ISOLATION AND STRUCTURAL ELUCIDATION OF A NEW FLAVONE GLYCOSIDE: 5,7,3',4'-TETRAHYDROXY-6-METHOXY FLAVONE-7-O-α-L-RHAMOPYRANOSYL(1→2)-O-β-D-GALACTOPYRANOSIDE FROM THE SEEDS OF CASSIA FISTULA LINN.

This work has been published in Journal of Asian Natural Product Research, China.
**Cassia fistula** Linn\textsuperscript{1–3} belongs to Leguminosae family. It is commonly known as 'Amaltas' in Hindi. The detailed description of this plant has already been given in chapter-1 on page no. 10 of this thesis.

**PREVIOUSLY WORK DONE**

Earlier workers have isolated several compounds from the different parts of this plant, which have been given in Table-III on page no. 14 of this thesis.

The present chapter deals with the isolation and structural elucidation of a new flavone glycoside from the acetone soluble fraction of the pet-ether extract of this plant.

**EXTRACTION AND ISOLATION OF THE COMPOUND**

Air-dried and powdered seeds of the plant (3kg) were extracted with pet-ether (40-60\textdegree C) in a Soxhlet extractor for 6-7 days. The total defatted seeds of the plant were further successively extracted with benzene, chloroform, ethyl acetate, acetone and methanol.

The benzene, chloroform, ethyl acetate and methanol fractions on the removal of the solvent yielded very small quantity of the residue, hence these fractions were discarded.

The study of acetone soluble part has been described in this chapter.

**STUDY OF THE ACETONE SOLUBLE PART**

The acetone soluble part of the defatted seeds of this plant was concentrated under reduced pressure, to yield a dark brown syrupy mass, which was subjected to TLC examination showed three spots. It was therefore, purified by column chromatography over Si-gel 'G' using EtOAc : MEOH in various proportions.

**STUDY OF THE ELUATES FROM EtOAc : MEOH (8:2)**

Eluates obtained from the (1-7) fractions were found to have the same \textit{R}_f values and hence mixed together. On evaporation of the
solvent, an amorphous compound CF was obtained which was recrystallized from methanol to yield pale yellow needles (2.04gm).

**STUDY OF COMPOUND CF**

It was analysed for m.f. C\textsubscript{28}H\textsubscript{32}O\textsubscript{16}, m.p. 252-254\textdegree C and [M]\textsuperscript{+} 624 (EIMS).

It responded to positive Molisch test for glycoside and all the characteristic colour reactions of flavonoids.\textsuperscript{4-6}

**UV SPECTRUM OF THE (CF)**

Its UV spectrum showed wavelengths of maximum absorption with various shift reagents, are given in Table-I

<table>
<thead>
<tr>
<th>S.No</th>
<th>Solvent + (Shift-reagents)</th>
<th>(\lambda_{\text{max}}) Values (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Band III</td>
</tr>
<tr>
<td>1.</td>
<td>MeOH</td>
<td>223</td>
</tr>
<tr>
<td>2.</td>
<td>MeOH+AlCl\textsubscript{3}</td>
<td>277</td>
</tr>
<tr>
<td>3.</td>
<td>MeOH+ AlCl\textsubscript{3}-HCl</td>
<td>294</td>
</tr>
<tr>
<td>4.</td>
<td>MeOH+NaOMe</td>
<td>277</td>
</tr>
<tr>
<td>5.</td>
<td>MeOH+NaOAC</td>
<td>-</td>
</tr>
</tbody>
</table>

**IR SPECTRUM OF THE GLYCOSIDE (CF)**

The significant peaks obtained in the IR spectrum (Fig.1) of the glycoside and the structural units inferred with the help of available literature\textsuperscript{7-8} are recorded in Table-II.

**TABLE-II**

**IR SPECTRUM OF GLYCOSIDE (CF)**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Wave Number Cm\textsuperscript{-1}</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3540</td>
<td>-OH group (s)</td>
</tr>
<tr>
<td>2</td>
<td>2945</td>
<td>-CH stretching vibration</td>
</tr>
<tr>
<td>3</td>
<td>2865</td>
<td>-OMe group (s)</td>
</tr>
<tr>
<td>4</td>
<td>1622</td>
<td>(\alpha,\beta)-Unsaturated C=O group</td>
</tr>
<tr>
<td>5</td>
<td>1535</td>
<td>Aromatic ring system</td>
</tr>
<tr>
<td>6</td>
<td>1260</td>
<td>C-O-C-stretching vibration</td>
</tr>
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<td>7</td>
<td>1116</td>
<td>C-O-C-bending vibration</td>
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<td>8</td>
<td>1025</td>
<td>O-glycosidic linkage</td>
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<tr>
<td>9</td>
<td>875</td>
<td>Two adjacent 'C' atoms in benzene ring</td>
</tr>
<tr>
<td>10</td>
<td>820</td>
<td>Two adjacent 'H' atoms in benzene ring</td>
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</tbody>
</table>
Fig. 1: IR SPECTRUM OF COMPOUND CF
PRESENCE OF -OH GROUP (S) IN GLYCOSIDE (CF)

A peak at $v_{\text{Max}}^{\text{KBr}} 3540 \text{ cm}^{-1}$ in the IR spectrum of the glycoside CF, revealed the presence of hydroxyl groups in it. On acetylation with Ac$_2$O/Pyridine, it yielded an acetyl derivative (CF-II), molecular formula C$_{46}$H$_{50}$O$_{25}$, m.p. 172-173°C and [M]$^+$ 1002. The percentage of the acetyl group (38.62%) was determined by Weisenberger method$^9$ as described by Belcher and Godbert$^{10}$ indicating the presence of nine acetylatable hydroxyl groups in the glycoside.

