ISOLATION AND CHARACTERISATION OF A NOVEL FLAVONE GLYCOSIDE: 2', 5, 7-TRIHYDROXY-3, 6-DIMETHOXY FLAVONE-7-O-\(\beta\)-D-GALACTOPYRANOSYL-(1\(\rightarrow\)4)-O-\(\alpha\)-L-RHAMNOPYRANOSIDE FROM THE SEEDS OF ECHINOPS ECHINATUS ROXB.

This paper has been communicated for publication in "Research Journal of Chemistry and Environment", 2005
Echinops echinatus Roxb\textsuperscript{1-3} belongs to Compositae family. It is known as 'Gokru' in Hindi. The detailed description of this plant has already been given in chapter-I on page no. 20 of this thesis.

PREVIOUSLY WORK DONE

Various workers\textsuperscript{4-5} have isolated several compounds from this plant which have been reported in Table-III on page no. 23 of this thesis. Since there is no systematic phytochemical examinations have been done on the seeds of this plant and due to its important therapeutic importance, it was therefore thought worthwhile to carryout further investigations of seeds of this plant phytochemically.

EXTRACTION AND ISOLATION OF THE COMPOUND (SM)

Powdered air-dried seeds (3kg) of *Echinops echinatus* were extracted with 95% methanol by Soxhlet apparatus. The methanolic extract was concentrated under reduced pressure to a brown viscous mass (4.46 gm) which was successively partitioned with petroleum ether (60-80\textdegree C), benzene, chloroform, ethyl acetate, acetone and methanol.

The pet-ether, benzene, ethyl acetate, acetone and methanol soluble fractions on removal of the solvent yielded very small quantity of residue and hence discarded.

The study of the chloroform soluble fraction has been described in this chapter.

STUDY OF THE CHLOROFORM SOLUBLE PART

The chloroform soluble part of the methanolic extract of the seeds of this plant was concentrated under reduced pressure to give light brown viscous residue (3.38 gm), which was examined by TLC examination, gave single spot. It was purified by column chromatography over silica-gel ‘G’ eluting with MeOH:CHCl\textsubscript{3} in different proportions (10:4, 8:6, 6:8).

STUDY OF THE ELUATES FROM MeOH:CHCl\textsubscript{3}(10:4)

The eluates obtained from MeOH:CHCl\textsubscript{3} (10:4) were found to have same \(R_t\) values and hence combined together. On removed of the solvent, it yielded a light brownish needles compound SM (2.18 gm). It was analysed for molecular formula \(C_{29}H_{34}O_{16}\), m.p.256-258\textdegree C and [M]\textsuperscript{+} 638 (FABMS) and gave positive
response to Molisch test for glycoside and various characteristic colour reactions for flavonoids.

**UV SPECTRUM OF THE GLYCOSIDE (SM)**

The wave lengths of maximum absorbance obtained with various shift reagents are given in Table-I

<table>
<thead>
<tr>
<th>S.No</th>
<th>SOLVENT + (Shift Reagents)</th>
<th>+ $\lambda_{max}$ Values (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Band II</td>
</tr>
<tr>
<td>1</td>
<td>MeOH</td>
<td>270</td>
</tr>
<tr>
<td>2</td>
<td>MeOH+AlCl₃</td>
<td>272</td>
</tr>
<tr>
<td>3</td>
<td>MeOH+ AlCl₃-HCl</td>
<td>270</td>
</tr>
<tr>
<td>4</td>
<td>MeOH+NaOMe</td>
<td>274</td>
</tr>
<tr>
<td>5</td>
<td>MeOH+NaOAc</td>
<td>268</td>
</tr>
</tbody>
</table>

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

The significant peaks obtained in the IR spectrum (Fig. 1) of the glycoside (SM) and functional groups assigned to the molecule with the help of available literature are given 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>3445</td>
<td>-OH group(s)</td>
</tr>
<tr>
<td>2</td>
<td>2982</td>
<td>-CH stretching vibration</td>
</tr>
<tr>
<td>3</td>
<td>2872</td>
<td>-OMe group(s)</td>
</tr>
<tr>
<td>4</td>
<td>1620</td>
<td>$\alpha$, $\beta$-Unsaturated C=O group</td>
</tr>
<tr>
<td>5</td>
<td>1652</td>
<td>Aromatic ring system</td>
</tr>
<tr>
<td>6</td>
<td>1244</td>
<td>C-O-C stretching vibration</td>
</tr>
<tr>
<td>7</td>
<td>1138</td>
<td>C-O-C bending vibration</td>
</tr>
<tr>
<td>8</td>
<td>1058</td>
<td>O-glycosidic linkage</td>
</tr>
<tr>
<td>9</td>
<td>873</td>
<td>Two adjacent 'H' atoms</td>
</tr>
<tr>
<td>10</td>
<td>825</td>
<td>Two adjacent 'C' atoms in benzene ring</td>
</tr>
</tbody>
</table>
Fig. 1: IR Spectrum of the compound SM

03/12/06 11:16 smg
X: 4 scans, 4.0cm⁻¹, flat, smooth, abex
PRESENCE OF -OH GROUP(S) IN GLYCOSIDE (SM)

In the IR spectrum of glycoside SM, a peak obtained at $\nu_{\text{max}}^{\text{KBr}}$ 3445 cm$^{-1}$ showed the presence of hydroxyl group(s) in it. On acetylation with AC$_2$O/pyridine yielded an acetyl derivative SM-(a) m.f. C$_{45}$H$_{50}$O$_{24}$, m.p. 192-194°C and $[M]^+$ 974 (EIMS). The estimation of the acetyl groups (34.49%) in the acetylated product was estimated by Weisenberger’s method$^{11}$ as described by Belcher and Godbert$^{12}$ that suggested the presence of eight hydroxyl groups in the glycoside.

