CHAPTER VI: PART A

ISOLATION AND STRUCTURAL STUDY OF THE ISOFLAVONOIDAL GLYCOSIDE; ISOQUERCETIN 7-O-β-D-GLUCOPYRANOSIDE FROM THE FLOWERS OF TRIDAX PROCUMBENS LINN. *

* This work has been communicated for publication in J. Chem. Sci., Indian Academy of Sciences, Bangalore.
Tridax procumbens Linn\textsuperscript{14} belongs to natural order Compositae and is commonly known as 'Ghamra' in Hindi.

The plant is medicinally important. The leaves of the plant are used in the treatment of bronchial catarrh, dysentery, diarrhoea, for restoring hairs and to check haemorrhage from cuts, bruises and wounds. An aqueous extract of the plant also has marked depressant action on respiration. It's leaves also possess antiseptic, insecticidal and parasiticidal properties\textsuperscript{5}.

Earlier workers\textsuperscript{67} have reported steroids and flavonol glycosides from this plant.

**ISOLATION OF THE ISOFLAVONE GLYCOSIDE AS-IV**

The flowers of *Tridax procumbens* Linn. were collected locally and identified by the reputed taxonomist. 2.0 Kg of air dried and powered flowers of *Tridax procumbens* Linn. were extracted with 95% ethanol. The ethanolic extract was concentrated under reduced pressure. The resultant brown mass was successively extracted with benzene, chloroform, acetone and ethyl acetate. The ethyl acetate soluble fraction was concentrated under reduced pressure. The residue obtained was subjected to TLC examination using B:A:W (4:1:5) as solvent system and I\textsubscript{2} vapours as visualizing agent, when it showed two spots. Therefore it was subjected to silica gel G column chromatography and eluted with MeOH: H\textsubscript{2}O mixture in varying proportions i.e. 2 : 4, 2 : 6 and 2 : 8. Eluates obtained from MeOH : H\textsubscript{2}O (2 : 8) were collected and found to have same R\textsubscript{t} values, so combined together. On removal of the solvent it gave a yellow crystalline compound AS-IV (0.0993\%) which was found to be
homogeneous on TLC examination using EtOAc : Ac₂O : AcOH : H₂O (5:3:1:1) as solvent system and I₂ vapours as visualizing agent. (Fig. 5.1).

**ISOLATION-CHART**

Air dried and powdered flowers (2.0kg.) of *Tridax procumbens* Linn.

1. **Extraction with 95% ethanol**
   - Ethanolic extract
   - Concentration under reduced pressure
   - Brown mass
   - Successive extraction with benzene, chloroform, acetone and ethyl acetate
   - Ethyl acetate soluble part
   - Concentration under reduced pressure
   - Crude compound
   - TLC [B : A : W (4 : 1 : 5)] and I₂ vapours
   - Two spots
   - Column chromatography over silica gel G
     - [MeOH : H₂O in 2 : 4, 2 : 6, 2 : 8 respectively]
     - Eluates from MeOH: H₂O (2 : 8)
     - 1. Removal of solvent
     - 2. Crystallisation from methanol

**AS-IV**

- Colourless needles (yield 0.0993%)
- m.f. : C₂₁H₂₀O₁₂
- m.p. : 226-228°C
- [M⁺] : 464 (FABMS)

**Fig. 5.1:** Fractionation protocol for isolation of compound AS-IV from plant *Tridax procumbens* Linn.
STUDY OF THE ISOFLAVONE GLYCOSIDE AS-IV

The glycoside AS-IV was found to be soluble in water, ethanol and methanol. It crystallised from MeOH and analysed for molecular formula C_{21}H_{20}O_{12}, m.p. 226–228°C and M^+ [464] (FABMS). It responded positive to all characteristic colour reactions of isoflavones and also gave positive Molisch's test for glycoside.

UV-SPECTRUM OF THE ISOFLAVONE GLYCOSIDE AS-IV

The wavelengths of maximum absorption as recorded with various shift regents were as follows:-

<table>
<thead>
<tr>
<th>S.No.</th>
<th>$\lambda_{max}$</th>
<th>Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>$\lambda_{max}^{\text{MeOH}}$</td>
<td>258, 365 nm</td>
</tr>
<tr>
<td>2.</td>
<td>$\lambda_{max}^{\text{AlCl}_3}$</td>
<td>240, 410 nm</td>
</tr>
<tr>
<td>3.</td>
<td>$\lambda_{max}^{\text{AlCl}_3+\text{HCl}}$</td>
<td>355, 425 nm</td>
</tr>
<tr>
<td>4.</td>
<td>$\lambda_{max}^{\text{NaOMe}}$</td>
<td>320, 412 nm</td>
</tr>
<tr>
<td>5.</td>
<td>$\lambda_{max}^{\text{NaOAc}}$</td>
<td>270, 325 nm</td>
</tr>
<tr>
<td>6.</td>
<td>$\lambda_{max}^{\text{NaOAc+H}_3\text{BO}_3}$</td>
<td>265, 385 nm</td>
</tr>
</tbody>
</table>

IR SPECTRUM OF ISOFLAVONE GLYCOSIDE AS-IV

The important bands obtained in the IR spectrum (Fig. 5.2) of AS-IV and structural units inferred with the help of available literature are recorded in table-5.2.
TABLE-5.2

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Wave number (cm⁻¹)</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3390.4</td>
<td>-OH group</td>
</tr>
<tr>
<td>2.</td>
<td>2936.7</td>
<td>C−H stretching vibration</td>
</tr>
<tr>
<td>3.</td>
<td>1640.8</td>
<td>&gt;C=O stretching</td>
</tr>
<tr>
<td>4.</td>
<td>1610.4</td>
<td>Aromating ring system</td>
</tr>
<tr>
<td>5.</td>
<td>1387.0</td>
<td>C−O−C bending vibration</td>
</tr>
<tr>
<td>6.</td>
<td>1050.6</td>
<td>C−O−C stretching vibration</td>
</tr>
<tr>
<td>7.</td>
<td>845.8</td>
<td>Two adjacent H-atoms in ring system</td>
</tr>
</tbody>
</table>

PRESENCE OF HYDROXYL GROUP(S) IN THE GLYCOSIDE AS-IV

In the IR spectrum of AS-IV a band at $\nu_{\text{max}}^{\text{KBr}}$ 3390.4 cm⁻¹ showed the presence of hydroxyl group(s) in it. On acetylation (Ac₂O/NaOAc) AS-IV furnished an acetylated product, which analysed for C₃₅H₃₄O₁₉, m.p. 241–243°C, [M⁺] 758 (FABMS). The quantitative estimation of acetyl group was done by Weisenberger¹⁹ method as described by Belcher and Godbert²⁰. The results showed the presence of seven hydroxyl groups in the glycoside AS-IV (38.5112%).

The IR spectrum of acetylated product showed a band at 3343.5 cm⁻¹, which revealed that all the hydroxyl groups were not acetylated under the condition (Ac₂O/NaOAc). The C−5 –OH group was not acetylated due to strong intramolecular hydrogen bonding with the carbonyl group at C−4.

Further structural elucidation of the glycoside AS-IV was carried out after the acid hydrolysis of AS-IV.
ACID HYDROLYSIS OF THE ISOFLAVONE GLYCOSIDE AS-IV

On acid hydrolysis with 7% $\text{H}_2\text{SO}_4$, the glycoside AS-IV provided the aglycone AS-IV (A) and sugar moiety (ies) in the hydrolysate. The aglycone AS-IV (A) and sugar moiety (ies) were separated by filtration and studied separately.