PRESENCE OF -OME GROUP (S) IN GLYCOSIDE (CF)

In the IR spectrum of CF, a peak at $v_{\text{Max}}^{\text{KBr}} 2865 \text{ cm}^{-1}$ showed the presence of methoxy group in it. Estimation of methoxy group was carried out by Zeisel’s method$^{11}$, which confirmed the presence of one methoxy group in it.

ACID HYDROLYSIS OF THE GLYCOSIDE (CF)

On acid hydrolysis of glycoside CF with 9% H$_2$SO$_4$ solution, yielded aglycone (CF-I), and sugar moiety (ies) which were separated by filtration and studied separately.

STUDY OF THE AGLYCONDE (CF-I)

It was crystallized from ethanol as a light brown amorphous compound. It was analysed for m.f. C$_{16}$H$_{12}$O$_7$, m.p. 218-220°C and [M]$^+$ 316 (EIMS). It gave all the characteristic colour reactions of flavon-oids$^{4-6}$.

UV SPECTRUM OF THE AGLYCONDE (CF-I)

UV spectrum of the aglycone showed wavelengths of maximum absorbance with various shift reagents are recorded in Table-III
TABLE-III

<table>
<thead>
<tr>
<th>S. No</th>
<th>SOLVENT + (Shift Reagents)</th>
<th>+ ( \lambda_{\text{max}} ) Values (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Band III</td>
</tr>
<tr>
<td>1</td>
<td>MeOH</td>
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<td>274</td>
</tr>
<tr>
<td>3</td>
<td>MeOH+AlCl₃-HCl</td>
<td>290</td>
</tr>
<tr>
<td>4</td>
<td>MeOH+NaOMe</td>
<td>274</td>
</tr>
<tr>
<td>5</td>
<td>MeOH+NaOAC</td>
<td>-</td>
</tr>
</tbody>
</table>

IR SPECTRUM OF THE AGLYCONE (CF-I)

The prominent peaks observed in the IR spectrum of **CF-I** (Fig. 2) and the structural units inferred with the help of available literature ¹²⁻¹³ are recorded in **Table-IV**

TABLE-IV

<table>
<thead>
<tr>
<th>S. No</th>
<th>Wave Number Cm⁻¹</th>
<th>Assignment</th>
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<tbody>
<tr>
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<td>3542</td>
<td>-OH group (s)</td>
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<td>2.</td>
<td>2943</td>
<td>-CH stretching vibration</td>
</tr>
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<td>3.</td>
<td>2862</td>
<td>-OMe group</td>
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<td>4.</td>
<td>1620</td>
<td>( \alpha,\beta )-Unsaturated C=O group</td>
</tr>
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<td>5.</td>
<td>1538</td>
<td>Aromatic ring system</td>
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<tr>
<td>6.</td>
<td>1265</td>
<td>C-O-C stretching vibration</td>
</tr>
<tr>
<td>7.</td>
<td>1120</td>
<td>C-O-C bending vibration</td>
</tr>
<tr>
<td>8.</td>
<td>873</td>
<td>Two adjacent 'C' atoms in benzene ring</td>
</tr>
<tr>
<td>9.</td>
<td>823</td>
<td>Two adjacent 'H' atoms in benzene ring</td>
</tr>
</tbody>
</table>

PRESENCE OF -OH GROUP (S) IN AGLYCONE

A peak at \( \nu_{\text{Max}}^\text{KBr} \) 3542 cm⁻¹ in the IR spectrum of **CF-I** suggested the presence of hydroxyl groups in it. On acetylation with Ac₂O/Pyridine it formed an acetyl derivative (**CF-III**) m.f. C₂₄H₂₀O₁₁, m.p. 137-139°C and [M]⁺ 484 (EIMS). The percentage of acetyl group (35.55%) was determined by Weisenberger's method⁹ as described by Belcher and Godbert¹⁰, which revealed the presence of four hydroxyl groups in it.
Fig. 2: IR SPECTRUM OF COMPOUND CF-1
PRESENCE OF OME GROUPS IN AGLYCONE (CF-I)

In the IR spectrum of CF-I, a significant peak obtained at $\nu_{\text{Max}}^\text{KBr} 2862$ cm$^{-1}$ showed the presence of methoxy group in it. Estimation of methoxy group by Zeitel's method$^{11}$ confirmed the presence of one methoxy group in it.

In the $^1$H-NMR spectrum of CF-I, a doublet at $\delta$ 3.83 integrating for three protons further confirmed the presence of one methoxy group in it.

On the basis of above facts, a tentative structure for the aglycone was assigned as (I)

![Chemical Structure](image)

(I)

ALKALINE DEGRADATION OF THE AGLYCONE (CF-I)

The aglycone on fusion with 30% ethanolic KOH$^{14}$, gave two products, identified as

(I). mono methoxy phloroglucinol (Ia) m.f. $C_7H_8O_4$, m.p. 179-180°C and $[M]^+$ 224

(II). 3,4-dihydroxy benzoic acid (Ib) m.f. $C_7H_6O_3$, m.p. 201-202°C and $[M]^+$ 128.
POSITION OF -OH GROUP(S) IN THE AGLYCON (CF-I)

(a) POSITION OF -OH GROUP AT C-5

(I) The aglycone (CF-I) gave a green colour with FeCl₃ solution showed the presence of -OH group at C-5 position¹⁵.

