s)</td>
</tr>
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<td>2.</td>
<td>2844</td>
<td>-OCH(_3) group(s)</td>
</tr>
<tr>
<td>3.</td>
<td>1661</td>
<td>C=O</td>
</tr>
<tr>
<td>4.</td>
<td>1120</td>
<td>-C-O-C bending vibration</td>
</tr>
<tr>
<td>5.</td>
<td>2930</td>
<td>-C-H-stretching vibration</td>
</tr>
<tr>
<td>6.</td>
<td>1650, 1586</td>
<td>Aromatic ring system</td>
</tr>
<tr>
<td>7.</td>
<td>822</td>
<td>Two adjacent hydrogen atom</td>
</tr>
</tbody>
</table>

PRESENCE OF HYDROXYL GROUP(S) IN THE AGLYCONE TA-1

The IR absorption at \( \nu_{KBr}^{\text{max}} \) 3421 cm\(^{-1}\) indicated the presence of free hydroxyl group(s) in the molecule. On acetylation with (Ac\(_2\)O/pyridine), it yielded an acetyl derivative; molecular formula C\(_{24}\)H\(_{22}\)O\(_{11}\), m.p. 199-200°C and \([M]^+\) 486 (EIMS). The percentage of the acetyl group (26.54%) was determined by Wiesenberger method\textsuperscript{11} as described by Belcher.
IR SPECTRUM OF THE AGLYCONE TA-1
and Godbert\textsuperscript{12}, indicating the presence of three hydroxyl groups in the aglycone.

**PRESENCE OF METHOXYL GROUP(S) IN THE AGLYCONE TA-1**

The IR absorption at $\nu_{\text{max}}^{\text{KBr}}$ 2844 cm\(^{-1}\) indicating the presence of $-\text{OCH}_3$ group(s) in the aglycone. Estimation of methoxyl group (25.83\%) by Zeisel's method, suggested the presence of three methoxyl groups in it.

**ALKALINE DEGRADATION OF THE AGLYCONE TA-1**

Alkaline degradation of the aglycone with 50\% ethanolic KOH, yielded two compounds which were identified as, 2,5,6-trihydroxy, 3,4-dimethoxy acetophenone (IA) (by Co-PC and Co-TLC) molecular formula $\text{C}_{10}\text{H}_{12}\text{O}_6$, [M]$^+$ 228, and p-hydroxy, 3-methoxy benzoic acid (IB) (by Co-PC and Co-TLC) molecular formula $\text{C}_8\text{H}_8\text{O}_4$, [M]$^+$ 168.
POSITION OF HYDROXYL GROUPS AT C-5 AND C-6

Fusion of aglycone with KOH, yielded the 2,5,6-trihydroxy-3,4-dimethoxy acetophenone, which indicated the presence of -OH groups at C-5 and C-6 in ring A.

The UV spectrum of the (TA-1) showed a bathochromic shift of 37 nm in band I with AlCl₃ (relative to MeOH) confirmed the presence the -OH group at C-5 position¹⁸.

Further, a bathochromic shift of 45 nm in band I with AlCl₃·HCl, relative to MeOH confirmed the presence of -OH group at C-6 position¹⁹.

POSITION OF HYDROXYL GROUP AT C-4'

The position of -OH group was fixed at C-4' position, because the pink coloured solution obtained in Shinoda reaction²⁰, became blue on addition of NaHCO₃, which indicated the presence of -OH group at C-4' in the ring B.

A bathochromic shift at 32 nm with NaOAc/H₃BO₃ in band I (relative to MeOH) further confirmed the presence of -OH group at C-4'²¹.

On the basis of above facts the structure of the aglycone assigned as; (I) 5,6,4'-trihydroxy, 7,8,3'-trimethoxy flavone.
The structure of the aglycone (TA-1) was further confirmed by the $^1$H NMR and mass spectral studies.

$^1$H NMR SPECTRUM OF THE ACETYL DERIVATIVE OF THE AGLYCONET TA-1

The prominent signals observed in the $^1$H NMR spectrum (Fig. 3) of the aglycone and structural units inferred with the help of available literature$^{22,23}$ are recorded in Table-III.