PRESENCE OF -OMe GROUP(S) IN GLYCOSIDE (SM)

In the IR spectrum of SM, a significant peak at $\nu_{\text{max}}^{\text{KBr}}$ 2872 cm$^{-1}$ revealed the presence of methoxy group(s) in it. The presence of methoxy group(s) was carried out by Zeisel’s method$^{13}$, which confirmed the presence of two methoxy groups in it.

ACID HYDROLYSIS OF THE GLYCOSIDE (SM)

Acid hydrolysis of the glycoside SM with 10 ml of 15% H$_2$SO$_4$ yielded a brownish needles of the aglycone (SM-A), which was separated by filtration and examined separately.

STUDY OF THE AGLYCONE (SM-A)

It was analysed for m.f. C$_{17}$H$_{14}$O$_7$, m.p. 180 -183°C and $[M]^+$ 330 (FABMS). It was crystallized from methanol to give brownish light needles (1.20 gm), which was found to be homogeneous on TLC examination. It gave pink colour with Mg/HCl, green colour with FeCl$_3$ and all the significant colour reactions of flavonoids$^{6-8}$.

UV SPECTRUM OF THE AGLYCONE (SM-A)

The UV spectrum of the aglycone (SM-A) showed wave lengths of maximum absorbance with various shift reagents are recorded in 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 II</td>
</tr>
<tr>
<td>1</td>
<td>MeOH</td>
<td>272</td>
</tr>
<tr>
<td>2</td>
<td>MeOH+AlCl$_3$</td>
<td>271</td>
</tr>
<tr>
<td>3</td>
<td>MeOH+ AlCl$_3$-HCl</td>
<td>273</td>
</tr>
<tr>
<td>4</td>
<td>MeOH+NaOMe</td>
<td>276</td>
</tr>
<tr>
<td>5</td>
<td>MeOH+NaOAc</td>
<td>270</td>
</tr>
</tbody>
</table>

TABLE –III
IR SPECTRUM OF THE AGLYCON (SM-A)

The prominent peaks obtained in the IR spectrum of aglycone SM-A (Fig.2) and the structural units inferred with the help of available literature\textsuperscript{14-16} are recorded in Table-IV

**TABLE-IV**

IR SPECTRUM OF AGLYCON (SM-A)

<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>3446</td>
<td>-OH group(s)</td>
</tr>
<tr>
<td>2</td>
<td>2984</td>
<td>-CH stretching vibration</td>
</tr>
<tr>
<td>3</td>
<td>2872</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>1654</td>
<td>Aromatic ring system</td>
</tr>
<tr>
<td>6</td>
<td>1245</td>
<td>C-O-C stretching vibration</td>
</tr>
<tr>
<td>7</td>
<td>1139</td>
<td>C-O-C bending vibration</td>
</tr>
<tr>
<td>8</td>
<td>875</td>
<td>Two adjacent 'H' atoms</td>
</tr>
<tr>
<td>9</td>
<td>828</td>
<td>Two adjacent 'C' atoms in benzene ring</td>
</tr>
</tbody>
</table>

PRESENCE OF -OH GROUP (S) IN THE AGLYCON (SM-A)

In the IR spectrum of SM-A, a peak at $\nu_{\text{max}}^{\text{KBr}}$ 3446 cm\textsuperscript{-1} showed the presence of free hydroxyl group(s) in it. The presence of hydroxyl group(s) in the aglycone was further confirmed by the acetylation of SM-A with AC\textsubscript{2}O/pyridine which afforded an acetyl derivative SM-A(a) of aglycone SM-A, m.f. C\textsubscript{23}H\textsubscript{20}O\textsubscript{10}, m.p. 186-188\textdegree C and [M]\textsuperscript{+} 456 (EIMS). The percentage of acetyl group (27.63\%) was done by Weisenberger's\textsuperscript{11} method as described by Belcher and Godbert\textsuperscript{12}, which showed the presence of three hydroxy groups in it.

PRESENCE OF -OMe GROUP (S) IN THE AGLYCON (SM-A)

The IR spectrum of SM-A showed a peak at $\nu_{\text{max}}^{\text{KBr}}$ 2872 cm\textsuperscript{-1} showed the presence of methoxy group(s) in it. Estimation of methoxy group(s) has been carried out by Zeisel's method\textsuperscript{13} confirmed the presence of two methoxy groups in it.
Fig. 2: IR Spectrum of the Compound SM-A

03/12/06 11:16 smg
X: 4 scans, 4.0cm⁻¹, flat, smooth, abex
Two singlets at δ 3.85 and 3.99 integrating for three protons of each in the
$^1$H-NMR spectrum of acetylated aglycone SM-A(a) further confirmed the presence
of two methoxy groups in it.

On the basis of above evidences, a tentative structure of the aglycone
(SM-A) was assigned as I.

![Chemical structure of I](image)

**ALKALINE DEGRADATION OF THE AGLYCONE (SM-A)**

The aglycone (SM-A) on alkaline degradation with 40% ethanolic KOH
furnished two compounds, which were identified as

(I) Mono-methoxy phloroglucinol (Ia) m.f. C$_7$H$_6$O$_4$, m.p. 217-220$^\circ$C and [M]$^+$
156 (EIMS) (by m.m.p. Co-PC).