(II) The aglycone gave bright yellow colour with boric acid in the presence of citric acid indicated the presence of -OH group at C-5 position¹⁶.

(III) A bathochromic shift at 62 nm in band I with AlCl₃ (relative to MeOH)¹⁷ further confirmed the presence of -OH group at C-5 in ring A.

(IV) Formation of mono methoxy phloroglucinol on alkaline degradation of the aglycone confirming the presence of -OH groups at C-5 and C-7 position in the ring A.

(b) POSITION OF -OH GROUPS AT C-3',4'

(I) Formation of 3,4-dihydroxy benzoic acid (IIIb) on alkaline degradation of the aglycon (CF-I) suggested the presence of hydroxy group at C-3' and C-4' position in ring B of CF-I.

(I) A bathochromic shift of 13nm in band I with NaoMe (relative to MeOH) further confirmed the presence of -OH group at C-3' and C-4' position respectively in the ring B of CF-I¹⁸.

(II) A pink colour was produced on addition of Mg-HCl to the CF-I which turned blue on adition of NaHCO₃ solution thereby confirming the presence of free -OH group C-4' position¹⁹.

POSITION OF -OCH₃ GROUP(S) IN CF-I

(a) POSITION OF -OCH₃ GROUP AT C-6

(I) Absence of bathochromic shift with SrSO₄ indicated the presence of a methoxyl group at C-6 position²⁰ in ring A of the aglycone (CF-I).
(II) Alkaline degradation of the aglycone (CF-I) yielded Ib which indicated the location of methoxy group at C-6 position in CF-I.

(III) A chemical shift at $\delta3.83$ integrating for three protons intensity in the $^1$H-NMR spectrum of the acetyl derivative (CF-I) further confirming the position of -OMe group at C-6 position in ring A.

Keeping all the above facts together, the following structure (II) of the aglycone was assigned as 5,7,3',4'-tetra-hydroxy-6-methoxy flavone.

\[
\text{HO} \quad \text{OH} \\
\text{H}_3\text{CO} \quad \text{OH} \quad \text{K} \\
\text{OH} \quad \text{OH}
\]

$^1$H-NMR SPECTRUM OF THE ACETYL DERivative OF AGLYCONE (CF-I)

The chemical shifts observed in the $^1$H-NMR spectrum of the aglycone (Fig.3) and structural units inferred with the help of available literature$^{21-22}$ are given in Table-V, which further supported its identity as structure II

<table>
<thead>
<tr>
<th>S.No</th>
<th>$\delta$ Value</th>
<th>Pattern</th>
<th>J value Hz</th>
<th>No. of Protons</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
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<td>6.06</td>
<td>dd</td>
<td>17.2,12.8</td>
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<td>H-3</td>
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<td>2.35</td>
<td>s</td>
<td></td>
<td>3</td>
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</tr>
<tr>
<td>3</td>
<td>3.83</td>
<td>s</td>
<td></td>
<td>3</td>
<td>6-OMe</td>
</tr>
<tr>
<td>4</td>
<td>2.87</td>
<td>s</td>
<td></td>
<td>3</td>
<td>7-OAc</td>
</tr>
<tr>
<td>5</td>
<td>6.74</td>
<td>s</td>
<td></td>
<td>1</td>
<td>H-8</td>
</tr>
<tr>
<td>6</td>
<td>7.72</td>
<td>s</td>
<td></td>
<td>1</td>
<td>H-2'</td>
</tr>
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<td>2.79</td>
<td>s</td>
<td></td>
<td>3</td>
<td>3'-OAc</td>
</tr>
<tr>
<td>8</td>
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<td>s</td>
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<td>3</td>
<td>4'-OAc</td>
</tr>
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<td>9</td>
<td>7.19</td>
<td>d</td>
<td>8.4</td>
<td>1</td>
<td>H-5'</td>
</tr>
<tr>
<td>10</td>
<td>7.64</td>
<td>d</td>
<td>8.4</td>
<td>1</td>
<td>H-6'</td>
</tr>
</tbody>
</table>
Fig-3: $^1$H-NMR SPECTRUM OF ACETYL DERIVATIVE (CF-III) OF AGLYCONE
MASS SPECTRUM OF THE AGLYCONES (CF-I)

The electron impact mass spectrum of the aglycone showed following fragment ion peaks:

\[ [M]^+ \ 316[M^+-sugar \ moiety], \ 315 \ [M^+-H], \ 301 \ [M^+-H-CH_2], \ 286 \ [M^+-H-COCH_2], \ 183[A_2]^+, \ 182[A_1]^+, \ 154[A_1-CO]^+, \ 137[B_1]^+, \ 134 \ [B_2]^+, \ 110 \ [B_1^+-CO]. \]

The several species obtained during the fragmentation of aglycone (CF-I) are shown in scheme-I, which further confirmed the structure II of the aglycone.

STUDY OF SUGAR MOIETY (IES)

The aqueous hydrolysate obtained after acid hydrolysis of the glycoside (CF), was neutralized with BaCO₃ and BaSO₄ filtered off. The filtrate was concentrated under reduced pressure and examined by paper chromatography, confirmed the presence of D-galactose (Rₗ 0.16) and L-rhamnose (Rₗ 0.37) (by Co-PC and Co-TLC).