STUDY OF THE AGLYCON AS-IV (A)

The aglycone was crystallised from methanol into yellow crystals. It was subjected to TLC to ensure its purity by using B:A:W (4 : 1 : 5) as solvent system and $\text{I}_2$ vapours as visualizing agent. It responded positive to all characteristic colour reactions of isoflavones\textsuperscript{21,22}. It analysed for molecular formula $\text{C}_{15}\text{H}_{10}\text{O}_7$, m.p. 318–320°C and [M\textsuperscript{+}] 302 (FABMS).

UV-SPECTRUM OF THE AGLYCON AS-IV (A)

The wavelengths of maximum absorption in the UV spectrum\textsuperscript{24} of AS-IV (A) were recorded in table-5.3.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>$\lambda_{max}$</th>
<th>Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>$\lambda_{\text{MeOH}}$</td>
<td>252, 302, 376 nm</td>
</tr>
<tr>
<td>2.</td>
<td>$\lambda_{\text{HCl}}$</td>
<td>273, 532, 421 nm</td>
</tr>
<tr>
<td>3.</td>
<td>$\lambda_{\text{HCl+HCl}}$</td>
<td>265, 300(sh), 357, 424 nm</td>
</tr>
<tr>
<td>4.</td>
<td>$\lambda_{\text{NaOMe}}$</td>
<td>322(dec), 423 nm</td>
</tr>
<tr>
<td>5.</td>
<td>$\lambda_{\text{NaOAc}}$</td>
<td>254, 270, 320, 393 nm</td>
</tr>
<tr>
<td>6.</td>
<td>$\lambda_{\text{NaOAc+HBO}_3}$</td>
<td>262, 393(dec) nm</td>
</tr>
</tbody>
</table>

IR SPECTRUM OF THE AGLYCON AS-IV (A)

The significant bands obtained in the IR spectrum (Fig. 5.3) of the aglycone AS-IV (A) and the structural units inferred with the help of available literature\textsuperscript{25-27} are recorded in table-5.4.
TABLE-5.4

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Wave number (cm⁻¹)</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3340.5</td>
<td>--OH group(s)</td>
</tr>
<tr>
<td>2.</td>
<td>2880.2</td>
<td>C–H stretching vibration</td>
</tr>
<tr>
<td>3.</td>
<td>1680.5</td>
<td>&gt;C = O</td>
</tr>
<tr>
<td>4.</td>
<td>1612.2</td>
<td>Aromating ring system</td>
</tr>
<tr>
<td>5.</td>
<td>1375.5</td>
<td>C–O–C bending vibration</td>
</tr>
<tr>
<td>6.</td>
<td>1035.5</td>
<td>C–O–C stretching vibration</td>
</tr>
<tr>
<td>7.</td>
<td>843.2</td>
<td>Two adjacent H-atoms in ring system</td>
</tr>
</tbody>
</table>

PRESENCE OF HYDROXYL GROUP(S) IN THE AGLYCONE AS-IV (A)

The IR absorption band at $\nu_{\text{max}}^{\text{KBr}} 3340.5 \text{ cm}^{-1}$ indicated the presence of --OH group(s) in AS-IV (A). Acetylation (Ac₂O/NaOAc) was done in order to determine the number of hydroxyl group(s). On acetylation AS-IV (A) furnished an acetylated derivative, which analysed for molecular formula $C_{23}H_{18}O_{11}$, m.p. 180-182°C and [M⁺] 470 (FABMS).

The percentage of acetyl group in the acetylated product was determined by Weisenberger¹⁹ method as described by Belcher and Godbert²⁸, which indicated the presence of four hydroxyl group (36.82%) in it.

In the IR spectrum of the acetylated product, a band at 3340 cm⁻¹ indicated that one of the hydroxyl groups was not acetylated. It must be C-5 --OH group which must have escaped acetylation because of strong intramolecular hydrogen bonding with C-4 carbonyl group.

¹H-NMR SPECTRUM OF THE TETRA ACETYL DERIVATIVE OF AS-IV (A)

The important signals obtained in the ¹H-NMR spectrum (Fig. 5.4) of acetylated product of the aglycone AS-IV (a) and structural units inferred with the help of available literature²⁸-²⁹ are recorded in table-5.5.
FIG. 5.3: IR SPECTRUM OF THE AGLYCON AS-IV (A)

03/08/09 15:45 smg
X: 4 scans, 4.0cm⁻¹, flat, smooth, abex
TABLE-5.5

<table>
<thead>
<tr>
<th>S.No</th>
<th>δ-value</th>
<th>Pattern</th>
<th>J-value</th>
<th>No. of protons</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>6.48</td>
<td>d</td>
<td>2.6 Hz</td>
<td>1</td>
<td>H-8</td>
</tr>
<tr>
<td>2.</td>
<td>6.04</td>
<td>d</td>
<td>2.4 Hz</td>
<td>1</td>
<td>H-6</td>
</tr>
<tr>
<td>3.</td>
<td>7.51</td>
<td>d</td>
<td>2.2 Hz</td>
<td>1</td>
<td>H-6'</td>
</tr>
<tr>
<td>4.</td>
<td>7.72</td>
<td>d</td>
<td>2.0 Hz</td>
<td>1</td>
<td>H-2'</td>
</tr>
<tr>
<td>5.</td>
<td>6.58</td>
<td>d</td>
<td>8.2 Hz</td>
<td>1</td>
<td>H-5'</td>
</tr>
<tr>
<td>6.</td>
<td>2.23</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>C-4' OAc</td>
</tr>
<tr>
<td>7.</td>
<td>2.26</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>C-7 OAc</td>
</tr>
<tr>
<td>8.</td>
<td>2.32</td>
<td>s</td>
<td>-</td>
<td>3</td>
<td>C-2 OAc</td>
</tr>
<tr>
<td>9.</td>
<td>2.26</td>
<td>s</td>
<td>--</td>
<td>3</td>
<td>C-3' OAc</td>
</tr>
</tbody>
</table>

ALKALINE DEGRADATION OF THE AGLYCONE AS-IV (A)

On fusion with 50% ethanolic KOH, aglycone AS-IV (A) furnished (i) phloroglucinol m.f. C₆H₅O₃, m.p. 216-217°C and (ii) 3, 4 dihydroxy phenyl acetic acid m.f. C₆H₅O₄, m.p. 182-183°C (By CoPC and CoTLC).

\[
\text{AS-IV (A)} \xrightarrow{50\% \text{ KOH}}
\]

\[
\begin{align*}
\text{HO} & \text{O} \quad \text{OH} \\
\text{m.f. C}_{6}\text{H}_{5}\text{O}_{3} & \quad \text{m.p. 216-217°C} \\
\text{HO} & \text{O} \quad \text{OH} \\
\end{align*}
\]

\[
\begin{align*}
\text{HOOC-C}_2\text{H}_4\text{HCO-} & \text{OH} \\
\text{m.f. C}_{6}\text{H}_{5}\text{O}_{4} & \quad \text{m.p. 182-183°C} \\
\end{align*}
\]

POSITION OF HYDROXYL GROUP IN THE AGLYCONE AS-IV (A)