(II) Salicylic acid (Ib) m.f. C$_7$H$_6$O$_3$, m.p. 156-158$^\circ$C and [M]$^+$ 138 (EIMS)
(by m.m.p., Co-PC).

Aglycone SM-A $\xrightarrow{40\% \text{ ethanolic KOH}}$ HO

![Chemical structures of Ia and Ib](image)

**POSITION OF -OH GROUP (S) IN AGLYCONE (SM-A)**

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

(I) When aglycone (SM-A) was treated with FeCl$_3$, a green colour was produced
which showed the presence of –OH group at C-5 position$^{17}$.

(II) The aglycone gave bright yellow colour with boric acid in the presence of
citric acid revealed the presence of –OH group at C-5 position$^{18}$.

(III) A bathochromic shift at 22 nm in band I with AlCl$_3$ (relative to MeOH) which
remained unchanged an addition of HCl, further confirmed the presence of
–OH group at C-5 position$^{19-20}$.
(IV) A chemical shift at $\delta$ 175.6 for C-4 carbon atom in the $^{13}$C-NMR spectrum of (SM) confirmed the presence of –OH group at C-5 position$^{21}$.

(b) POSITION OF –OH GROUP AT C-7
(i) The solubility of the aglycone in 10% aqueous sodium carbonate showed the presence of –OH group at C-7$^{22}$.
(ii) A bathochromic shift at 32 nm in band I with NaOAc (relative to MeOH) confirmed the presence of –OH group at C-7 position$^{23}$.

(c) POSITION OF -OH GROUP AT C-2′
(i) Formation of 2-hydroxy benzoic acid on alkaline degradation of SM-A showed the presence of –OH group at C-2′ position.
(ii) A chemical shift at $\delta$ 155.6 in the $^{13}$C-NMR spectrum of the glycoside, further confirmed the –OH group at C-2′ position in ring B.

POSITION OF -OMe GROUP (S) IN AGLYGONE (SM-A)

(a) POSITION OF -OMe GROUP AT C-3
(i) In $^{13}$C-NMR spectrum, chemical shifts at $\delta$ 155 for C-2, 137.4 for C-3 and $\delta$ 175.6 for C-4 were indicative of the 3-O-methyl etherification$^{21}$.
(ii) The dark purple colour of the compound was produced under UV light further confirmed the substitution of methoxy group at C-3 in ring C$^{24}$.
(iii) In the UV spectrum of the aglycone (SM-A), no induced bathochromic shift in band II with AlCl$_3$ (relative to MeOH) was observed which clearly showed the presence of a methoxy group at C-3 position in ring C$^{25}$.

(b) POSITION OF -OMe GROUP AT C-6
(i) Absence of green precipitate with SrSO$_4$ indicated the presence of a methoxy group at C-6 position$^{26}$ in ring A of the aglycone SM-A.
(ii) No induced bathochromic shift in band II with AlCl$_3$-HCl (relative to MeOH), was observed in the UV spectrum of SM-A, further confirmed the presence of second methoxy group at C-6 position$^{27}$.

On the basis of all the above discussions, the structure of the aglycone was assigned as II.
\[ \text{II} \]

\(^1\)H-NMR SPECTRUM OF THE ACETYLATED DERIVATIVE SM-A (a) OF THE AGLYCONES (SM-A)

\(^1\)H-NMR spectrum (Fig. 3) of the acetylated derivative of the aglycone and the structural units inferred with the help of available literature\(^{28-29}\) are recorded in Table-V, which further supported the above assigned structure-II of the aglycone.

**TABLE-V**

\(^1\)H-NMR SPECTRUM (300MHz, CDCl\(_3\)) OF THE ACETYLATED DERIVATIVE SM-A(a) OF THE AGLYCONES (SM-A)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>(\delta) value</th>
<th>Pattern</th>
<th>J-value Hz</th>
<th>No. of proton</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.86</td>
<td>s</td>
<td></td>
<td>3</td>
<td>OMe-3</td>
</tr>
<tr>
<td>2</td>
<td>2.53</td>
<td>s</td>
<td></td>
<td>3</td>
<td>OAc-5</td>
</tr>
<tr>
<td>3</td>
<td>4.02</td>
<td>s</td>
<td></td>
<td>3</td>
<td>OMe-6</td>
</tr>
<tr>
<td>4</td>
<td>2.61</td>
<td>s</td>
<td></td>
<td>3</td>
<td>OAc-7</td>
</tr>
<tr>
<td>5</td>
<td>6.56</td>
<td>s</td>
<td>8.2</td>
<td>1</td>
<td>H-8</td>
</tr>
<tr>
<td>6</td>
<td>2.69</td>
<td>s</td>
<td>8.2</td>
<td>3</td>
<td>OAc-2'</td>
</tr>
<tr>
<td>7</td>
<td>7.26</td>
<td>d</td>
<td>8.2</td>
<td>1</td>
<td>H-3'</td>
</tr>
<tr>
<td>8</td>
<td>7.45</td>
<td>dt</td>
<td>8.2</td>
<td>1</td>
<td>H-4'</td>
</tr>
<tr>
<td>9</td>
<td>7.08</td>
<td>br t</td>
<td>8.2</td>
<td>1</td>
<td>H-5'</td>
</tr>
<tr>
<td>10</td>
<td>7.65</td>
<td>dd</td>
<td>8.2, 2.1</td>
<td>1</td>
<td>H-6'</td>
</tr>
</tbody>
</table>

**MASS SPECTRUM OF THE AGLYCONES (SM-A)**

The prominent fragment ion peaks observed in the FABMS of the aglycone are given below:

\([M^+]\) 330 \(\text{[M}^+\text{-sugar moity]}, \ 329 \ [M}^+\text{-H}], \ 315 \ [M}^+\text{-CH}_2], \ 302 \ [M}^+\text{-CO}], \ 299 \ [M}^+\text{-CH}_3], \ 182 \ [A}_1^+], \ 183 \ [A}_2^+], \ 154 \ [A}_1^+\text{-CO}], \ 121 \ [B}_1^+], \ 118 \ [B}_2^+], \ 94 \ [B}_1^+\text{-CO}].
Fig. 3: $^1$H-NMR spectrum of acetylated derivative SM-A(a) of aglycone SM-A
The different species obtained during fragmentation of aglycone are described in Scheme-I which were found to be in complete conformity with assigned Structure-II for aglycone.