QUANTITATIVE ESTIMATION OF SUGAR

The quantitative estimation of sugar(s) in the glycoside was estimated by the procedure of Mishra and Rao²³, which revealed that both the sugars were present in the equimolar ratio.

PERIODATE OXIDATION OF THE GLYCOSIDE (CF)

The glycoside (CF) on sodium metaperiodate oxidation²⁴ consumed 3.02 moles of periodate with the liberation of 1.04 moles of formic acid, showed that both the sugars were present in pyranose form.

POSITION OF ATTACHMENT OF THE SUGAR TO THE AGLYCONE

The position of attachment of the sugar moiety (ies) to the aglycone (CF-I) at C-7 position in ring-A was confirmed by comparing the UV spectral data of aglycone and glycoside (CF) on the basis of the following facts.
SCHEME I

C_{16}H_{12}O_{7} m/z 316

C_{15}H_{9}O_{7} m/z 301

[M'-H-CH_{2}]^{-}

C_{16}H_{11}O_{7} m/z 315

[M'-H-CO]^{-}

C_{15}H_{10}O_{6} m/z 286

Pathway I: without H transfer

Pathway II: with H transfer

Fragment (C_{16}H_{12}O_{7} m/z 316)

RC DA

C_{8}H_{6}O_{5} m/z 182 [A_{1}]^{+}

C_{7}H_{6}O_{4} m/z 154 [A_{1}^{-}-CO]

C_{8}H_{7}O_{5} m/z 183 [A_{2}]^{+}

C_{9}H_{6}O_{2} m/z 134 [B_{2}]^{+}

C_{9}H_{6}O_{2} m/z 110 [B_{1}^{-}-CO]

C_{7}H_{5}O_{3} m/z 137 [B_{1}]^{+}
(I). The sugar moiety (ies) was attached at C-7 position because the aglycone yielded red colour with p-toluene sulphonic acid, where as glycoside did not give above test, this is the specific reaction for free-OH group at C-7 position in ring A$^{25}$. 

(II). UV spectra of aglycone showed a bathochromic shift of 32 nm in band I with NaOAC where as absent in glycoside (CF), which confirmed the attachment of sugar moiety at C-7 position in ring A$^{26}$.

Keeping all the above facts together, a tentative structure of the glycoside was assigned as (III).

![Chemical Structure](image)

**SEQUENCE OF THE SUGAR RESIDUE (S)**

On graded hydrolysis of the glycoside (CF) with Kiliani mixture$^{27}$ (HCl-HOAC-H$_2$O, 15:35:50) gave L-rhamnose first followed by D-galactose indicated that L-rhamnose was the terminal sugar and D-galactose was attached to the aglycone.

The isolation and study of two proaglycones **PCF-I** and **PCF-II**, which were produced by the partial hydrolysis of the glycoside with Kiliani mixture further supported the above sequence of the sugars in the glycoside. The two proaglycones were separated by column chromatography and examined separately.

**STUDY OF THE PROAGLYCONE (PCF-I)**

It was analysed for m.f. C$_{22}$H$_{22}$O$_{12}$, m.p. 213-215$^\circ$C and [M]+466 (EI-MS). On hydrolysis with 9% methanolic H$_2$SO$_4$, it yielded aglycone (CF-I) and D-galactose.
The proaglycone **PCF-I** on enzymatic hydrolysis with almond emulsin showed the presence of β-linkage between D-galactose and aglycone.

**PERMETHYLATION AND HYDROLYSIS OF THE PROAGLYCONOME (PCF-I)**

The proaglycone **PCF-I** on permethylation by Kuhn's method28 followed by acid hydrolysis yielded methylated aglycone identified as 7-hydroxy-5,6,3',4'-tetramethoxy flavone and methylated sugar which was identified as 2,3,4,6-tetra-O-methyl-D-galactose (Co-PC and Co-TLC) thereby revealing that C_1''-OH of the D-galactose was linked to the C-7 of aglycone and also suggested that D-galactose was present in pyranose form.

Thus the structure of the proaglycone **(PCF-I)** was assigned as **(IV)** 5,7,3',4'-tetrahydroxy-6-methoxy flavone-7-O-β-D-galactopyranoside.

![Chemical Structure](image)

**STUDY OF THE PROAGLYCONOME (PCF-II)**

It was analysed for m.f. C_{28}H_{32}O_{16}, mp 252-254°C and [M]^+ 624 (EIMS). On acid hydrolysis with 9% methanolic H_2SO_4, it gave aglycone identified as proaglycone **(PCF-I)** and L-rhamnose.

**PERMETHYLATION AND HYDROLYSIS OF THE PROAGLYCONOME (PCF-II)**

Permethylation of proaglycone **(PCF-II)** by Kuhn's method28 followed by acid hydrolysis gave methylated aglycone identified as 7-hydroxy-5,6,3',4'-tetramethoxy flavone and methylated sugars were identified as 3,4,6-tri-O-methyl-D-galactose and 2,3,4-tri-O-methyl-L-rhamnose (by Co-PC and Co-TLC) which showed that C_1''-OH of the L-rhamnose was linked to the C_2''-OH of the D-galactose. Thus
the intersugar glycosidic linkage (1→2) was found between both the sugars.