**TABLE - III**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>$^3$ value (Hz)</th>
<th>Pattern</th>
<th>$^3$ value (Hz)</th>
<th>Num.of protons</th>
<th>Assignment</th>
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<td>3.90</td>
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<td>7-OCH$_3$</td>
</tr>
<tr>
<td>2.</td>
<td>4.05</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>8-OCH$_3$</td>
</tr>
<tr>
<td>3.</td>
<td>3.65</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>3'-OCH$_3$</td>
</tr>
<tr>
<td>4.</td>
<td>7.53</td>
<td>dd</td>
<td>8.6, 2.5</td>
<td>2</td>
<td>H-3.5'</td>
</tr>
<tr>
<td>5.</td>
<td>6.56</td>
<td>dd</td>
<td>8.6, 2.5</td>
<td>d</td>
<td>H-2', 6'</td>
</tr>
<tr>
<td>6.</td>
<td>2.47</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>5-OAc</td>
</tr>
<tr>
<td>7.</td>
<td>2.32</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>6-OAc</td>
</tr>
</tbody>
</table>
$^1$H NMR SPECTRUM OF THE ACETYL DERIVATIVE OF THE AGLYCONE TA-1
MASS SPECTRUM$^{24}$ OF THE AGLYCONES TA-1

The EIMS of the aglycone gave following fragments ion:

\[ [M]^+ \ 360 \ m/e \ 332, \ 331, \ 212, \ 213, \ 184, \ 164, \ 163. \]

The various species obtained during fragmentation are described in the Scheme-I.

STUDY OF THE SUGAR MOIETY (IES)

The hydrolysate of the glycoside was neutralised with BaCO$_3$ and BaSO$_4$ filtered off. The filtrate was concentrated to a brownish gummy mass, which was found to contain xylose (Rf = 0.29) and glucose (Rf = 0.17) (confirmed by Co-PC and Co-TLC).

QUANTITATIVE ESTIMATION OF THE SUGAR

Quantitative estimation of the sugar was carried out by the procedure of Mishra and Rao$^{25}$, which showed that both the sugars were present in equimolar ratio (1:1).

PERIODATE OXIDATION OF THE GLYCOSIDE TA-1

Oxidation of the glycoside with sodium meta periodate$^{26}$ consumed 3.01 moles of periodate and liberated 1.15 moles of formic acid thereby indicating the presence of one molecule of D-glucose and one molecule of D-xylose attached to the molecule of
Scheme I
aglycone and also showed that D-glucose in pyranose form and D-xylose in furanose form in the glycoside.

POSITION OF ATTACHMENT OF THE SUGARS TO THE AGLYCONЕ

There were the two possibilities of attachment of both the sugars to the aglycone:

1. Either, both the sugars were attached on different carbon atoms of the aglycone.

2. or, both glucose and xylose were attached on the same carbon atom as disaccharide. Out of these possibilities the linkage of the sugars as disaccharide was confirmed, by the fact that during the periodate oxidation; the glycoside consumed 3.01 moles of periodate with the liberation of 1.15 moles of formic acid. These values were in accordance with the disaccharide nature of the sugar and not monosaccharide. Hence the sugar must be attached with the same carbon atom of the aglycone.

The position of sugars were fixed at C-4' in the glycoside, by comparing the UV absorption data of the glycoside and that of aglycone, as follows;
A bathochromic shift of 32 nm in band I was obtained on addition of NaOAc/H₃BO₃ in the UV spectrum of the aglycone, confirmed the presence of a free hydroxyl group at C-4' position. In the UV spectrum of the glycoside, above bathochromic shift was not observed, thereby revealing that C₄'-OH in the glycoside was not free whereas in the aglycone it was free and confirming that it was involved in the glycosylation.

Above fact further supported by the permethylation of the glycoside followed by hydrolysis, when it yielded 4'-hydroxy, 5,6,7,8,3'-pentamethoxy flavone confirmed (by Co-PC and Co-TLC) and 2,3,5-tri-O-methyl xylose and 2,3,4,6-tetra-O-methyl glucose (by Co-PC and Co-TLC).