STUDY OF THE SUGAR MOIETY (IES)

The hydrolysate obtained after the acid hydrolysis of the glycoside, was neutralized with BaCO$_3$ and BaSO$_4$ filtered off. The filtrate was concentrated and subjected to paper chromatography examination, showed the presence of D-galactose (R$_f$ 0.18) and L-rhamnose (R$_f$ 0.36) (by Co-PC).

QUANTITATIVE ESTIMATION OF SUGARS

The quantitative estimation of sugars in the glycoside was carried out by Mishra and Rao procedure$^{30}$, which showed that both the sugars were present in an equimolar ratio.

PERIODATE OXIDATION OF THE GLYCOSIDE (SM)

On oxidation of the glycoside SM with sodium meta periodate$^{31}$ consumed 3.03 moles of periodate and liberated 1.02 moles of formic acid, thereby suggesting the presence of one molecule each of D-galactose and L-rhamnose per molecule of the aglycone and also confirmed that both the sugars were present in pyranose form$^{32}$.

POSITION OF ATTACHMENT OF THE SUGAR TO THE AGLYCONES

The position of attachment of the sugar moiety(ies) to the aglycone (SM-A) was fixed at C-7 position by comparing the UV spectral data of aglycone and glycoside on the basis of the following facts.

(I) A bathochromic shift of 32 nm in band I on addition of NaOAc (relative to MeOH) in the UV spectrum of the aglycone (SM-A) confirmed the presence of free -OH at C-7 position. No such bathochromic shift was observed in the UV spectrum of the glycoside (SM) thereby suggesting that −OH group at C-7 position in SM was involved in the glycosidation.

(II) The glycoside (SM) on permethylation followed by acid hydrolysis gave a permethylated aglycone identified as 7-hydroxy-2', 3, 5, 6-tetramethoxy flavone, further confirmed that −OH at C-7 position was involved in the glycosidation.
On the basis of above facts, the following structure (III) was assigned for glycoside.

![Chemical structure](image)

(III)  \( R = \text{Sugar moiety} \)

**SEQUENCE OF THE SUGAR MOIETY (IES) IN THE GLYCOSIDE (SM)**

The glycoside (SM) on graded hydrolysis with Kiliani mixture (HOAc-HCl-H\(_2\)O; 35:15:50\(^{33}\)) liberated D-galactose first followed by L-rhamnose indicating that D-galactose was terminal sugar and L-rhamnose was attached to the aglycone.

The above sequence of sugars in the glycoside were further supported by the isolation and study of two proaglycones designated as PSM-I and PSM-II, which were obtained from the partial hydrolysis of the glycoside with Kiliani mixture. The two proaglycones were separated by column chromatography and studied separately.

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

It was analysed for m.f. C\(_{23}\)H\(_{24}\)O\(_{11}\), m.p. 232-234\(^{0}\)C and [M]\(^+\) 476 (FABMS). PSM-I on hydrolysis with 10% H\(_2\)SO\(_4\) gave the aglycone and L-rhamnose. Enzymatic hydrolysis of the proaglycone PSM-I with takadiastase, revealed the presence of \(\alpha\)-linkage between aglycone and L-rhamnose (R\(_f\) 0.36) (by Co-PC).

**PERMETHYLATION AND HYDROLYSIS OF THE PROAGLYCONE (PSM-I)**

The proaglycone PSM-I on permethylation by Kuhn's method\(^{34}\) followed by acid hydrolysis yielded the methylated aglycone identified as 7-hydroxy-2',3,5,6-tetramethoxy flavone and 2,3,4-tri-O-methyl-L-rhamnose (by Co-PC) thereby suggesting that C-1" of L-rhamnose was involved in the formation of glycosidic linkage and also showed that the L-rhamnose was present in the pyranose form.

Thus, the structure (IV) of the proaglycone PSM-I was assigned as 2',5,7-trihydroxy-3,6-dimethoxy flavone-7-O-\(\alpha\)-L-rhamnopyranoside.
STUDY OF THE PROAGLYCONE (PSM-II)

It was analysed for m.f. C_{29}H_{34}O_{16}, m.p. 256-258\degree C and [M]+ 638 (FABMS). PSM-II on acid hydrolysis with 10% H_{2}SO_{4} yielded aglycone (SM-A) and sugars, which were identified as D-galactose (R_{f} 0.18) and L-rhamnose (R_{f} 0.36) (by Co-PC).

PERMETHYLATION AND HYDROLYSIS OF THE PROAGLYCONE (PSM-II)

Permethylolation of the proaglycone PSM-II by Kuhn's method^{34} followed by acid hydrolysis yielded the methylated aglycone identified as 7-hydroxy-2',3,5,6-tetramethoxy flavone and methylated sugars which were identified as 2,3-di-O-methyl-L-rhamnose and 2,3,4,6-tetra-O-methyl-D-galactose according to Petek\(^{35}\) therefore it was concluded that C-1"''-OH of D-galactose was attached with C-4" "-OH of the L-rhamnose and C-1"'-OH of L-rhamnose was linked with C-7 -OH of the aglycone, thereby revealing the (1→4) inter linkage was found between D-galactose and L-rhamnose.