**ENZYMATIC HYDROLYSIS OF THE GLYCOSIDE(CF)**

The glycoside (CF) was hydrolysed with enzyme takadiastase gave proglycone PCF-I and L-rhamnose confirming the presence of α-linkage between L-rhamnose and proglycone PCF-I. The PCF-I on further hydrolysis with enzyme almond emulsin liberated aglycone(CF-I), and D-galactose (Rf 0.34) (by Co-PC), confirmed the presence of β-linkage between D-galactose and aglycone.

Therefore it was concluded that C1"'-OH of L-rhamnose was linked to C2"' -OH of D-galactose via α-linkage and C1"' -OH of D-galactose was attached with C7-OH of the aglycone through β-linkage.

On the basis of above discussions, the structure of the glycoside was assigned as (VI) 5,7,3',4'-tetrahydroxy-6-methoxy flavone-7-O-β-D-galactopyranosyl (1→2)-O-α-L-rhamnopyranoside.

![Structure of glycoside (VI)](image)

The above structure (V) of glycoside (CF) was further confirmed by its 1H-NMR, 13C-NMR and Mass spectral analysis.
$^1$H-NMR SPECTRUM OF ACETYL DERIVATIVE (CF-II) OF THE GLYCOSIDE (CF)

The chemical shift obtained in the $^1$H-NMR spectrum (Fig.4) of CF and structural units inferred with the help of available literature are recorded in Table-VI.

$^1$H-NMR SPECTRUM (300MHz,CDCls) OF ACETYL DERIVATIVE (CF-II) OF THE GLYCOSIDE (CF)

<table>
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<tr>
<th>S.No.</th>
<th>$\delta$ Value</th>
<th>Pattern</th>
<th>J value Hz</th>
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<td>d</td>
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<td>H-1&quot;</td>
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<td>11</td>
<td>5.47</td>
<td>d</td>
<td>6.2</td>
<td>1</td>
<td>H-2&quot;</td>
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<td></td>
<td>3</td>
<td>H-3&quot;, H-4&quot;, H-5&quot;</td>
</tr>
<tr>
<td>13</td>
<td>4.46</td>
<td>dd</td>
<td>2.2, 8.4</td>
<td>1</td>
<td>H-6&quot;</td>
</tr>
<tr>
<td>14</td>
<td>5.25</td>
<td>s</td>
<td></td>
<td>1</td>
<td>H-1&quot;&quot;</td>
</tr>
<tr>
<td>15</td>
<td>4.25</td>
<td>dd</td>
<td>2.2, 4.6</td>
<td>1</td>
<td>H-2&quot;&quot;</td>
</tr>
<tr>
<td>16</td>
<td>3.26-3.83</td>
<td>m</td>
<td></td>
<td>3</td>
<td>H-3&quot;&quot;, H-4&quot;&quot;, H-5&quot;&quot;</td>
</tr>
<tr>
<td>17</td>
<td>1.16</td>
<td>d</td>
<td>2.5</td>
<td>3</td>
<td>Rham-Me</td>
</tr>
<tr>
<td>18</td>
<td>2.16-2.32</td>
<td>m</td>
<td></td>
<td>18</td>
<td>Sugar-acetoxyls</td>
</tr>
</tbody>
</table>

$^{13}$C-NMR SPECTRUM OF THE GLYCOSIDE (CF)

The chemical shifts obtained in the $^{13}$C-NMR spectrum (Fig.5) of the glycoside CF and structural units inferred with the help of available literature are recorded in Table-VII which further confirmed the assigned structure (V) of the glycoside.
Fig-4: $^1$H-NMR SPECTRUM OF ACETYL DERIVATIVE (CF-II) OF COMPOUND CF
### TABLE-VII

**13C-NMR SPECTRUM (90MHz,DMSO-d$_6$) OF THE GLYCOSIDE(CF)**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>δ Value</th>
<th>Atom</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>149.3</td>
<td>C-2</td>
</tr>
<tr>
<td>2</td>
<td>108.2</td>
<td>C-3</td>
</tr>
<tr>
<td>3</td>
<td>177.4</td>
<td>C-4</td>
</tr>
<tr>
<td>4</td>
<td>152.5</td>
<td>C-5</td>
</tr>
<tr>
<td>5</td>
<td>135.0</td>
<td>C-6</td>
</tr>
<tr>
<td>6</td>
<td>164.5</td>
<td>C-7</td>
</tr>
<tr>
<td>7</td>
<td>95.8</td>
<td>C-8</td>
</tr>
<tr>
<td>8</td>
<td>154.2</td>
<td>C-9</td>
</tr>
<tr>
<td>9</td>
<td>107.2</td>
<td>C-10</td>
</tr>
<tr>
<td>10</td>
<td>125.0</td>
<td>C-1'</td>
</tr>
<tr>
<td>11</td>
<td>116.8</td>
<td>C-2'</td>
</tr>
<tr>
<td>12</td>
<td>146.9</td>
<td>C-3'</td>
</tr>
<tr>
<td>13</td>
<td>150.0</td>
<td>C-4'</td>
</tr>
<tr>
<td>14</td>
<td>116.9</td>
<td>C-5'</td>
</tr>
<tr>
<td>15</td>
<td>123.2</td>
<td>C-6'</td>
</tr>
<tr>
<td>16</td>
<td>97.9</td>
<td>C-1''</td>
</tr>
<tr>
<td>17</td>
<td>76.8</td>
<td>C-2''</td>
</tr>
<tr>
<td>18</td>
<td>77.5</td>
<td>C-3''</td>
</tr>
<tr>
<td>19</td>
<td>72.0</td>
<td>C-4''</td>
</tr>
<tr>
<td>20</td>
<td>77.2</td>
<td>C-5''</td>
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<tr>
<td>21</td>
<td>61.2</td>
<td>C-6''</td>
</tr>
<tr>
<td>22</td>
<td>111.2</td>
<td>C-1'''</td>
</tr>
<tr>
<td>23</td>
<td>71.2</td>
<td>C-2'''</td>
</tr>
<tr>
<td>24</td>
<td>69.8</td>
<td>C-3'''</td>
</tr>
<tr>
<td>25</td>
<td>73.0</td>
<td>C-4'''</td>
</tr>
<tr>
<td>26</td>
<td>67.9</td>
<td>C-5'''</td>
</tr>
<tr>
<td>27</td>
<td>21.0</td>
<td>C-6'''</td>
</tr>
<tr>
<td>28</td>
<td>58.8</td>
<td>O-Me</td>
</tr>
</tbody>
</table>

