CHAPTER-3
CHEMICAL EXAMINATION OF STEM OF RHINACANTHUS COMMUNIS

The air dried and finely crushed stem of Rhinacanthus communis was extracted with hot ethanol. The alcoholic extract was concentrated under reduced pressure. The concentrated alcoholic extract was poured into excess of ice cold water with constant stirring whereby an aqueous solution and a water insoluble residue was obtained. The water insoluble residue was successively extracted with hexane, benzene, chloroform, ethyl acetate and methanol in a soxhlet extractor. The individual fraction was worked separately. Concentrated hexane was chromatographed with dry column using different solvents in increasing polarity, viz. hexane, benzene, dichloromethane, ethylacetate and methanol respectively. Hexane: dichloromethane (1:1 v/v) fraction was found to contain two spots on thin layer chromatography marked as C-5 and C-6. These two were separated by preparative thin layer chromatography H : B (6:4 v/v) and crystallized from ethanol. Homogeneity and purity of these compounds were checked by thin layer chromatography.

Compounds isolated from stem of Rhinacanthus communis characterised as:

C-5 : Lupene – 20(29) ene-3-ol.

C-6 : 19α, 24-dihydroxy urs-12-ene, 28-oic acid-3-O-β-D-xylopyranoside.

The detailed study of compound C-5 and C-6 has been given in this chapter under section-I and section-II respectively.
SCHEME OF ISOLATION AND PURIFICATION OF COMPOUNDS FROM STEM OF RHINACANTHUS COMMUNIS

Stem of Rhinacanthus communis

Dried and crushed plant material extracted with ethanol.

Dark brown extract

Extract concentrated and poured into excess of ice cold water with continuous stirring.

Water insoluble residue

FRACTION - 1

Water soluble solution

FRACTION - 2
Fraction - 1

Extracted in a soxhlet extractor, with solvents of increasing polarity

Hexane Fraction

Concentrated and chromatographed over dry column and eluted with different organic solvents of increasing polarity

Hexane  ▼  Benzene  ▼  Hexane : DCM (1:1 v/v)  ▼  Dichloromethane  ▼  Ethylacetate

Two spots present separated by preparative TLC

Preparative TLC solvent system
Hexane:Benzene (6:4 v/v) two compounds C-5 and C-6 separated at different Rt using methanol as eluting solvent

C- 5  ▼  m.p. 214°  ▼  C- 6  ▼  m.p. 280°
EXPERIMENTAL

The stem of Rhinacanthus communis was obtained from Chitrakoot (U.P.) and adjoining area.

Air dried and finely crushed stem of Rhinacanthus communis was extracted with ethyl alcohol under reflux. The alcoholic extract was concentrated under reduced pressure in rotator evaporator. The concentrated extract was poured into an excess of ice-cold water with constant stirring whereby a water insoluble (Fraction -1) and water soluble solution (Fraction -2) were obtained which were separated by filtration.

Fraction-1

Fraction -1 was extracted with different solvents in increasing polarity viz., hexane, benzene, dichloromethane, ethylacetate and methanol in soxhlet extractor.

Hexane fraction

It was concentrated under reduced pressure in a rotatory evaporator and was chromatographed with dry column using different solvents in increasing polarity. Hexane: Dichloromethane (1:1 v/v) fraction was found two spot on TLC. These two were separated by preparative thin layer chromatography in solvent system Hexane : Benzene (6:4 v/v) at different Rf using methanol as eluting solvent, were marked as C-5 m.p. 214° (1 g) and C-6 m. p. 280° (900 mg). These two compounds were triterpenoid in nature. Purity and homogeneity of these compounds were checked by silica gel ‘g’ plates.
SECTION-I

STRUCTURAL STUDIES OF COMPOUND C-5

(Lupene-20 (29)-ene-3-ol)
Compound C-5, m.p. 214° was analyzed for C\textsubscript{30} H\textsubscript{50}O. On the basis of elemental analysis and molecular wt. determination (M', 426), compound gave all the colour reactions characteristic of triterpenes.