ENZYMATIC HYDROLYSIS OF THE GLYCOside (SM)

Enzymatic hydrolysis of the glycoside(SM) with almond emulsin yielded PSM-I and D-galactose (R_{f} 0.18) (by Co-PC) showed the presence of β-linkage between D-galactose and L-rhamnose. PSM-I on further hydrolysis with enzyme takadiastase gave aglycone and L-rhamnose (R_{f} 0.36) (by Co-PC) confirming the presence of α-linkage between L-rhamnose and aglycone.

On the basis of all the above facts, the following structure (V) of the glycoside (SM) was assigned as 2',5,7-trihydroxy-3, 6-dimethoxy flavone-7-O-β-D-galactopyranosyl-(1→4)-O-α-L-rhamnopyranoside.
\[ \text{V} \]

**$^1$H-NMR SPECTRUM OF THE ACETYLATED DERIVATIVE SM- (a) OF THE GLYCOSIDE (SM)**

The significant chemical shifts obtained in the $^1$H-NMR spectrum of the acetylated derivative of the glycoside (Fig.4) and the structural units inferred with the help of available literature\textsuperscript{36-37} are given in Table-VI

**TABLE-VI**

$^1$H-NMR SPECTRUM (300 MHz, CDCl$_3$) OF THE ACETYLATED DERIVATIVE SM- (a) OF THE GLYCOSIDE (SM)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>( \delta ) Value</th>
<th>Pattern</th>
<th>J value ( H_2 )</th>
<th>No. of protons</th>
<th>Assignment</th>
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<tbody>
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</tr>
<tr>
<td>2.</td>
<td>2.53</td>
<td>s</td>
<td></td>
<td>3</td>
<td>5-OAc</td>
</tr>
<tr>
<td>3.</td>
<td>3.99</td>
<td>s</td>
<td></td>
<td>3</td>
<td>6-OMe</td>
</tr>
<tr>
<td>4.</td>
<td>6.56</td>
<td>s</td>
<td></td>
<td>1</td>
<td>H-8</td>
</tr>
<tr>
<td>5.</td>
<td>2.69</td>
<td>s</td>
<td></td>
<td>3</td>
<td>OAc-2'</td>
</tr>
<tr>
<td>6.</td>
<td>7.32</td>
<td>d</td>
<td>8.2</td>
<td>1</td>
<td>H-3'</td>
</tr>
<tr>
<td>7.</td>
<td>7.56</td>
<td>dt</td>
<td>8.2, 2.1</td>
<td>1</td>
<td>H-4'</td>
</tr>
<tr>
<td>8.</td>
<td>7.06</td>
<td>br t</td>
<td>8.2</td>
<td>1</td>
<td>H-5'</td>
</tr>
<tr>
<td>9.</td>
<td>7.94</td>
<td>dd</td>
<td>8.2, 2.2</td>
<td>1</td>
<td>H-6'</td>
</tr>
<tr>
<td>10.</td>
<td>5.45</td>
<td>d</td>
<td>7.3</td>
<td>1</td>
<td>H-1''</td>
</tr>
<tr>
<td>11.</td>
<td>4.05-4.28</td>
<td>m</td>
<td></td>
<td>4</td>
<td>H-2'', H-3'', H-4'', H-5''</td>
</tr>
<tr>
<td>12.</td>
<td>1.16</td>
<td>d</td>
<td>2.5</td>
<td>3</td>
<td>Rham-Me-6''</td>
</tr>
<tr>
<td>13.</td>
<td>5.54</td>
<td>d</td>
<td>5.8</td>
<td>1</td>
<td>H-1'''</td>
</tr>
<tr>
<td>14.</td>
<td>4.62-5.37</td>
<td>m</td>
<td></td>
<td>4</td>
<td>H-2'', H-3'', H-4'', H-5''</td>
</tr>
<tr>
<td>15.</td>
<td>4.48</td>
<td>dd</td>
<td>2.2, 8.4</td>
<td>2</td>
<td>H-6'''</td>
</tr>
<tr>
<td>16.</td>
<td>2.16-2.32</td>
<td>m</td>
<td></td>
<td>18</td>
<td>sugar acetoxyls</td>
</tr>
</tbody>
</table>

\[ \text{CH}_3 \]
Fig. 4: ¹H-NMR spectrum of acetylated derivative SM-(a) of glycone SM
$^{13}$C-NMR SPECTRUM OF THE GLYCOSIDE (SM)

The significant chemical shifts observed in the $^{13}$C-NMR spectrum (Fig.5) of glycoside and structural units inferred with the help of available literature$^{36}$ are given in Table-VII.