### MASS SPECTRUM OF GLYCOSIDE (CF)

The prominent fragment ion peaks observed in the EIMS of CF are given below:


The different species obtained during the fragmentation are shown in Scheme-II, which further confirmed the above assigned structure (V) of glycoside (CF).
EXPERIMENTAL

The seeds of the *Cassia fistula* Linn. were collected from the Bandari forest in Sagar Distt. (M.P.) and authenticated by the Taxonomist, Department of Botany, Dr. H.S. Gour University, Sagar, M.P., India.

ISOLATION OF THE COMPOUND

Air-dried and powdered seeds (3kg) of *Cassia fistula* were extracted with 95% pet-ether in Soxhlet extractor for 7-8 days. The total defatted seeds of the plant was further successively partitioned with various solvents of increasing polarity such as benzene, chloroform, ethyl acetate, acetone and methanol.

The benzene, chloroform ethyl acetate and methanol soluble fractions were found in very small quantity on the removal of the solvent and hence can not be used for further chemical examinations. The study of acetone soluble part has been described in this chapter.

STUDY OF THE ACETONE SOLUBLE PART

The acetone soluble fraction of the defatted seeds of this plant was concentrated under reduced pressure to give a light brown syrupy mass (2.85gm) which was subjected to TLC examination using solvents (EtOAc-MeOH-H2O, 10:5:3) and I2 vapours as visualizing agent, gave three spots which were separated by preparative TLC and gave three compound **CF, CFa, CFb**. The quantity of the compounds **CFa** and **CFb** were found in very small quantity and hence rejected for further examination. Coumpound **CF** was purified by column chromatography over Si-gel 'G' using EtOAc:MeOH in various proportions. The details of the coloumn chromatography are given in Table-VIII.
(I). Length of the column : 55 cm
(II). Diameter of the column : 3.5 cm
(III). Weight of the crude extract : 2.85 gm
(IV). Weight of the Si-Gel G : 140 gm

TABLE-VIII

<table>
<thead>
<tr>
<th>S. No</th>
<th>Fraction No.</th>
<th>Eluants</th>
<th>Spot on TLC</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1-7</td>
<td>EtOAC:MeOH (8:2)</td>
<td>One</td>
<td>Compound(CF)</td>
</tr>
<tr>
<td>2.</td>
<td>8-14</td>
<td>EtOAC:MeOH (7:3)</td>
<td>One</td>
<td>Compound (CFa)</td>
</tr>
<tr>
<td>3.</td>
<td>15-21</td>
<td>EtOAC:MeOH (6:4)</td>
<td>One</td>
<td>Compound (CFb)</td>
</tr>
</tbody>
</table>

The eluants obtained from the fractions (8-14) and fractions (15-21) were found in very small quantity therefore it was not possible for further examination.

The fractions (1-7) collected were found to have same Rf values, hence combined together. After removal of the solvent, an amorphous compound was obtained which was recrystallized from methanol to yield compound CF as pale yellow needles (2.04 gm), which was found to be homogeneous on TLC examination.

STUDY OF THE COMPOUND (CF)

It was soluble in methanol and water. It was analysed for m.f. C28H32O16, m.p. 252-254°C and [M]+ 624 (EIMS).

It gave positive Molisch test for its glycosidic nature and following characteristic colour reactions of the flavonoids:

(I). Green colour with FeCl3,

(II). Pink colour with Mg/HCl.

(III). Orange red colour with Zn/HCl
ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C_{28}H_{32}O_{16}</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 53.83%</td>
<td>C = 53.84%</td>
</tr>
<tr>
<td>H = 5.12%</td>
<td>H = 5.13%</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>624 (by EIMS)</td>
</tr>
</tbody>
</table>

ACETYLATION OF THE GLYCOSIDE (CF)

75mg of glycoside was treated with acetic anhydride (5ml) and pyridine (3.5ml) in a 150 ml round bottomed flask for about 9-10 hours at 110-115°C. The total reaction mixture was added in ice cold water when a white precipitate was produced. Which was extracted with solvent ether in a separating funnel. The ethereal layer was washed with anhydrous sodium bicarbonate and ether was removed by evaporation.

It was analysed for molecular formula C_{46}H_{50}O_{25}, m.p.172-173°C and [M]+ 1002 (EIMS).

ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C_{46}H_{50}O_{25}</th>
</tr>
</thead>
<tbody>
<tr>
<td>C=55.08%</td>
<td>C=55.09%</td>
</tr>
<tr>
<td>H=4.98%</td>
<td>H=4.99%</td>
</tr>
<tr>
<td>Acetyl group= 38.65%</td>
<td>Acetyl group=38.62%</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>1002 (by EIMS)</td>
</tr>
</tbody>
</table>

ACID HYDROLYSIS OF THE GLYCOSIDE (CF)

150 mg of compound CF was refluxed with 10 ml of 9% methanolic H_2SO_4 in a 100 ml RB flask fitted with an air condensor on a water bath for 8-9 hours. The contents were deposited as a crystalline product on cooling which was separated by filtration. The aglycone and hydrolysate were studied separately.

STUDY OF THE AGLYCOME (CF-I)

It was crystallized from ethanol as a light brown amorphous compound, which was found to be homogeneous on TLC examina-
tion. It was analysed for m.f. C₁₆H₁₂O₇, m.p. 218-220⁰C and [M]⁺ 316 (EIMS). It showed following colour reactions for flavonoidal nature.

(I). Green colour with FeCl₃
(II). Orange red colour with Zn/Hcl
(III). Pink colour with Mg/HCl

ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C₁₆H₁₂O₇</th>
</tr>
</thead>
<tbody>
<tr>
<td>C=60.07%</td>
<td>C=60.08%</td>
</tr>
<tr>
<td>H=3.78%</td>
<td>H=3.79%</td>
</tr>
</tbody>
</table>

Molecular weight 316 (by EIMS)

ACETYLATION OF THE AGLYCOME (CF-I)

The acetylation of the compound CF-I was done in a similar procedure as described for glycoside CF on page no. 45 of this thesis. It was analysed for molecular formula C₂₄H₂₀O₁₁, m.p. 137-139⁰C and [M]⁺ 484 (EIMS).

ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C₂₄H₂₀O₁₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>C=59.49%</td>
<td>C=59.50%</td>
</tr>
<tr>
<td>H=4.12%</td>
<td>H=4.13%</td>
</tr>
</tbody>
</table>

Acetyl group= 35.56%  Acetyl group= 35.55%
Molecular weight 484 (by EIMS)

ESTIMATION OF METHOXY GROUP IN THE AGLYCOME

The estimation of methoxy groups was carried out by Zeisel's method. About 25 mg of the aglycone CF-I, 0.4 gm of A.R. Phenol, 3 ml of propionic anhydride and a few small carborundum chips were taken in a 150 ml round bottomed flask. About 10 ml of A.R. hydroiodic acid was added to it. The neck of the flask was connected to the source of CO₂ and rate of passage of CO₂ adjusted. The contents were heated on oil bath for 6-7 hours gradually, and cooled.
The total reaction mixture was transferred to a 250ml conical flask containing 10 ml of 25% sodium acetate solution and (85%) bromine. A.R. formic acid was then added drop by drop to the contents till smell of bromine was destroyed. The reaction mixture was diluted to 100 ml and 1 gm of A.R. KI and 6 ml of 10% H₂SO₄ were added to it. The contents were shaken and allowed to stand for 10-12 minutes. The liberated iodine was titrated against 0.05N sodium thiosulphate solution using starch as an indicator.

**ALKALINE DEGRADATION OF THE AGLYCONE (CF-I)**

70 mg. of the aglycone CF-I was treated with 10ml of ethanol and 15ml of 50% KOH in a 250 ml round bottomed flask on water bath. The reaction mixture was allowed to cool and then acidified with dil. HCl. The contents were further extracted with solvent ether in a separating funnel. The ethereal layer was washed with water and divided into two portions.

(I). The first portion was treated with 50% NaHCO₃ solution and the aqueous part on acidification yielded a compound (Ib). It was analysed for 3,4-dihydroxy benzoic acid (Ib) m.f. C₇H₆O₃ m.p. 201-202°C and [M]+ 128.

(II). The second portion of the ethereal part was treated with 10% NaOH solution and the aqueous part on acidification gave another compound (Ia). It was analysed for mono methoxy phloroglucinol (Ia) m.f. C₇H₈O₄, m.p. 179-180°C and [M]+ 224.

**PARTIAL HYDROLYSIS OF THE GLYCOSIDE (CF)**

The glycoside (200mg) was hydrolysed with Kiliani mixture (HCl-OHAc-H₂O; 15:35:50) in a 100 ml round bottomed flask fitted with an air condensor. The reaction mixture was left at room temperature for a week and then extracted with n-butanol. The n-butanol soluble part on paper chromatography examination showed two spots.
The n-butanol soluble portion was concentrated under reduced pressure and subjected to column chromatography over Si-Gel ‘G’ using chloroform and methanol in various proportions. The details of column chromatography are given in Table-IX.

COLUMN CHROMATOGRAPHY

(I). Length of the column - 55cm
(II). Diameter of the column - 3.5cm
(III). Weight of the butanol soluble part - 145mg
(IV). Weight of the Si-gel G - 140gm

<table>
<thead>
<tr>
<th>S.N o</th>
<th>Fraction No.</th>
<th>Eluants</th>
<th>Spot on TLC</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-5</td>
<td>CHCl3 : MeOH(6:2)</td>
<td>One</td>
<td>PCF-I</td>
</tr>
<tr>
<td>2</td>
<td>6-12</td>
<td>CHCl3 : MeOH(6:3)</td>
<td>Two</td>
<td>PCF-I&amp;PCF-II</td>
</tr>
<tr>
<td>3</td>
<td>13-19</td>
<td>CHCl3 : MeOH(6:4)</td>
<td>One</td>
<td>PCF-II</td>
</tr>
</tbody>
</table>

STUDY OF THE FRACTIONS (1-5)

The eluants obtained from the fractions (1-5) were found to have same Rf values and hence mixed together. On evaporation of the solvent, it yielded proaglycone (PCF-I).