All the above facts, keeping together, a tentative structure to the glycoside (TA) was assigned as (II); which was further supported by its $^1$H NMR and mass spectral analysis.
SEQUENCE OF THE SUGAR RESIDUE

On graded hydrolysis of the glycoside with Kiliani Mixture\textsuperscript{28} liberated D-xylose first followed by D-glucose suggesting that D-xylose was the terminal sugar and D-glucose was fixed to the aglycone.

It was further supported by the isolation and study of two proaglycones designated as: TAP-1 and TAP-2 which were obtained by the partial hydrolysis of the glycoside with Kiliani Mixture and separated by column chromatography and examined separately.

STUDY OF THE PROAGLYCON TAP-1

The proaglycone (TAP-1) analysed for the molecular formula \( \text{C}_{24}\text{H}_{26}\text{O}_{13} \), m.p. 230°C, \([M]^+\) 522. On hydrolysis with 7\% \( \text{H}_2\text{SO}_4 \) it yielded the aglycone and D-glucose (mmp and Co-TLC).

The proaglycone (TAP-1) was hydrolysed with enzyme almond emulsin and afforded D-glucose and aglycone thereby confirming the presence of \( \beta \)-linkage between the aglycone and D-glucose.

PERMETHYLATION AND HYDROLYSIS OF THE PROAGLYCON TAP-1

The proaglycone TAP-1 on permethylation by Kuhn's procedure\textsuperscript{27} followed by acid hydrolysis yielded aglycone and 2,3,4,6-tetra-O-methyl glucose (Co-PC)
which indicated that C-1 of D-glucose was involved in the formation of glycosidic linkage and also suggested that the D-glucose was present in the pyranose form.

Thus the proaglycone (TAP-1) was assigned the structure (III) as; 5,6-dihydroxy, 7,8,3'-trimethoxy flavone-4'-O-β-D-glucose.

![Chemical Structure](image)

(III)

**STUDY OF THE PROAGLYCONE TAP-2**

The proaglycone TAP-2 analysed for \( C_{29}H_{34}O_{17} \), m.p. 335°C [M] + 654. On acid hydrolysis with 7% alcoholic sulphuric acid, it yielded aglycone, D-glucose and D-xylose (Co-PC and Co-TLC).
PERMETHYLATION AND HYDROLYSIS OF THE PROAGLYCONE TAP-2

The proaglycone (TAP-2) on permethylation and followed by hydrolysis gave the aglycone and 2,3,5-tri-O-methyl xylose and 2,3,4,6-tetra-O-methyl glucose indicating that D-glucose was present in pyranose form and D-xylose was present in furanose form and also suggested that C₄-OH group of D-glucose was linked with C₁-OH of D-xylose.

ENZYMATIC HYDROLYSIS OF THE GLYCOSIDE TA

Enzymatic hydrolysis²⁹ of the glycoside with almond emulsin yielded D-xylose and D-glucose by Co-PC and Co-TLC indicating that the D-xylose was attached to D-glucose through β-linkage and further confirmed that D-glucose was attached to the aglycone by β-linkage.

The above facts when taken together concluded that the C₄₁-OH of the aglycone was linked with C₁-OH of the D-glucose via β-linkage and C₄-OH of the D-glucose was attached to the C₁-OH of D-xylose via β-linkage.

Thus the structure of the glycoside (TA) was assigned as (IV); 5,6-dihydroxy,7,8,3'-trimethoxy flavone-4'-O-β-D-xylopyranosyl (1→4)-O-β-D-glucopyranoside.
$^1$H NMR SPECTRUM OF THE OCTA ACETYL DERIVATIVE OF THE GLYCOSIDE TA

The chemical shifts observed in the $^1$H NMR of octaacetyl derivative of the glycoside (Fig. 4) and structural units inferred with the help of available literature$^{30,31}$ are recorded in Table-IV; which further supported the above assigned structure (IV);
### TABLE - IV