TABLE-VII

$^{13}$C-NMR SPECTRUM (90MHZ, DMSO-d$_6$) OF THE GLYCOSIDE (SM)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>$\delta$ value</th>
<th>Atom</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>155.9</td>
<td>C-2</td>
</tr>
<tr>
<td>2</td>
<td>137.4</td>
<td>C-3</td>
</tr>
<tr>
<td>3</td>
<td>175.6</td>
<td>C-4</td>
</tr>
<tr>
<td>4</td>
<td>151.5</td>
<td>C-5</td>
</tr>
<tr>
<td>5</td>
<td>130.6</td>
<td>C-6</td>
</tr>
<tr>
<td>6</td>
<td>155.3</td>
<td>C-7</td>
</tr>
<tr>
<td>7</td>
<td>93.3</td>
<td>C-8</td>
</tr>
<tr>
<td>8</td>
<td>153.4</td>
<td>C-9</td>
</tr>
<tr>
<td>9</td>
<td>106.5</td>
<td>C-10</td>
</tr>
<tr>
<td>10</td>
<td>118.4</td>
<td>C-1'</td>
</tr>
<tr>
<td>11</td>
<td>155.6</td>
<td>C-2'</td>
</tr>
<tr>
<td>12</td>
<td>120.4</td>
<td>C-3'</td>
</tr>
<tr>
<td>13</td>
<td>133.5</td>
<td>C-4'</td>
</tr>
<tr>
<td>14</td>
<td>119.2</td>
<td>C-5'</td>
</tr>
<tr>
<td>15</td>
<td>129.8</td>
<td>C-6'</td>
</tr>
<tr>
<td>16</td>
<td>102.1</td>
<td>C-1''</td>
</tr>
<tr>
<td>17</td>
<td>72.2</td>
<td>C-2''</td>
</tr>
<tr>
<td>18</td>
<td>72.7</td>
<td>C-3''</td>
</tr>
<tr>
<td>19</td>
<td>73.7</td>
<td>C-4''</td>
</tr>
<tr>
<td>20</td>
<td>70.0</td>
<td>C-5''</td>
</tr>
<tr>
<td>21</td>
<td>18.6</td>
<td>C-6''</td>
</tr>
<tr>
<td>22</td>
<td>108.4</td>
<td>C-1'''</td>
</tr>
<tr>
<td>23</td>
<td>76.3</td>
<td>C-2'''</td>
</tr>
<tr>
<td>24</td>
<td>79.5</td>
<td>C-3'''</td>
</tr>
<tr>
<td>25</td>
<td>74.2</td>
<td>C-4'''</td>
</tr>
<tr>
<td>26</td>
<td>64.3</td>
<td>C-5'''</td>
</tr>
<tr>
<td>27</td>
<td>63.2</td>
<td>C-6'''</td>
</tr>
</tbody>
</table>
Fig. 5: $^{13}$C-NMR Spectrum of the Compound (SM)
MASS SPECTRUM OF THE GLYCOside (SM)

The important fragment ion peaks obtained in FABMS of glycoside (SM) are given below which further confirmed the assigned structure (V) of the glycoside.

\[ [M^+] \text{ 638 (absent), 476 [M\textsuperscript{+}-galactose], 330 [M\textsuperscript{+}-rhamnose, aglycone], 329 [M\textsuperscript{+}-H], 315 [M\textsuperscript{+}-CH}_2\textsuperscript{, 302 [M\textsuperscript{+}-CO], 299 [M\textsuperscript{+}-CH}_3\textsuperscript{, 182 [A}_1\textsuperscript{+}, 183 [A}_2\textsuperscript{+}, 154 [A}_1\textsuperscript{+}-CO], 121 [B}_1\textsuperscript{+}, 118 [B}_2\textsuperscript{+}, 94 [B}_3\textsuperscript{+}-CO}.\]

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

_Echinops echinatus_ Roxb. is commonly known as 'Gokru' in Hindi. It belongs to Compositae family. The seeds of this plant were collected around the Sagar region, and taxonomically authenticated by Taxonomist, Department of Botany, Dr. H.S. Gour University, Sagar (M.P.).

EXTRACTION AND ISOLATION OF THE COMPOUND (SM)

Powdered air-dried seeds (3kg) of _E. echinatus_ were extracted with 95% methanol by a Soxhlet apparatus. The total methanolic extract obtained was concentrated under reduced pressure to give a brown viscous mass (4.46 gm), which was successively partitioned with pet-ether (60-80°C), benzene, chloroform, ethyl acetate, acetone and methanol.

On removal of the solvent from various fractions except chloroform soluble part, gave negligible amount of residue therefore any phytochemical examination on these soluble portions was not possible.

The detailed investigation of the chloroform soluble part has been described in this chapter.

STUDY OF THE CHLOROFORM SOLUBLE FRACTION

The chloroform soluble fraction of the methanolic extract of the seeds of the plant was concentrated under reduced pressure to give brown viscous (3.38 gm). It showed single spot on TLC examination using (EtOAc:MeOH:H₂O, 6:4:2) as solvent system and I₂ vapours as visualizing agent. It was further purified by column chromatography over silica-gel-'G' and eluted with MeOH:CHCl₃ in various proportions (10:4,8:6,6:8). The details of column chromatography are given in Table-IX.

COLUMN CHROMATOGRAPHY

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of the column</td>
<td>155 cm</td>
</tr>
<tr>
<td>Diameter of the column</td>
<td>3.5 cm</td>
</tr>
<tr>
<td>Weight of the crude extract</td>
<td>3.38 gm</td>
</tr>
<tr>
<td>Weight of the silica-gel</td>
<td>155 gm</td>
</tr>
</tbody>
</table>
TABLE-IX

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Fraction No.</th>
<th>Eluants</th>
<th>Spot on TLC</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-8</td>
<td>MeOH:CHCl₃ (10:4)</td>
<td>one</td>
<td>Compound SM</td>
</tr>
<tr>
<td>2</td>
<td>9-18</td>
<td>MeOH:CHCl₃ (8:6)</td>
<td>nil</td>
<td>Gummy</td>
</tr>
<tr>
<td>3</td>
<td>19-28</td>
<td>MeOH:CHCl₃ (6:8)</td>
<td>nil</td>
<td>Sticky mass</td>
</tr>
</tbody>
</table>

STUDY OF THE FRACTIONS (1-8)

Eluants obtained from the fractions (1-8) were found to have same Rᵣ values, hence mixed together. On evaporation of the solvent it yielded a light brownish needles compound (2.18 gm), which was found to be homogeneous on TLC examination.