The positions of all five hydroxyl groups were ascertained by observing and comparing the UV spectral data of AS-IV (A) in methanol and in various shift reagents.
FIG. 5.4: $^1$H-NMR SPECTRUM OF THE AGLYCONE AS-IV (A)

![NMR Spectrum Image]

**AS-IVA**
**RSIC No. 8568**

**Current Data Parameters**
- **NAME:** 6590-RSIC
- **FIDNO:** 10
- **PROCNO:** 1

**F2 - Acquisition Parameters**
- **Date:** 8/04/91
- **Time:** 11:44
- **SPECTRUM:** 00:200
- **Pulseprog:** 5-6 Dual 13
- **PULPROG:** p010
- **ID:** 06705
- **SOLVENT:** DMSO
- **NS:** 16
- **DS:** 2
- **SW:** 4100.073 Hz
- **T1DEL** 0.126214 Hz
- **T0:** 2.0064242 sec
- **AG:** 84
- **DEP:** 50.000000 sec
- **TD:** 600.00 sec
- **DT:** 1.00000000 sec

**--------------- CHANNEL F1 ---------------**
- **RES:** 60.00 ppm
- **P1:** 300.00 ppm
- **P2:** 4.00 ppm
- **P3:** 300.00 ppm

**F2 - Processing parameters**
- **SI:** 102354
- **SF:** 500 1500277 MHz
- **SSB:** 0.0 Hz
- **SSB:** 0.30 Hz
- **DS:** 10
- **PC:** 1.00

**10 ppm plot parameters**
- **CZ:** 10.00 cm
- **F2P:** 14.50 ppm
- **F1:** 2935.83 Hz
- **F2:** 2.783 ppm
- **F3:** 2.0 ppm
- **F4:** 0.0 ppm
- **IC:** 0.05 ppm

**ppm**

- 12
- 10
- 8
- 6
- 4
- 2
- 0
Position of Hydroxyl Groups at C-2, C-3' and C-4':

The aglycone AS-IV (A) displayed degenerated peaks with NaOMe, thereby indicating the presence of 2, 3' and 4' hydroxyl group in it.\(^{38}\)

The bathochromic shift of 48 nm in band I with AlCl\(_3\) (relative to MeOH) on addition of HCl indicated the presence of orthodihydroxy groups in B ring (3', 4' hydroxyl groups).\(^{31}\)

The position of hydroxyl group at C-3' and C-4' was also confirmed by the formation of 3, 4 dihydroxy phenyl acetic acid, m.f. C\(_6\)H\(_8\)O\(_4\), m.p. 182-183°C on alkaline degradation of aglycone AS-IV (A).

Position of Hydroxyl Group at C-7:

The bathochromic shift of 18nm in band II with NaOAc (relative to MeOH) indicated the presence of hydroxyl group at C-7\(^{32}\).

Position of Hydroxyl Group at C-5 :

The bathochromic shift of 19 nm in band I (in MeOH) to band II (in AlCl\(_3\)/HCl) indicated the presence of C-5 –OH group.\(^{33}\)

The presence of hydroxyl group at C-5 was also confirmed by the partial acetylation of the aglycone AS-IV (A). Instead of penta acetyl derivative, tetra acetyl derivative was formed, showing that C-5 –OH group was not acetylated because of being strongly associated with intramolecular hydrogen bonding with keto group at C-4.

On alkaline degradation the aglycone AS-IV (A) yielded phloroglucinol, m.f. C\(_6\)H\(_6\)O\(_3\) and m.p. 216-217°C, which further confirmed the position of hydroxyl groups at C-5 and C-7.

On the basis of above facts following structure (I) was assigned to aglycone AS-IV (A), thereby established its identity as 2, 5, 7, 3', 4' penta hydroxyl isoflavone (Isoquercetin)\(^{34-36}\).
MASS SPECTRUM OF THE AGLYCONE AS-IV (A)

Important fragmentation peaks in the FABMS$^{37}$ of the aglycone AS-IV (A) were at:

m/z 302 [M$^+$], 274, 153, 152, 151 and 124.

The various species assigned to the fragments are shown in scheme-I, which further confirmed its identity as AS-IV (A).

STUDY OF THE HYDROLYSATE

The filtrate obtained by the acid hydrolysis of glycoside AS-IV was neutralised by adding BaCO$_3$ and BaSO$_4$ was filtered off. After filtration, the filtrate was concentrated to a syrupy mass, which was examined by paper chromatography. The sugar was identified as D-glucose (R$_f$ 0.18).

POSITION OF ATTACHMENT OF THE SUGAR TO THE AGLYCONE AS-IV (A)

The sugar moiety is attached to the C-7 –OH group of aglycone, which is confirmed by comparing the UV spectra of aglycone AS-IV (A) and glycoside AS-IV. The facts$^{38}$, which confirmed the site of attachment of sugar to aglycone, are as follows:

(i) The presence of C-4' hydroxyl group was confirmed by the characteristic colour reactions. Both AS-IV (A) and AS-IV produced pink colour with Mg/HCl, which turned blue on the addition of sodium bi carbonate indicating the presence of C-4' –OH group in both the compounds.
A bathochromic shift of 47 nm in band I on addition of NaOMe (relative to MeOH) was observed in the UV spectra of aglycone AS-IV (A) and glycoside AS-IV, which further confirmed the presence of a free hydroxyl group at C-4'.

(ii) A bathochromic shift of 45 nm in band I on addition of AlCl₃ (relative to MeOH) was observed in the UV spectra of aglycone AS-IV (A) and glycoside AS-IV, which indicated the presence of a free hydroxyl group at C-5 in both the compounds.
(iii) In the UV spectrum of aglycone AS-IV (A) a bathochromic shift of 18 nm was observed in band II by the addition of NaOAc which indicated the presence of free hydroxyl group at C-7, but such a shift was absent in the UV spectrum of the glycoside AS-IV. It clearly indicated that C-7 has a free -OH group in aglycone whereas in glycoside the sugar moiety is attached to C-7 of AS-IV.

On the basis of above facts a tentative structure (II) was assigned to the isoflavonol glycoside AS-IV.

![Structure II]

---

**QUANTITATIVE ESTIMATION OF SUGAR**

The hydrolysate obtained from the acid hydrolysis of the glycoside AS-IV was neutralised by adding BaCO₃ and BaSO₄ was filtered off. After filtration and concentration a syrupy mass was obtained which was subjected to CoPC and CoTLC and was found to contain D-glucose.

The sugar was estimated quantitatively by the procedure of Mishra and Rao⁴⁶, which revealed that the sugar and aglycone were present in equimolar ratio (1 : 1) in the glycoside (AS-IV).

**PERMETHYLATION AND HYDROLYSIS OF THE GLYCOSIDE AS-IV**

The glycoside AS-IV on permethylation by Kuhn's procedure⁴⁷ followed by acid hydrolysis yielded aglycone AS-IV (A) and 2, 3, 4, 6 tetra-O-methyl-D-glucose (CoPC and CoTLC) thereby indicating that C-1 of D-glucose was involved in glycosylation.
PERIODATE OXIDATION OF THE GLYCOSIDE AS-IV

On treatment with sodium meta periodate\(^1\) one mole of glycoside AS-IV required 3.02 moles of periodate and liberated 1.06 moles of formic acid indicating that the sugar was in pyranose form\(^3\) in the glycoside AS-IV. Thus the tentative structure (III) was assigned to the glycoside AS-IV.