STUDY OF THE PROAGLYCONE PCF-I

It was crystallized from methanol and was analysed for molecular formula C_{22}H_{22}O_{12}, m.p. 213-215°C and [M]+ 466 (EIMS).

ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C_{22}H_{21}O_{12}</th>
</tr>
</thead>
<tbody>
<tr>
<td>C=54.08%</td>
<td>C=54.09%</td>
</tr>
<tr>
<td>H=4.72%</td>
<td>H=5.73%</td>
</tr>
</tbody>
</table>

Molecular weight 466 (by EIMS)
PERMETHYLATION AND HYDROLYSIS OF THE PROAGLYCONE (PCF-I)

50mg of the proaglycone was dissolved in 5 ml of DMF and treated with 5 ml of Mel and 12 mg of Ag₂O in a 150ml conical flask and the contents were stirred for 24 hours at room temperature, and filtered. The reaction mixture was washed with DMF. The filtrate was concentrated under reduced pressure and hydrolysed by refluxing with 10% ethanolic H₂SO₄ to give the methylated aglycone identified as 7-hydroxy-5,6,3',4'-tetramethoxy flavone.

The aqueous hydrolysate obtained after the removal of aglycone was neutralised with BaCO₃ and BaSO₄ filtered off. The filtrate was concentrated and subjected to paper chromatography examination on Whatman filter paper no.1 using B:A:W (4:1:5) as solvent system and aniline hydrogen pthalate as detecting reagent. The methylated sugar was identified as 2,3,4,6-teta-O-methyl- D-galactose (Co-PC and Co-TLC)

STUDY OF THE FRACTIONS (13-19)

The fractions (13-19) were found to have the same Rf values and so combined together. It yielded compound (PCF-II) on evaporation of the solvent. It was analysed for m.f. C₂₈H₃₂O₁₆, m.p. 252-254°C and [M]+ 624 (EIMS).

ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C₂₈H₃₂O₁₆</th>
</tr>
</thead>
<tbody>
<tr>
<td>C=53.83%</td>
<td>C=53.84%</td>
</tr>
<tr>
<td>H=5.12%</td>
<td>H=5.13%</td>
</tr>
</tbody>
</table>

Molecular weight 624(by EIMS)
PERMETHYLATION AND HYDROLYSIS OF THE PROAGLYCONE (PCF-II)

Permethylation and hydrolysis of the proaglycone PCF-II was carried out in the similar method as described for PCF-I. It yielded methylated aglycone identified as 7-hydroxy-5,6,3',4'-tetra-methoxy flavone and methylated sugars which were identified as 2,3,4-tri-O-methyl-L-rhamnose and 3,4,6-tri-O-methyl-D-galactose (by Co-PC and Co-TLC).

IDENTIFICATION OF SUGAR AFTER HYDROLYSIS

The aqueous hydrolysate obtained after the acid hydrolysis of CF was neutralized with BaCO₃ and BaSO₄ filtered off. The filtrate was concentrated and subjected to paper chromatography examination on Whatman filter paper no. 1 using following solvent systems and aniline hydrogen phthalate as detecting agent. The results are recorded in Table-X.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Solvent System</th>
<th>R_f values</th>
<th>Sugar Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reported⁳²</td>
<td>Found</td>
</tr>
<tr>
<td>1</td>
<td>n-BuOH-AcOH-H₂O</td>
<td>0.16</td>
<td>0.17 D-galactose</td>
</tr>
<tr>
<td></td>
<td>(4:1:5)</td>
<td>0.37</td>
<td>0.38 L-rhamnose</td>
</tr>
<tr>
<td></td>
<td>s-collidine</td>
<td>0.34</td>
<td>0.35 D-galactose</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.59</td>
<td>0.57 L-rhamnose</td>
</tr>
</tbody>
</table>

PRIODATE OXIDTION OF THE GLYCOSIDE (CF)

30 mg of the glycoside was dissolved in 25 ml of MeOH, and treated with 15 ml of sodium meta periodate for three days at room temperature in a 150 ml conical flask fitted with a glass stopper. Simultaneously a blank was also run in the similar way. The quantity of consumed sodium mataperiodate and liberated formic acid were estimated by Jone's method.
ENZYMATIC HYDROLYSIS OF THE GLYCOSIDE (CF)

The glycoside (35mg) was dissolved in 20 ml of methanol and mixed with equal volume of enzyme takadiastase yielded proaglycone PCF-I and sugar was identified as L-rhamnose (Rf 0.37) (Co-PC and Co-TLC).

On hydrolysis of PCF-I with 25 ml of enzyme almond emulsin in a 100 ml conical flask attached with a stopper. The contents were allowed to left for 48 hours at room temperature and then filtered. The aglycone was identified CF-I, m.p. 218-220°C. The hydrolysate was concentrated and subjected to C0-PC, revealing the presence of D-galactose (Rf 0.16).
REFERENCE