<table>
<thead>
<tr>
<th>S. No.</th>
<th>$\delta$ value</th>
<th>Pattern</th>
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<th>Assignment</th>
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<td>-</td>
<td>3</td>
<td>8-OCH$_3$</td>
</tr>
<tr>
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<td>3</td>
<td>3'-OCH$_3$</td>
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<td>dd</td>
<td>8.5,2.5</td>
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<td>H-2',6'</td>
</tr>
<tr>
<td>5.</td>
<td>6.65</td>
<td>dd</td>
<td>8.5,2.5</td>
<td>2</td>
<td>H-3,5'</td>
</tr>
<tr>
<td>6.</td>
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<td>12.</td>
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<td>16.</td>
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<td>-</td>
<td>9</td>
<td>Protons of sugar residue</td>
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</table>

$^{13}$C NMR SPECTRUM$^{32}$ OF THE GLYCOSIDE TA

$^{13}$C NMR spectrum of the glycoside revealed the presence of 29 carbon atoms in the compound and confirmed the structure of glycoside as IV; (Table-V) (400 MHz, CDCl$_3$); (Fig. 5).
$^1$H NMR SPECTRUM OF THE ACETYL DERIVATIVE OF THE GLYCOSIDE TA
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</tr>
<tr>
<td>C-3&quot;</td>
<td>76.81</td>
</tr>
<tr>
<td>C-4&quot;</td>
<td>78.61</td>
</tr>
<tr>
<td>C-5&quot;</td>
<td>61.99</td>
</tr>
<tr>
<td>C-1&quot;'</td>
<td>105.63</td>
</tr>
<tr>
<td>C-2&quot;'</td>
<td>75.47</td>
</tr>
<tr>
<td>C-3&quot;'</td>
<td>76.59</td>
</tr>
<tr>
<td>C-4&quot;'</td>
<td>78.97</td>
</tr>
<tr>
<td>C-5&quot;'</td>
<td>64.39</td>
</tr>
<tr>
<td>C-6&quot;'</td>
<td>50.56</td>
</tr>
</tbody>
</table>
$^{13}\text{C} \text{ NMR SPECTRUM OF THE GLYCOSIDE TA}$
MASS SPECTRUM OF THE GLYCOSIDE TA

The important fragment ion peaks, obtained in the EIMS, are given below:

\[ [M]^+ 654 \text{ (absent)}, \ m/z \ 521, 522, \ 360, 332, 331, 212, \]
\[ 213, 184, 164, 163. \]

The different species obtained during the fragmentation is shown in scheme II and were found to be in complete accord with the structure of the glycoside being 5-6 dihydroxy 7,8,3'-trimethoxy flavone-4'-O-\(\beta\)-D-xylofuranosyl (1→4)-O-\(\beta\)-D-glucopyranoside (IV).
EXPERIMENTAL

Trichosanthes anguina Linn. (N.O. cucurbitaceae) was procured by M/s United Chemicals and Allied Products, Calcutta and authenticated by Department of Botany, Dr. Hari Singh Gour University, Sagar. A herbarium specimen has been deposited in the room no. 36 of the Chemistry Department.

The air dried and powdered seeds (3 kg) of T. anguina Linn. were extracted with 95% ethanol in a 5 litre round bottom flask fitted with a condenser. The total extract was concentrated under reduced pressure to a pale yellow syrupy mass (125 gm), which was dissolved in distilled water and the water soluble and water insoluble part were separated. The water soluble part was concentrated to viscous mass and successively extracted with the solvents of increasing basicity; i.e., petroleum ether, benzene, chloroform, ethyl acetate, acetone and methanol.

The petroleum ether, benzene, chloroform and methanol soluble fractions were insufficient to provide for any substantive investigations.

The study of acetone soluble fraction has been described in Chapter IV of the thesis. The
study of ethyl acetate soluble fraction has been reported in this chapter.

STUDY OF ETHYL ACETATE SOLUBLE PART

The ethyl acetate soluble fraction of the water soluble part of the ethanolic extract was concentrated under reduced pressure, to yield a yellow syrupy mass (3.4 gm). It gave two spots on TLC examination using solvent system EtOAc-Acetone-H₂O (9:3:1) and I₂ vapours as visualising agent, which were separated by column chromatography over Si-gel G and eluted with chloroform-methanol in various proportions (6:1), (6:2), (6:4) and (6:8).