![Structure (III)](image)

ENZYMATIC HYDROLYSIS OF THE GLYCOSIDE AS-IV

The glycoside AS-IV when hydrolysed with enzyme almond emulsion\(^4\) yielded aglycone AS-IV (A) and D-glucose (CoPC) which confirmed that C-7 \(-\text{OH}\) of the aglycone was linked to C-1 \(-\text{OH}\) of D-glucose via \(\beta\)-linkage.

Thus, the glycoside AS-IV was assigned the following structure and was found to be 2, 5, 3', 4' tetra hydroxy isoflavone 7-O-\(\beta\)-D glycopyranoside (Isoquercetin 7-O-\(\beta\)-D-glucopyranoside).

![Structure AS-IV](image)

The above structure was further supported by its \(^1\)H-NMR, \(^13\)C-NMR and mass spectral studies.
1H-NMR SPECTRUM OF THE GLYCOSIDE AS-IV

The significant chemical shifts in the 1H-NMR spectrum (Fig. 5.5) of the glycoside AS-IV and structural units inferred with the help of available literature\(^{46}\) are recorded in table-5.6.

**TABLE-5.6**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>δ-Value</th>
<th>Pattern</th>
<th>J-Value</th>
<th>No. of Protons</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>7.62</td>
<td>s</td>
<td>-</td>
<td>1</td>
<td>H-2'</td>
</tr>
<tr>
<td>2.</td>
<td>7.49</td>
<td>d</td>
<td>7.8 Hz</td>
<td>1</td>
<td>H-6'</td>
</tr>
<tr>
<td>3.</td>
<td>6.71</td>
<td>d</td>
<td>7.9 Hz</td>
<td>1</td>
<td>H-5'</td>
</tr>
<tr>
<td>4.</td>
<td>6.35</td>
<td>d</td>
<td>2.1 Hz</td>
<td>1</td>
<td>H-8</td>
</tr>
<tr>
<td>5.</td>
<td>6.30</td>
<td>d</td>
<td>2.0 Hz</td>
<td>1</td>
<td>H-6</td>
</tr>
<tr>
<td>6.</td>
<td>5.15</td>
<td>d</td>
<td>8.0 Hz</td>
<td>1</td>
<td>H-1&quot;</td>
</tr>
<tr>
<td>7.</td>
<td>3.50</td>
<td>Complex signal</td>
<td>-</td>
<td>6</td>
<td>Glucose Protons</td>
</tr>
</tbody>
</table>

13C-NMR SPECTRUM OF THE GLYCOSIDE AS-IV

The significant signals obtained in the 13C-NMR spectrum (Fig. 5.6) of the glycoside AS-IV and structural units inferred with the help of available literature\(^{46,35}\) are recorded in the table 5.7.

**Table - 5.7**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>δ-Value</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>133.5</td>
<td>C-2</td>
</tr>
<tr>
<td>2.</td>
<td>156.4</td>
<td>C-3</td>
</tr>
<tr>
<td>3.</td>
<td>177.0</td>
<td>C-4</td>
</tr>
<tr>
<td>4.</td>
<td>160.5</td>
<td>C-5</td>
</tr>
<tr>
<td>5.</td>
<td>98.6</td>
<td>C-6</td>
</tr>
<tr>
<td>6.</td>
<td>164.2</td>
<td>C-7</td>
</tr>
<tr>
<td>7.</td>
<td>92.8</td>
<td>C-8</td>
</tr>
<tr>
<td>S.No.</td>
<td>δ-Value</td>
<td>Assignments</td>
</tr>
<tr>
<td>-------</td>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>8.</td>
<td>156.2</td>
<td>C-9</td>
</tr>
<tr>
<td>9.</td>
<td>103.8</td>
<td>C-10</td>
</tr>
<tr>
<td>10.</td>
<td>120.5</td>
<td>C-1'</td>
</tr>
<tr>
<td>11.</td>
<td>115.3</td>
<td>C-2'</td>
</tr>
<tr>
<td>12.</td>
<td>146.4</td>
<td>C-3'</td>
</tr>
<tr>
<td>13.</td>
<td>147.7</td>
<td>C-4'</td>
</tr>
<tr>
<td>14.</td>
<td>116.0</td>
<td>C-5'</td>
</tr>
<tr>
<td>15.</td>
<td>120.2</td>
<td>C-6'</td>
</tr>
<tr>
<td>16.</td>
<td>90.0</td>
<td>C-1''</td>
</tr>
<tr>
<td>17.</td>
<td>73.2</td>
<td>C-2''</td>
</tr>
<tr>
<td>18.</td>
<td>77.5</td>
<td>C-3''</td>
</tr>
<tr>
<td>19.</td>
<td>69.9</td>
<td>C-4''</td>
</tr>
<tr>
<td>20.</td>
<td>76.0</td>
<td>C-5''</td>
</tr>
<tr>
<td>21.</td>
<td>66.8</td>
<td>C-6''</td>
</tr>
</tbody>
</table>

**MASS SPECTRUM OF THE GLYCOSIDE AS-IV**

The important fragmentation peaks\(^7\) obtained in the mass spectrum (Fig. 5.7) of the glycoside AS-IV were at: m/z 464 [M\(^+\)], 302, 274, 153, 152, 151 and 124.

The different species obtained during the fragmentation are shown in the scheme II and further supported the identity of AS-IV as; 2, 5, 3', 4' tetra hydroxy isoflavone 7-O-β-D glucopyranoside (Isoquercetin 7-O-β-D-glucopyranoside).
FIG. 5.5: \(^1\text{H}-\text{NMR SPECTRUM OF THE GLYCOSIDE AS-IV}\)

**AS-IV**

**RSIC NO. 6568**

Current Date Parameters
- NAME: 6568.rsic
- ZAPNO: 10
- MODUAL: 1

**R2 - Acquisition Parameters**
- Date: 20040709
- Time: 21.38
- RESPH: 60 x 200
- HPRWM: 5 ms Dual 13
- PULPROG: R032
- TD: 32768
- SOLVENT: CH3OD
- NS: 16
- DS: 2
- DM: 430.073 Hz
- TR: 0.126214 sec
- TC: 3.9556642 sec
- TC: 84
- SW: 120.629 Hz
- D: 0.08 sec
- TE: 300.0 Hz
- D1: 1.00000000 sec

********** CHANNEL II **********

**UC**
- 10.00 usec

**RC**
- -4.00 deg

**PR**
- 200.131258 Hz

**R2 - Processing parameters**
- SI: 4335
- SF: 300 190097 Hz
- MCR: 18
- S1: 0
- LB: 0.30 Hz
- GB: 0
- AC: 1.00

**1D NMR plot parameters**
- CA: 20.00 cm
- TR: 14.670 sec
- TE: 293.85 Hz
- FP: -0.795 sec
- FT: -0.39 sec
- T0: 0.72279 sec/cm
- TCM: 154.60758 Hz/cm
FIG. 5.6: $^{13}$C-NMR SPECTRUM OF THE GLYCOSIDE AS-IV

AS-IV
RSIC NO: 0568

Current data Parameters
NAME 0568-FRSC
END 21

FD - Acquisition Parameters
Data 26500000
Time 11.90
INCUB 60000
POWO 15 ms (delay 10)
POLAR 160000 Hz
FD 0000 Hz
SOLVENT D2O
N1 0