STUDY OF THE COMPOUND (SM)

It was analysed for m.f. C₂₉H₃₄O₁₆, m.p. 256-258°C and [M]+ 638 (FABMS). It gave Molisch test for glycoside and the following characteristic colour reactions of flavonoids.

(I) Pink colour with Mg/HCl.
(II) Green colour with FeCl₃
(III) Yellow green fluorescent with NH₃ under UV.

ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C₂₉H₃₄O₁₆</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 53.81%</td>
<td>C = 53.84%</td>
</tr>
<tr>
<td>H = 5.14%</td>
<td>H = 5.12%</td>
</tr>
</tbody>
</table>

Molecular weight = 638 (by FABMS)

ACETYLATION OF THE GLYCOSIDE (SM)

The acetylation of the glycoside (SM) was carried out by similar method as described for glycoside (SM) in chapter-2 on page no. 56 of this thesis. The acetyl derivative of the glycoside was crystallized from methanol as light yellowish needles and analysed for m.f. C₄₅H₅₆O₂₄, m.p. 192-194°C and [M]+ 974 (EIMS).
ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C_{45}H_{50}O_{24}</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 55.41%</td>
<td>C = 55.44%</td>
</tr>
<tr>
<td>H = 5.11%</td>
<td>H = 5.13%</td>
</tr>
</tbody>
</table>

**Found**: % of acetyl group = 34.47  
**Calculated**: % of acetyl group = 34.49  
**Molecular weight** = 974 (by EIMS)

ACID HYDROLYSIS OF THE GLYCOSIDE (SM)

The glycoside (200mg) (SM) was treated with 10% H_{2}SO_{4} in a 100 ml round bottomed flask fitted with an air condenser on a water bath for about 7-8 hours at 100\(^{0}\)C. The residue was recrystallized from methanol to yield light yellow needles aglycone (SM-A). The aqueous hydrolysate, on removal of the solvent, was neutralized with BaCO_{3} and subjected to PC for the analysis of sugar moiety(ies).

STUDY OF THE AGLYCON (SM-A)

It was crystallized from methanol as brownish needles (1.20 gm), which was found to be homogeneous on TLC examination. It was analysed for molecular formula, C_{17}H_{14}O_{7}, m.p. 180-183\(^{0}\)C and [M]\(^{+}\) 330 (FABMS) and yielded all the following colour reactions of the flavonoids.

(I) Orange red colour with Zn/HCl.
(II) Green colour with FeCl_{3}
(III) Pink colour with Mg/HCl

ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C_{17}H_{14}O_{7}</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 60.77%</td>
<td>C = 60.75 %</td>
</tr>
<tr>
<td>H = 3.82 %</td>
<td>H = 3.78 %</td>
</tr>
</tbody>
</table>

**Molecular weight** = 330 (by FABMS)

ACETYLATION OF THE AGLYCON (SM-A)

The acetylation of the aglycone was done by similar procedure as described for glycoside (SM) on page no. 56 of this thesis. The acetyl derivative SS-A (a) of the aglycone was analysed for m.f. C_{25}H_{20}O_{10}, m.p. 186-183\(^{0}\)C and [M]\(^{+}\) 456 (EIMS).
ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C_{22}H_{20}O_{10}</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 60.50%</td>
<td>C = 60.52%</td>
</tr>
<tr>
<td>H = 4.38%</td>
<td>H = 4.38%</td>
</tr>
</tbody>
</table>

Found : % of acetyl group = 27.61
Calculated : % of acetyl group = 27.63
Molecular weight = 456 (by EIMS).

ESTIMATION OF -OMe GROUP (S) IN AGLYCON (SM-A)

The estimation of methoxy groups in SM-A was carried out by similar method as described in chapter-3 on page no. 87 of this thesis.

ALKALINE DEGRADATION OF THE AGLYCON (SM-A)

Aglycone (SM-A) (50 mg) was refluxed with 25 ml of 50% ethanolic KOH in a 150 ml B-14 ground joint flask. The reaction mixture was cooled and then acidified by dil. HCl. The contents were extracted with solvent ether in a separating funnel. The ethereal layer was washed with water and separated into two portions.

(I) The first part was treated with 10% NaOH solution and on acidification yielded a compound (Ia), m.f. C_{7}H_{8}O_{4}, m.p. 217-220\(^{\circ}\)C and [M]\(^+\) 156 (EIMS), identified as monomethoxy phloroglucinol (by m.m.p., CO-PC).

(II) The second part was treated with 50% NaHCO\(_3\) solution and on acidification gave a compound (Ib), m.f. C_{7}H_{6}O_{3}, m.p. 156-158\(^{\circ}\)C and [M]\(^+\) 138 (EIMS), identified as salicylic acid (by m.m.p., CO-PC).