COLUMN CHROMATOGRAPHY

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of the column</td>
<td>150 cm.</td>
</tr>
<tr>
<td>Diameter of the column</td>
<td>4.0 cm.</td>
</tr>
<tr>
<td>Weight of the crude extract</td>
<td>3.4 gm.</td>
</tr>
<tr>
<td>Weight of the Si-gel G</td>
<td>250 gm</td>
</tr>
</tbody>
</table>

**TABLE - VI**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Fraction No.</th>
<th>Eluant</th>
<th>Spot on TLC</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 - 6</td>
<td>Chloroform:Methanol (6:1)</td>
<td>Nil</td>
<td>No solid mass</td>
</tr>
<tr>
<td>2</td>
<td>7 - 14</td>
<td>Chloroform:Methanol (6:2)</td>
<td>One</td>
<td>Compound (TA)</td>
</tr>
<tr>
<td>3</td>
<td>15 - 24</td>
<td>Chloroform:Methanol (6:4)</td>
<td>One</td>
<td>Compound (TB)</td>
</tr>
<tr>
<td>4</td>
<td>25 - 36</td>
<td>Chloroform:Methanol (6:8)</td>
<td>Nil</td>
<td>Sticky mass</td>
</tr>
</tbody>
</table>
STUDY OF FRACTION (7-14)

Eluates obtained from chloroform:Methanol (6:2) were found to have same Rf value, hence combined. On removal of the solvent, a yellow coloured amorphous powder (1.95 gm) was obtained, which was found to be homogenous on TLC examination using solvent system, BuOH-AcOH-H₂O (4:1:5) and I₂ vapours as visualising agent.

STUDY OF THE FLAVONE GLYCOSIDE TA

The flavone glycoside (TA) was a yellow amorphous powder which was crystallised from solvent ether. It was soluble in ethanol.

It analysed for molecular formula, \( \text{C}_{29}\text{H}_{34}\text{O}_{17} \), m.p. 336°C and [M]⁺ 654 (EIMS).

It gave positive Molisch test for glycoside and responded to following characteristic colour reactions of flavonoids:

(i) Brown colour with \( \text{FeCl}_3 \)
(ii) Red colour with Na-Hg/HCl

ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for ( \text{C}<em>{29}\text{H}</em>{34}\text{O}_{17} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 53.20%</td>
<td>C 53.21%</td>
</tr>
<tr>
<td>H 5.0%</td>
<td>H 5.1%</td>
</tr>
</tbody>
</table>

Molecular weight - 654 (By EIMS)
ACETYLATION OF THE COMPOUND TA

100 mg of the glycoside (TA), 8 ml. of acetic anhydride and 3 ml. of pyridine were taken in a 100 ml. round bottom flask, fitted with an air condensor. The reaction mixture was refluxed at 120° for about 7-8 hours. After cooling, the contents were poured into cold water, when a white precipitate was obtained, which was dissolved in solvent ether and separated by separating funnel. The ethereal layer was washed with anhydrous sodium bicarbonate and ether was removed on evaporation. The residue was crystallised from methanol as white needles, which analysed for molecular formula C_{45}H_{50}O_{25}, m.p. 198°C and [M]^+ 990.

ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C_{45}H_{50}O_{25}</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 54.6%</td>
<td>C 54.5%</td>
</tr>
<tr>
<td>H 5.2%</td>
<td>H 5.0%</td>
</tr>
</tbody>
</table>

Molecular weight - 990
(By EIMS)

ACID HYDROLYSIS OF THE GLYCOSIDE TA

The glycoside (400 mg) and 7% ethanolic H_2SO_4 was refluxed in a 250 ml. RB flask at 100° for 8-10 hours. Then 100 ml. of water was added to the reaction mixture and after removal of alcohol
it gave an aglycone (TA-l) as a precipitate, which was separated by filtration. The aglycone and hydrolysate were studied separately.

STUDY OF THE AGLYCONES TA-1

The aglycone was crystallised from methanol and obtained as yellow coloured needles (200 mg). It was found to be homogenous on TLC examination (Benzene-Methanol) 6:3, I₂ vapours as visualizing agent. It analysed for molecular formula C₁₈H₁₆O₈, m.p. 235°C and [M]⁺ 360 (EIMS).

It showed the following colour reactions:

(i) Yellow colour with Mg/HCl.
(ii) Green colour with FeCl₃.

ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C₁₈H₁₆O₈</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 60.02%</td>
<td>C 60.0%</td>
</tr>
<tr>
<td>H 4.3%</td>
<td>H 4.4%</td>
</tr>
</tbody>
</table>

Molecular weight - 360 (By EIMS).

ACETYLATION OF THE AGLYCONES TA-1

85 mg. of aglycone (TA-l) was mixed with acetic anhydride (5 ml) and pyridine (2 ml) as described on page no. of thesis. The acetyl derivative
crystallised from methanol as silkey needles, m.p. 165°C. It analysed for molecular formula C_{24}H_{22}O_{11}, [M]^+ 486.

**ELEMENTAL ANALYSIS**

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C_{24}H_{22}O_{11}</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 59.22%</td>
<td>C 59.25%</td>
</tr>
<tr>
<td>H 4.54%</td>
<td>H 4.52%</td>
</tr>
</tbody>
</table>

Molecular weight - 486  
(By EIMS).

**ESTIMATION OF METHOXYL GROUPS IN THE AGLYCONE TA-1**

The aglycone (30 mg), few quantity of phenol and 2 ml of propionic anhydride were taken in a 100 ml round bottom flask. The total mixture was heated and 10 ml. of A.R. hydroiodic acid was added to the flask. The flask was connected to the source of CO\textsubscript{2} and rate of passage of CO\textsubscript{2} adjusted and contents were heated on a oil bath for six to seven hours, gradually. The delivery tube was washed with distilled water and the contents collected in the receiver and then contents were transferred to a 100 ml. conical flask containing 2 ml. of 25% sodium acetate solution. Formic acid was added drop by drop to the solution to remove the smell of bromine. The contents were diluted and 1 gm. of A.R.KI and 10% H\textsubscript{2}SO\textsubscript{4} were added to it. The contents were shaked and
allowed to stand for 15 minutes. The liberated iodine was titrated against 0.05 N sodium thiosulphate solution using starch as an indicator.

PARTIAL HYDROLYSIS OF THE GLYCOSIDE

The glycoside (210 mg) was treated with Kiliani mixture (HCl-CH₃COOH-H₂O, 15:35:50) in a 100 ml. conical flask and left for a week at room temperature. The contents were extracted with n-butanol. The n-butanol soluble part on PC examination using B:A:W (4:1:5) and aniline hydrogen phthalate as spraying reagent, showed two spots (Rf 0.54 and 0.38). The n-butanol soluble part was concentrated and separated by column chromatography over Si-gel G and eluted with CHCl₃:MeOH in different proportions.

The details of column chromatography is as followed:

- Length of the column: 100 cm.
- Diameter of the column: 3.0 cm.
- Weight of the n-butanol soluble part: 200 mg.
- Weight of Si-gel G: 225 g
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Fraction No.</th>
<th>Eluant</th>
<th>Spot on TLC</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1 - 5</td>
<td>CHCl₃-MeOH (4:1)</td>
<td>One</td>
<td>TAP-1</td>
</tr>
<tr>
<td>2.</td>
<td>6 - 10</td>
<td>CHCl₃-MeOH (4:2)</td>
<td>Two</td>
<td>TAP-1 and TAP-2</td>
</tr>
<tr>
<td>3.</td>
<td>11 - 15</td>
<td>CHCl₃-MeOH (4:3)</td>
<td>One</td>
<td>TAP-2</td>
</tr>
</tbody>
</table>

STUDY OF FRACTIONS (1-5)

The fractions (1-5) being of the same Rf value were mixed together and solvent removed to furnish the compound TAP-1. It crystallised from ethanol and analysed for $C_{24}H_{26}O_{13}$ m.p. 230°C and $[M]^+$ 522.