-=-=-=- CHANNEL 11 -=-=
W001 1386
W1 0.00 Hz
W1 0.00 dB
W1 50.3282424 MHz

-=-=-=- CHANNEL 12 -=-=
W001 3000 Hz
W1 10000 Hz
W2 10.00 dB
W1 10.00 dB
W1 300 1300000 MHz

FD - Processing parameters
S1 32486
S2 50.328734 MHz
OM 10000 Hz
S2 10000 Hz
L0 1.00 Hz
L1 1.40

1D NMR data parameters
LS 30.00 cm
FD 215 000 Hz
F1 10075 30 Hz
F2 5.000 Hz
F1 10075 30 Hz
FD 5 050 Hz
F1 10075 30 Hz
F2 5 500 Hz
F1 10075 30 Hz
F2 25 500 Hz

Department of Chemistry, Dr. H.S. Gour University, Sagar
FIG. 5.7: MASS SPECTRUM OF THE GLYCOSIDE AS-IV

Mass Spectrum
Data File: 2EAO26AM
Sample: AS-4 PROF VK SAKERA SAGAR & 656B
RT 0.12" FAB(POS+) GC 1.4c BP: m/z 274.0000 Int. 31.5587 LV 0.00
Scan# (1 to 3)

[Graph showing mass spectrum with peaks at 124, 152, 151, 153, 274, 302, and 464]
EXPERIMENTAL

The flowers of *Tridax procumbens* Linn. (N.O. Compositae) were collected locally and identified by reputed taxonomist. The air dried and powdered flowers (2.0 Kg.) of *Tridax procumbens* Linn. were extracted with aqueous methanol. The ethanolic extract was concentrated under reduced pressure. The resultant brown mass was successively extracted with benzene, chloroform, acetone, ethyl acetate and methanol.

ISOLATION OF THE ISOFLAVONE GLYCOSIDE AS-IV

The ethyl acetate soluble part was concentrated under reduced pressure and the residue obtained was subjected to silica gel G column chromatography.

Elution was done with MeOH : H₂O in varying proportions. After the removal of the solvents a solid mass was obtained which was subjected to preparative TLC [ethyl acetate : acetic anhydride : acetic acid : water, (5 : 3 : 3 : 1)]. After developing the chromatogram in I₂, vapours a single homogeneous spot was observed due to a yellow crystalline compound obtained by the removal of solvent.

STUDY OF THE ELUATES FROM METHANOL : WATER (2 : 8)

Eluates from methanol : water (2 : 8) were collected and were found to have the same Rₛ values and so were combined together. on evaporation of the solvent, it gave a solid mass, which crystallised from methanol to get yellow crystals of compound AS-IV (yield 0.0993%). The compound AS-IV was found to be homogeneous on TLC examination using EtOAc : Ac₂O : AcOH : H₂O (5 : 3 : 1 : 1) as solvent system and I₂ vapours as visualizing agent.
COLUMN CHROMATOGRAPHY

The details of the column chromatography is tabulated below:-

<table>
<thead>
<tr>
<th>S.No</th>
<th>Parameter</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Length of column</td>
<td>100 cm</td>
</tr>
<tr>
<td>2.</td>
<td>Diameter of column</td>
<td>4.0 cm</td>
</tr>
<tr>
<td>3.</td>
<td>Weight of crude extract</td>
<td>4.0 g</td>
</tr>
<tr>
<td>4.</td>
<td>Weight of silica gel G</td>
<td>100 g</td>
</tr>
</tbody>
</table>

The results obtained in the column chromatography are recorded in table 5.8 :-

<table>
<thead>
<tr>
<th>S.No</th>
<th>Fraction</th>
<th>Eluants</th>
<th>Spot on TLC</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1 – 5</td>
<td>Methanol : Water (2 : 4)</td>
<td>Nil</td>
<td>No solid mass</td>
</tr>
<tr>
<td>2.</td>
<td>6 – 12</td>
<td>Methanol : Water (2 : 6)</td>
<td>Nil</td>
<td>No solid mass</td>
</tr>
<tr>
<td>3.</td>
<td>13 – 18</td>
<td>Methanol : Water (2 : 8)</td>
<td>One</td>
<td>Compound AS-IV</td>
</tr>
</tbody>
</table>

STUDY OF THE FRACTIONS (13–18)

The eluates obtained from methanol : water (2 : 8) were found to have same Rf values and hence were combined together. On evaporation of the solvent and crystallization from methanol it yielded yellow crystals of compound AS-IV. The compound AS-IV gave a single spot on TLC examination using EtOAc : Ac₂O : AcOH : H₂O (5 : 3 : 3 : 1) as solvent system and I₂ vapours as visualizing agent.

STUDY OF THE ISOFLAVONE GLYCOSIDE AS-IV

The glycoside AS-IV analysed for molecular formula C₂₁H₂₀O₁₂, m.p. 226-228°C and [M⁺] 464 (FABMS). It was found to be soluble in water, ethanol and methanol. It responded positive to all characteristic
colour reactions of isoflavones and also gave positive Molisch's test for glycoside.

1. It produced intense green colour with FeCl₃.
2. It gave red colour with Na-Hg/HCl.
3. It gave pink colour with Na-Hg/HCl.
4. It produced deep yellow colour with liquid ammonia and
5. It produced red-violet ring with Molisch's reagent.

Elemental analysis

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C₂₁H₂₀O₁₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 54.33%</td>
<td>C = 54.31%</td>
</tr>
<tr>
<td>H = 4.32%</td>
<td>H = 4.31%</td>
</tr>
</tbody>
</table>

Molecular weight 464 by FABMS

ACETYLYATION OF THE ISOFLAVONE GLYCOSIDE AS-IV

50 mg of AS-IV was taken in a 100 ml conical flask and mixed with 4ml of acetic anhydride and 25 mg of sodium acetate. The mixture was refluxed on a sand bath for about 10 hrs. After cooling the contents of the flask were transferred to a beaker filled with ice cold water, when the sticky mass settled down in the beaker which was separated by decantation. The mass was recrystallized with ethanol to an acetylated product (40 mg). It analysed for C₃₅H₃₄O₁₉, m.p. 241-243°C and [M⁺] 758 (for hepta acetate derivative as 5-OH was not acetylated).

Elemental analysis

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C₃₅H₃₄O₁₉</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 55.42%</td>
<td>C = 55.40%</td>
</tr>
<tr>
<td>H = 4.47%</td>
<td>H = 4.48%</td>
</tr>
</tbody>
</table>

Molecular weight 758 by FABMS
ACID HYDROLYSIS OF THE GLYCOSIDE AS-IV

250 mg of the glycoside AS-IV was taken with 30 ml of 7% H₂SO₄ in a round bottomed flask fitted with a reflux condenser. The reaction mixture was heated for about 8 hrs. on a water bath. The contents of the flask were extracted with water and aqueous layer was shaken with solvent ether in a separating funnel. The ethereal layer was separated, washed and dried over anhydrous sodium sulphate. After removal of solvent ether yellow crystals of aglycone AS-IV (A) were obtained.

The aqueous layer in the hydrolysate was worked up separately for the identification of sugar (s).

STUDY OF THE AGLYCONES AS-IV (A)

The aglycone AS-IV (A) analysed for molecular formula C₁₅H₁₀O₇, m.p 318-320°C and [M⁺] 302 (FABMS).

It gave all characteristic colour reactions of isoflavones described on page 170, it did not give Molisch's test.