1) It gave yellow colour changing to red in Salkawski reaction.

2) A deep red colour in Liebermann-Burchard reaction.

3) A red colour with a greenish fluorescence in Tschgajew reaction.

4) A solution of compound developed red color on treatment with Nollers reagent.

5) A reddish colour with Rosenhein reagent.

6) A deep purple colour in Kohlenberg's reaction on treatment with chlorosulphonic acid in glacial acetic acid compound gave reddish violet colour (Brieskorn test)

Compound gave violet colour with 2, 6 – ditertiary butyl – p – cresol\textsuperscript{1,2} in ethanol, indicating it to be a pentacyclic triterpene.

IR absorption peaks at $V_{\text{max}}$ 3540 cm\textsuperscript{-1}, 1640, 880 cm\textsuperscript{-1} and 1610 cm\textsuperscript{-1} corresponded for hydroxyl group, exo=CH\textsubscript{2} group and a trisubstituted double bond respectively\textsuperscript{3}.

\textsuperscript{1}H NMR spectrum showed peaks at δ 0.88, 0.91, 0.93, 1.02, 1.11 and 1.15 (3H, S) corresponded for six tertiary methyl groups. Compound in \textsuperscript{1}H NMR showed a multiplet at δ 5.32 (1H) which is a characteristic of C-12 olifinic proton. Compound gave positive color test with tetranitromethane, indicating the presence of double bond at position C-12 and C-13.

Compound gave signals in \textsuperscript{1}H NMR spectrum at δ 2.30 (1H, S) and 3.90 (1H). Correspond for hydroxylic proton and a carbinol methine proton respectively. The \textsuperscript{1}H
NMR spectrum also showed peaks at δ 4.50 and δ 4.65 (1H, d, J=1.5 Hz) which is a characteristic of exo-CH₂ group.

The mass spectrum of compound showed [M⁺] at m/z 426. Peaks at m/z 385 due to loss of C₃H₅ unit, m/z 411 (M-15), m/z 408 (M-18), m/z 393 [M-(Me + H₂O)] and two intense peaks at m/z 218 and m/z 207 were due to retro-dieals-aldor cleavage.

On the basis of above facts the compound was found to be a pentacyclic triterpene of lupine – 12 – ene type containing one –OH group.

Thus the partial structure of the compound can be represented as

![Partial structure of the compound]

**Position of hydroxyl group**

Mass spectrum of compound showed two intense peaks at m/z 207 and m/z 218 indicating that – OH group is present either in ring A or B. The ¹³C NMR spectrum of compound showed deshielding effect at C-3 due to presence of OH group substituent which appeared at δ 79.0.
SCHEME-1

- C₃H₅

m/z 385

\[ \text{[M}^+ 426] \]

- H₂O

m/z 408

- CH₃

m/z 411

m/z 393

m/z 218

m/z 207

- H₂O

m/z 393

m/z 218

m/z 207

m/z 189

m/z 190
**TABLE**

$^{13}$C NMR data of compound C-5

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<td>C-30</td>
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Finally the structure of compound C-5 was confirmed by its mass fragmentation peaks present at m/z 426 [M⁺], m/z 385, m/z 411, m/z 408, m/z 393, m/z 218, m/z 207, m/z 219, m/z 189. (scheme 1).

Thus the structure of the compound C-5 represented as lupene – 20 (29) – ene – 3 – ol.

This is a known compound and isolated from many natural sources.