ELEMENTAL ANALYSIS OF THE PROAGLYCONE (TAP-1)

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculate</th>
<th>Calculated for $C_{24}H_{26}O_{13}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 54.3%</td>
<td>C 55.1%</td>
<td></td>
</tr>
<tr>
<td>H 4.6%</td>
<td>H 4.9%</td>
<td></td>
</tr>
</tbody>
</table>

Molecular weight - 522
(By EIMS)

PERMETHYLATION AND HYDROLYSIS OF THE PROAGLYCONE (TAP-1)

The proaglycone (TAP-1) 75 mg was treated with dimethyl formamide (6 ml) in a 100 ml. conical flask. Then CH₃I (10 ml) and Ag₂O (100 mg) were
added and the reaction mixture was kept for two days at room temperature. The reaction mixture was filtered and washed with DMF. The filtrate was concentrated under reduced pressure and hydrolysed by refluxing with 7% H₂SO₄ to yield the aglycone and the methylated sugar.

The aqueous hydrolysate after the removal of the aglycone, was neutralised with BaCO₃ and BaSO₄ filtered off. The filtrate was concentrated and studied by PC on Whatman filter paper No.1 using BAW (4:1:5) as solvent system and aniline hydrogen phthalate as spraying reagent. The methylated sugar was identified as 2,3,4,6-tetra-O-methyl D-glucose.

**STUDY OF FRACTIONS (11-15)**

The fractions (11-15) were of the same Rf value, so they were combined together. On evaporation of the solvent afforded compound TAP-2, which crystallised from ethanol. It analysed for C₂₉H₃₄O₁₇, m.p. 335°C and [M]⁺ 654 (EIMS).

**ELEMENTAL ANALYSIS OF PROAGLYCONE (TAP-2)**

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C₂₉H₃₄O₁₇</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 53.3%</td>
<td>C 53.21%</td>
</tr>
<tr>
<td>H 5.4%</td>
<td>H 5.1%</td>
</tr>
<tr>
<td>Molecular weight - 654</td>
<td>(By EIMS)</td>
</tr>
</tbody>
</table>
PERMETHYLATION AND HYDROLYSIS OF THE PROAGLYCONE TAP-2

The permethylation and hydrolysis of the proaglycone (TAP-2) was carried out by similar procedure as described on page no. 53, which revealed the presence of aglycone (by mmp, Co-PC and Co-TLC) and methylated sugars (by Co-PC and Co-TLC with authentic samples).

IDENTIFICATION OF SUGARS

The aqueous hydrolysate obtained after the hydrolysis of the glycoside was neutralised with BaCO₃ and BaSO₄ filtered off. The filterate after concentration was examined by PC by using solvent systems (i) Isopropanol-pyridine-water (8:8:4:1) and (ii) n-butanol-aceticacid-water (4:1:5) and aniline hydrogen phthalate as spraying agent. The results are shown in Table-VIII.

### TABLE - VIII

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Solvent system</th>
<th>RF value Reported</th>
<th>Found</th>
<th>Sugar identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Isopropanol-pyridine-water (8:8:4:1)</td>
<td>0.64</td>
<td>0.65</td>
<td>D-glucose</td>
</tr>
<tr>
<td>2.</td>
<td>n-butanol-acetic acid-water (4:1:5)</td>
<td>0.18</td>
<td>0.17</td>
<td>D-glucose</td>
</tr>
</tbody>
</table>

|                    |                                  | 0.73              | 0.70  | D-xylose        |
|                    |                                  | 0.28              | 0.29  | D-xylose        |
PERIODATE OXIDATION OF THE GLYCOSIDE TA

The glycoside (100 mg) was dissolved in methanol (50 ml) in a 100 ml. conical flask, and treated with sodium metaperiodate (20 ml) and then reaction mixture left for 48 hours at room temperature. Simultaneously a blank was also run in the same way. The quantity of sodium metaperiodate consumed and formic acid liberated was estimated by Jone's method.

ENZYMATIC HYDROLYSIS OF THE GLYCOSIDE TA

The glycoside (30 mg) was dissolved in ethanol (25 ml) & treated with almond emulsin (35 ml) in a conical flask (100 ml), fitted with a stopper. The content was allowed to stand for 3 days at room temperature and filtered. The aglycone and hydrolysate were studied separately.

After concentration the hydrolysate was examined by PC on Whatman filter paper No.1, using BAW (4:1:5) solvent system, confirming the presence of D-xylose and D-glucose and also suggested the presence of β-linkage between both the sugar moieties and between sugar and aglycone.
REFERENCES