Elemental analysis

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C₁₅H₁₀O₇</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 59.61%</td>
<td>C = 59.60 %</td>
</tr>
<tr>
<td>H = 3.32%</td>
<td>H = 3.31 %</td>
</tr>
</tbody>
</table>

**Molecular weight 302 by FABMS**

ALKALINE DEGRADATION OF AGLYCONES AS-IV (A)

50 mg of the aglycone AS-IV (A) was taken in B-14 ground joint flask and dissolved in 80 ml of ethanol and 20 ml of 50% KOH in ethanol was added. The mixture was refluxed for 6 hrs. and allowed to cool and acidified by HCl. Degraded products were extracted with ether. The ethereal layer was washed with water and separated into two parts:
(a) First part of the ethereal layer was treated with small amount of 50% NaHCO₃ solution yielding a compound, m.p. 182-183°C and identified as 3, 4 dihydroxy phenyl acetic acid (By mixed m.p. and CoTLC with authentic sample).

(b) Second part of the ethereal layer was treated with small amount of 1% NaOH solution when another compound was obtained. It was identified as phloroglucinol m.p. 216-217°C (By mixed m.p. and CoTLC).

PREPARATION OF ACETYLATED PRODUCT OF THE AGLYcone AS-IV (A)

30 mg of aglycone was mixed with 2.5 ml of acetic anhydride and 17.5 mg of sodium acetate and acetylated by the procedure described earlier.

STUDY OF THE ACETYLATED PRODUCT OF THE AGLYcone AS-IV (A)

Acetyl derivative analysed for C₂₅H₁₈O₁₁, m.p. 180-182°C and [M⁺] 470 (FABMS) for tetra acetate derivative [one of the hydroxyl groups (5 –OH) was not acetylated under the conditions].

Elemental analysis

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C₂₅H₁₈O₁₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 56.71%</td>
<td>C = 56.72 %</td>
</tr>
<tr>
<td>H = 3.83%</td>
<td>H = 3.82 %</td>
</tr>
</tbody>
</table>

Molecular weight 470 by FABMS

STUDY OF THE SUGAR HYDROLYSATE

The hydrolysate obtained after acid hydrolysis of glycoside AS-IV, was neutralised by adding BaCO₃ and BaSO₄, was filtered off. After
filtration the filtrate was concentrated to a syrupy mass, which was subjected to paper chromatography on Whatman filter paper No. 1 and aniline hydrogen phthalate was used as visualizing agent.

The solvent systems with corresponding $R_t$ values are tabulated in table-5.9:-

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Solvent system</th>
<th>$R_t$ values</th>
<th>Sugar identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Phenol, NH$_3$ (1% w/v)</td>
<td>0.38, 0.39</td>
<td>D-glucose</td>
</tr>
<tr>
<td>2.</td>
<td>s-collidine</td>
<td>0.40, 0.40</td>
<td>D-glucose</td>
</tr>
</tbody>
</table>

**PERMETHYLATION FOLLOWED BY HYDROLYSIS OF GLYCOSIDE AS-IV**

40 mg of the glycoside AS-IV was treated with methyl iodide (5 ml) and Ag$_2$O (20mg) in DMF (5ml) in a conical flask. The reaction mixture was left for about 48 hrs. at room temperature. The precipitate obtained was filtered and filtrate was treated with 7% H$_2$SO$_4$ when aglycone and methylated sugar were obtained.

The hydrolysate was neutralised by adding BaCO$_3$ and BaSO$_4$, was filtered off. After filtration the concentrated mass was examined by paper chromatography using B:A:W (4:1:5) as solvent system and aniline hydrogen phthalate as visualizing agent. The methylated sugar was identified as 2, 3, 4, 6 tetra-O-methyl D-glucose.

**PERIODATE OXIDATION OF THE GLYCOSIDE AS-IV**

The glycoside AS-IV (50mg) was dissolved in methanol (40ml) in a conical flask (100ml) fitted with a glass stopper. Sodium meta periodate (15ml) was then added and reaction mixture was left standing for 48 hrs.
Simultaneously, a blank experiment was run with the same procedure. The amount of sodium meta periodate consumed and formic acid liberated was estimated by Jone's method.

**ENZYMATIC HYDROLYSIS OF THE GLYCOSIDE AS-IV**

The glycoside AS-IV (30mg) was dissolved in ethanol (10ml) and mixed with enzyme Almond emulsion (25ml) in a 100 ml conical flask. The flask was allowed to stand for 8 hrs. at room temperature, and then filtered. The aglycone was identified as isoquercetin by super imposable spectral analysis. The sugar in the hydrolysate was identified as D-glucose by paper chromatography using Whatman filter paper No. 1 and B:A:W: (4:1:5) as solvent system (Rf 0.18).
CHAPTER-II: PART-B

STUDIES ON ANTIFUNGAL ACTIVITY OF THE COMPOUNDS AS-I, AS-II, AS-III AND AS-IV. *

* This work was done by courtesy of Dr. (Mrs.) A. Mehta, Senior Lecturer, Department of Botany, Dr. H.S. Gour University, Sagar (M.P.).
Fungal infection of plants have greater significance for tropical countries like India, where temperature and humidity are more favourable for their onset and persistence. A proper understanding of the mode of action of the fungitoxic compounds on the fungal pathogens will definitely be of great help in anticipating the efficacy of these compounds.

Fungal infection of plants play an important role in damages caused by pest to crop plants. Although numerous chemicals are fungicidal or fungistatic in vitro, many chemicals cause adverse effect on the host plant. The antifungal activity in plants may be due to their active components, which are present in them in combination with other substance of varied chemical nature. Several reports on the antifungal activity of some plant species have been reported by various workers, in the recent past. Most of the flavonoids and saponins which have been isolated from the plants have been found to have their fungitoxic activity and showing non toxicity to the host plant

*Nerium indicum* Linn. belongs to family Apocyanaceae and is commonly known as 'Kaner' in Hindi. *Tridax procumbens* Linn. belongs to family Compositae and is commonly known as “Ghamra” in Hindi. The details of these plants along with their medicinal importance have been reported in the earlier chapters of this thesis.

**ISOLATION OF THE COMPOUNDS**

Air dried and powdered flowers of *Nerium indicum* Linn. and *Tridax procumbens* Linn. were extracted with 95% hot ethanol, and the ethanolic
extract was concentrated under reduced pressure separately. The residues obtained were successively extracted with benzene, chloroform, acetone, ethyl acetate and methanol.

The different soluble fractions were subjected to column chromatography over silica gel G/alumina to get the compounds AS-I, AS-II, AS-III, and AS-IV. The description about these plants are given below in table-5.10.

**ANTIFUNGAL SCREENING OF THE COMPOUNDS**

The evaluation of antifungal activity was done by fitter paper disc method.\textsuperscript{56-63}

**Standard Drug:** The standard drug used in the present study was griseofulvin.

**Sample Preparation:** All the compounds and standard drug were made as 4 percent (w/v) solution in DMF. Then, two dilution 1:5 and 1:10 were made from this 4\% solution to test the antifungal activity of the compounds.