PARTIAL HYDROLYSIS OF THE GLYCOSIDE (SM)

The glycoside (150mg) was treated with Kiliani reagent (AcOH- HCl-H\(_2\)O; 35:15:50) in a 250 ml round bottomed flask and the reaction mixture was left at room temperature for a week. The contents were further extracted with n-butanol. The n-butanol soluble part was concentrated and subjected to TLC examination showed two spots, which were separated by column chromatography over silica-gel-'G' and eluted with MeOH:CHCl\(_3\) in various proportions. The observations are given in the following Table-X
COLUMN CHROMATOGRAPHY

Length of the column : 155 cm
Diameter of the column : 3.5 cm
Weight of the silica-gel : 90 gm
Weight of the n-butanol soluble part : 140 mg

TABLE-X

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Fraction No.</th>
<th>Eluants</th>
<th>Spot on TLC</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-8</td>
<td>MeOH:CHCl₃(10:4)</td>
<td>One</td>
<td>PSS-I</td>
</tr>
<tr>
<td>2</td>
<td>9-18</td>
<td>MeOH:CHCl₃(9:5)</td>
<td>One</td>
<td>PSS-II</td>
</tr>
</tbody>
</table>

STUDY OF FRACTIONS (1-8)

The eluants collected from fractions (1-8) were found to have same R̂ values and so combined together. On removal of the solvent yielded proaglycone PSM-I

STUDY OF THE PROAGLYCONE PSM-I

It was crystallized from methanol and analysed for m.f. C₂₂H₂₄O₁₁, m.p. 232-234°C and [M]+476(FABMS). On hydrolysis with 10% H₂SO₄ gave aglycone and L-rhamnose (R̂ 0.36) (by Co-PC).

ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for C₂₂H₂₄O₁₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 57.97%</td>
<td>C = 57.98%</td>
</tr>
<tr>
<td>H = 5.02%</td>
<td>H = 5.04%</td>
</tr>
</tbody>
</table>

Molecular weight = 476 (by FABMS)

PERMETHYLATION AND HYDROLYSIS OF THE PROAGLYCONE PSM-I

Permethylation followed by acid hydrolysis of the proaglycone PSM-I was carried out by similar method as described in chapter-2 on page no. 59 of this thesis. It yielded methylated aglycone which was identified as 7-hydroxy-2',3,5,6,-tetramethoxy flavone and methylated sugar which was identified as 2,3,4-tri-O-methyl-L-rhamnose (by Co-PC).
STUDY OF FRACTIONS (9-18)

The fractions (9-18) collected were found to have the same \( R_f \) values and hence mixed together. On evaporation of solvent, it gave proaglycone PSM-II. It was crystallized from methanol.

STUDY OF THE PROAGLYCONE PSM-II

It was analysed for m.f. \( C_{29}H_{34}O_{16} \), m.p. 256-258\(^{0}\)C and \([M]^+\) 638 (FABMS). On hydrolysis with 10% H\(_2\)SO\(_4\), yielded aglycone, D-galactose (\( R_f \) 0.18) and L-rhamnose (\( R_f \) 0.36) (by Co-PC).

ELEMENTAL ANALYSIS

<table>
<thead>
<tr>
<th>Found</th>
<th>Calculated for ( C_{29}H_{34}O_{16} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 53.81%</td>
<td>C = 53.84%</td>
</tr>
<tr>
<td>H = 5.14%</td>
<td>H = 5.12%</td>
</tr>
</tbody>
</table>

Molecular weight = 638 (by FABMS).

PERMETHYLATION AND HYDROLYSIS OF THE PROAGLYCONE PSM-II

The permethylation and hydrolysis of the proaglycone PSM-II was done by similar procedure as described in chapter-2 on page no. 59 of this thesis. It gave methylated aglycone identified as 7-hydroxy-2', 3, 5, 6-tetramethoxy flavone and methylated sugars which were identified as 2,3-di-O-methyl-L-rhamnose and 2,3,4,6-tetra-O-methyl-D-galactose (by Co-PC).

IDENTIFICATION OF SUGARS AFTER HYDROLYSIS

The aqueous hydrolysate obtained after acid hydrolysis of the glycoside was neutralised with BaCO\(_3\) and BaSO\(_4\) filtered off. The filtrate was concentrated and subjected to PC on Whatman filter paper no.1 using following solvent systems and aniline hydrogen phathalate as spraying reagent. The results are recorded in Table-XI.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Solvent system</th>
<th>( R_f ) Value</th>
<th>Sugar identified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reported(^{38})</td>
<td>Found</td>
</tr>
<tr>
<td>1</td>
<td>n-BuOH-AcOH-H(_2)O (4:1:5)</td>
<td>0.16</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.37</td>
<td>0.36</td>
</tr>
<tr>
<td>2</td>
<td>EtOAc-( C_6H_5N-H_2O ) (2:1:2)</td>
<td>0.23</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.38</td>
<td>0.36</td>
</tr>
</tbody>
</table>
PERIODATE OXIDATION OF THE GLYCOSIDE (SM)

Periodate oxidation of the glycoside was carried out by similar procedure as described in chapter-2 on page no. 61 of this thesis.

ENZYMATIC HYDROLYSIS OF THE GLYCOSIDE (SM)

The glycoside (60 mg) was dissolved in 30 ml of methanol and treated with equal volume of almond emulsin in a 150 ml round bottomed flask fitted with an air condenser. The contents were then allowed to stand at room temperature for three days and filtered. The aglycone was identified as 2',5,7-trihydroxy-3,6-dimethoxy flavone, m.p. 180-183°C (by m.m.p) and the hydrolysate was studied separately.

The hydrolysate was concentrated and subjected to paper chromatography examination on Whatman filter paper No. 1 using BAW (4:1:5) solvent system and aniline hydrogen phthalate as spraying reagent. The sugars were identified as D-galactose (Rf 0.18) and L-rhamnose (Rf 0.36) (by Co-PC).
REFERENCES