**Culture Media for the Study of Antifungal Activity of Compounds:**
In the present investigation the Sabourand's Dextrose Broth medium\textsuperscript{64} having the following composition was used-

\begin{align*}
\text{Agar-Agar} & = 20\text{gm} \\
\text{Peptone} & = 200\text{gm} \\
\text{Dextrose} & = 25\text{gm} \\
\text{Distilled water} & = 100\text{ml}
\end{align*}
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Plant</th>
<th>Part of the plant</th>
<th>Isolated compounds</th>
<th>Molecular formula</th>
<th>Melting point</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Nerium indicum</em> Linn.</td>
<td>Flowers</td>
<td>Kanerocin-3-O-β-D glucopyranosyl (1→4)-O-α-L arabino pyranosyl (28→1)-β-D-glucopyranosyl ester</td>
<td>C_{47}H_{74}O_{17}</td>
<td>270-271°C</td>
<td>AS-I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oleandrigenin 3-O-α-L-rhamnopyranoside</td>
<td>C_{31}H_{46}O_{10}</td>
<td>178-180°C</td>
<td>AS-II</td>
</tr>
<tr>
<td>2.</td>
<td><em>Tridax procumbens</em> Linn.</td>
<td>Flowers</td>
<td>β-Sitosterol-3-O-β-D-xylopyranoside</td>
<td>C_{34}H_{58}O_{5}</td>
<td>196-198°C</td>
<td>AS-III</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Isoquercetin 7-O-β-D-Glucopyranoside</td>
<td>C_{21}H_{20}O_{12}</td>
<td>226-228°C</td>
<td>AS-IV</td>
</tr>
</tbody>
</table>
Sterilization: The sterilization of cultural media, cultural tubes and other materials was done by autoclaving them at 15 lbs/sq dishes were sterilized by keeping them over night in an electrically heated air over at 140°C.

Test Organism: All the four compounds have been screened in vitro against the following fungi using griseofulvin as standard.

1. *Aspergillus niger*
2. *Penicillium digitatum*
3. *Fusarium oxysporum*
4. *Trichoderma viride*.

Preparation of Paper Discs: Discs of 6mm in diameter were prepared by Whatman filter paper No. 1. These discs were sterilized by heating at 140°C.

Preparation of Inoculum: The inoculum of organisms was prepared by transferring the corresponding organism from the stock culture into the sterile broth and incubated at 37°C for 72 hours. The organism was subcultured on the nutrient agar slants. The inoculum was shaken very well to break the colony. The organism was inoculated in the petridish containing nutrient media.

Preparation of Nutrient Plates: 4% (w/v) of the spore suspension of each broth cultured organism was mixed thoroughly in the sterilized nutrient agar media. 20 ml of this media was poured in each sterilized petridishes of 4 inch in diameter and allowed to solidify.
DETERMINATION OF ACTIVITY

The antifungal activities of the compounds AS-I, AS-II, AS-III, and AS-IV were determined by filter paper disc method. Sterilized filter paper discs were thoroughly moistened with the solutions of different compounds to be tested and then placed over the seeded medium with the help of well sterilized forceps. Then it was pressed so that every part of the disc comes in contact with the seeded medium. These seeded plates were then incubated at 37°C for 72 h. Same procedure was followed for the standard drug. Solvent DMF was also tested for its activity against all the tested organisms and found that it had no activity.

All the experiments were conducted in duplicate for each test sample. The average zones of inhibition were noted and are given in table-5.11:-

**TABLE-5.11**

Antifungal activity of the compounds

**AS-I, AS-II, AS-III, and AS-IV**

Diameters of Zone of inhibition (in mm)*

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Fungi species</th>
<th>AS-I 1:5</th>
<th>AS-1 1:10</th>
<th>AS-II 1:5</th>
<th>AS-II 1:10</th>
<th>AS-III 1:5</th>
<th>AS-III 1:10</th>
<th>AS-IV 1:5</th>
<th>AS-IV 1:10</th>
<th>Griseofulvin**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Aspergillus niger</em></td>
<td>9.5</td>
<td>8.5</td>
<td>11.5</td>
<td>10.5</td>
<td>7.5</td>
<td>6.0</td>
<td>3.5</td>
<td>2.5</td>
<td>14.5</td>
</tr>
<tr>
<td>2.</td>
<td><em>Penicillium digitatum</em></td>
<td>7.5</td>
<td>6.5</td>
<td>7.0</td>
<td>6.5</td>
<td>9.5</td>
<td>6.5</td>
<td>7.5</td>
<td>5.5</td>
<td>16.5</td>
</tr>
<tr>
<td>3.</td>
<td><em>Fusarium oxysporum</em></td>
<td>10.5</td>
<td>8.5</td>
<td>10.0</td>
<td>9.7</td>
<td>12.5</td>
<td>10.5</td>
<td>8.5</td>
<td>6.0</td>
<td>21.0</td>
</tr>
<tr>
<td>4.</td>
<td><em>Trichoderma viride</em></td>
<td>9.5</td>
<td>8.5</td>
<td>6.5</td>
<td>5.5</td>
<td>9.0</td>
<td>4.5</td>
<td>6.0</td>
<td>7.0</td>
<td>23.0</td>
</tr>
</tbody>
</table>

* The diameters of zone of inhibition taken as average of four determinations in four different directions.

** Griseofulvin used as standard antifungal agent.
RESULTS AND DISCUSSION

The compound AS-I was found to be more active against *Fusarium oxysporum* *Aspergillus niger*, and *Trichoderma viride* while minimum activity was observed against *Penicillium digitatum*. Similarly maximum activity of compound AS-II was observed against *Aspergillus niger* and *Fusarium oxysporum* whereas it was found to be minimum active against *Penicillium digitatum* and *Trichoderma viride*. The compound AS-III was found to be more active against *Fusarium oxysporum*, *Penicillium digitatum* and *Trichoderma viride* whereas it was found to have minimum activity against *Aspergillus niger*.

Compound AS-IV was observed to be more active against *Fusarium oxysporum* and *Penicillium digitatum* while minimum activity was found against *Trichoderma viride* and *Aspergillus niger*.

The various results are recorded in the table-5.11. These results concluded that all the compounds AS-I, AS-II, AS-III and AS-IV have considerable activity against most of the fungi, which were tested, the investigation thus revealed that the above compounds may potentially be used as fungitoxic agent against several fungi.

Thus, it is evident from the above study that the phytochemical constituents show very strong fungitoxic activity and this valuable property may be utilized for the cure of widely spread fungal diseases of varied nature.
CHAPTER-V: PART-C

STUDIES ON INSECTICIDAL SCREENING OF THE COMPOUNDS AS-I, AS-II, AS-III AND AS-IV.
Plants have a history of usage as folk remedies and are still used to kill or repel insects. Continued search of compounds concerning to pesticidal activity isolated from plants has revealed that some compounds which are extracted form plants and possess insecticidal properties are used to kill insects. To date, by far the most important contributions of natural products to commercial pesticides have been made by insecticides of plant origin.

Though synthetic pesticides are increasingly used to reduce or prevent the harmful pest, they can pose serious problems to crop plants. The pesticides will need to be highly potent, target pest specific and harmless to the environment. To make these features possible natural products and bio pesticides are being increasingly explored.

Working on the same line two pesticidal plants in *Nerium indicum* Linn. and (ii) *Tridax procumbens* Linn. were selected for their insecticidal efficacy.

**EXPERIMENTAL**

**ISOLATION OF THE COMPOUNDS**

Air dried and powdered flowers of *Nerium indicum* Linn. were extracted with of 95% ethanol and ethanolic extract was concentrated under reduced pressure. The residue obtained after concentration, was partitioned into petroleum ether, benzene, chloroform, acetone, ethyl acetates and methanol. The benzene soluble fraction when worked up yielded compound AS-I while ethyl acetate soluble fraction yielded compound AS-II.
Air dried and powdered flowers of *Tridax procumbens* Linn. were extracted with 95% ethanol. The concentrated ethanolic extract was successively extracted with various solvents. The methanol soluble fraction when worked up yielded compound AS-III, whereas ethyl acetate soluble fraction yielded compound AS-IV.

A brief account of the isolated compounds is tabulated in table-5.10.

**INSECTICIDAL SCREENING OF ISOLATED COMPOUNDS**

Insecticidal screening of the isolated compounds was carried out by residual film method. A detailed method is described below:-

1. **Test Insects:**

   Two common household insect pests used in the insecticidal screening were-

   (a) *Sitophilus oryzae* and  (b) *Periplaneta americana*.

2. **Test Sample Preparation:**

   All the four isolated compounds AS-I, AS-II, AS-III, and AS-IV were made as 1% (w/v) and 5% (w/v) solutions separately in solvent acetone: petroleum ether 60-80°C (4:1). These sample solutions were employed for testing insecticidal activity.

3. **Insecticidal Screening:**

   A contact bioassay by residual film method was used to test the toxicity of the compounds. The lethality of compounds 5%(w/v) in acetone: petroleum ether, 60-80°C (4:1) of *N. indicum* Linn. and *T. procumbens* Linn. were determined by residual film method. Thin uniform film of compounds were prepared in petridishes (10 cm diameter) separately. After evaporation of solvents 10 young adult insects of each
type were introduced into the petridishes. Knockdown activity of insects (i.e. the insects that no longer maintained normal posture and were unable to move or were on their backs) was recorded at 1 min. intervals until total mortality was achieved. Mortality was observed and recorded after the covered dishes were left standing for 24 hours. The mortality was determined when they did not respond to mechanical stimulation. Petridishes having acetone, methanol and no solvent were used as control dishes. This treatment was repeated three times.

RESULTS AND DISCUSSION

The tested compounds (at two different concentrations) possess definite insecticidal activity against insect pests *S. oryzae* and *P. americana*. The results of present investigation are summarized in table 5.12-5.15.

**TABLE-5.12**

Insecticidal activity of compound AS-I

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Insects</th>
<th>Groups</th>
<th>Knockdown (KD₉₀) in min.</th>
<th>Mortality% (after 24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>S. oryzae</em></td>
<td>(a) Compound AS-I 1% (w/v)</td>
<td>30.6+1.1*</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) Compound AS-I 5% (w/v)</td>
<td>20.8+1.2*</td>
<td>80%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(c) Control-1 (Acetone)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d) Control-2 (Methanol)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(e) Control-3 (No solvent)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td><em>P. americana</em></td>
<td>(a) Compound AS-I 1% (w/v)</td>
<td>56.1+1.4*</td>
<td>40%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) Compound AS-I 5% (w/v)</td>
<td>59.9+1.9*</td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(c) Control-1 (Acetone)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d) Control-2 (Methanol)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(e) Control-3 (No solvent)</td>
<td>0.0*</td>
<td>-</td>
</tr>
</tbody>
</table>

*After 24 hours mortality was observed.

*No mortality of the insect pests was found in any of the controls upto 100 hours.*
### TABLE-5.13

Insecticidal activity of compound AS-II

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Insects</th>
<th>Groups</th>
<th>Knockdown (KD₅₀) in min.</th>
<th>Mortality% (after 24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>S. oryzae</em></td>
<td>(a) Compound AS-II 1% (w/v)</td>
<td>35.6+1.8*</td>
<td>30%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) Compound AS-II 5% (w/v)</td>
<td>15.3+1.1*</td>
<td>60%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(c) Control-1 (Acetone)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d) Control-2 (Methanol)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(e) Control-3 (No solvent)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td><em>P. americana</em></td>
<td>(a) Compound AS-II 1%(w/v)</td>
<td>54.2+1.5*</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) Compound AS-II 5%(w/v)</td>
<td>59.7+1.2*</td>
<td>30%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(c) Control-1 (Acetone)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d) Control-2 (Methanol)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(e) Control-3 (No solvent)</td>
<td>0.0*</td>
<td>-</td>
</tr>
</tbody>
</table>

* After 24 hours mortality was observed.
* No mortality of the insect pests was found in any of the controls upto 100 hours.

### TABLE-5.14

Insecticidal activity of compound AS-III

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Insects</th>
<th>Groups</th>
<th>Knockdown (KD₅₀) in min.</th>
<th>Mortality% (after 24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>S. oryzae</em></td>
<td>(a) Compound AS-III 1% (w/v)</td>
<td>25.4+1.0*</td>
<td>30%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) Compound AS-III 5% (w/v)</td>
<td>20.6+1.4*</td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(c) Control-1 (Acetone)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d) Control-2 (Methanol)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(e) Control-3 (No solvent)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td><em>P. americana</em></td>
<td>(a) Compound AS-III 1%(w/v)</td>
<td>50.2+1.6*</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) Compound AS-III 5%(w/v)</td>
<td>58.9+1.2*</td>
<td>40%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(c) Control-1 (Acetone)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d) Control-2 (Methanol)</td>
<td>0.0*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(e) Control-3 (No solvent)</td>
<td>0.0*</td>
<td>-</td>
</tr>
</tbody>
</table>

* After 24 hours mortality was observed.
* No mortality of the insect pests was found in any of the controls upto 100 hours.
### TABLE-5.15

Insecticidal activity of compound AS-IV

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Insects</th>
<th>Groups</th>
<th>Knockdown (KD₅₀) in min.</th>
<th>Mortality% (after 24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>S. oryzae</em></td>
<td>(a) Compound AS-IV 1% (w/v)</td>
<td>17.5+1.3*</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) Compound AS-IV 5% (w/v)</td>
<td>15.3+1.2*</td>
<td>30%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(c) Control-1 (Acetone)</td>
<td>0.0*</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d) Control-2 (Methanol)</td>
<td>0.0*</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(e) Control-3 (No solvent)</td>
<td>0.0*</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td><em>P. americana</em></td>
<td>(a) Compound AS-IV 1%(w/v)</td>
<td>55.6+1.8*</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) Compound AS-IV 5%(w/v)</td>
<td>59.5+1.2*</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(c) Control-1 (Acetone)</td>
<td>0.0*</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d) Control-2 (Methanol)</td>
<td>0.0*</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(e) Control-3 (No solvent)</td>
<td>0.0*</td>
<td>–</td>
</tr>
</tbody>
</table>

* After 24 hours mortality was observed.
* No mortality of the insect pests was found in any of the controls upto 100 hours.

**Discussion:**

The results reveal that the compounds AS-I, AS-II, and AS-III produced significant knockdown activity (KD₅₀) for *S. oryzae* and *P. americana* at 5% (w/v) concentration. Maximum mortality was observed with AS-I, in both the concentrations for both the insect pests, whereas AS-IV was found to be least active to produce knockdown activity.

It can be concluded that the compounds AS-I and AS-II isolated from the flowers of *Nerium indicum* Linn. and AS-III isolated from the flowers of *Tridax procumbens* Linn. have definite insecticidal activity. Thus, they can have a very potent insecticidal effect and so, may potentially be explored for preventing many insect pests.
REFERENCES