**EXPERIMENTAL**

It was crystallized from methanol as a white compound, m.p. is 214°

**Solubility**  :  Hexane, Benzene, chloroform, Ethyl acetate, methanol.

**Rf**  :  0.49 Hexane  :  dichloromethae (7:3 v/v)
Elemental analysis

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<th>Found</th>
<th>Calculated for C₈H₁₅O₂</th>
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<td>H=12.20%</td>
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Spectral studies

IR $V_{\text{KBr}}^{\text{max}}$

3540, 1640, 1610, 880

$^1$H NMR [CDCl₃, 200 MHz]

0.88 (3H, S), 0.91 (3H, S), 0.93 (3H, S), 1.02(3H, S), 1.11 (3H, S), 1.15 (3H, S), 1H, S, 3.90 (1H, d, J=2.5 cps), 4.50 and 4.65 (1H, d, J=1.5 Hz), 5.32 (1H, m)

Mass Spectrum

$m/z$ 426, [M+], 411, 408, 393, 385, 219, 218, 189, 38.7, 27.5, 79.0, 38.9, 55.3, 18.3, 34.3, 40.9, 50.5, 37.2, 21.0, 125.0, 137.0, 42.9, 27.5, 35.6, 43.0, 48.0, 48.3, 150.9, 29.1, 40.0, 28.0, 15.3, 16.1, 16.0, 14.6, 18.0, 19.3, 109.3
SECTION-II

STRUCTURAL STUDIES OF COMPOUND C-6

(19α, 24-dihydroxy urs-12-ene, 28-oic acid-3-O-β-D-xylopyranoside)
A white compound, m.p. 280° was analyzed for C₃₅H₅₆O₉ on the basis of elemental analysis. Compound gave positive Molisch’s test but neither reduces Fehling’s solution nor gave characteristic colour with aniline hydrogen phthalate reagent indicating that the reducing group is not free and probably involved in glycosidic linkage.

On acid hydrolysis aglycone was obtained from hydrolysate. The sugar has been identified as D-xylose by co-chromatography with an authentic sample.

**Aglycone**

On the basis of elemental analysis and molecular weight determination [M⁺, 488] the aglycone was analyzed for C₃₀H₄₈O₅. The compound gave all the colour reactions characteristic of triterpenoids.

1) It gave yellow colour changing to red in Salkawski reaction.

2) A deep red colour in Liebermann-Burchard reaction.

3) A red colour with a greenish fluorescence in Tschugajew reaction.

4) A solution of compound developed red colour on treatment with Noller’s reagent.

5) A reddish colour with Rosenhein reagent.

6) A deep purple colour in Kohlenberg’s reaction.

7) On treatment with chlorosulphonic acid in glacial acetic acid compound gave reddish violet colour (Brieskorn).

The aglycone gave violet colour with 2, 6-diter-butyl-p-cresol in ethanol indicating it to be a pentacyclic triterpene.
IR absorption of aglycone at $\nu_{\text{max}}$ 3600 cm$^{-1}$ showed the presence of hydroxyl group. The aglycone has absorption peaks at $\nu$ max 1110, 1050, 1000, and 940 cm$^{-1}$. These peaks are for the primary, secondary and tertiary hydroxyl groups respectively. A peak in IR spectrum at $\nu$ max 1695 cm$^{-1}$ showed the presence of –COOH group in the compound. The aglycone also gave absorption peak at $\nu_{\text{max}}$ 1610 cm$^{-1}$ for the trisubstituted double bond$^{5,6}$.

$^1$H NMR spectra of aglycone showed peaks at $\delta$ 0.86-1.27 (18H) corresponded for six methyl groups. A doublet at $\delta$ 0.98, J=7 cps indicated that a secondary, methyl group was present in aglycone. A multiplet appeared at $\delta$ 5.31 (1H) ppm corresponds for C-12 olifinic proton. Aglycone gave positive colour test with tetra-nitromethane (Ruzicka reaction)$^7$ indicated the presence of double bond at position C-12 and C-13 which is a characteristic of $\alpha$ and $\beta$ amyrin series. A broad signal present at $\delta$ 2.60 which corresponds for H-18.

The fragment at M$^+$, 488,m/z 264 and m/z 23 in the mass spectrum were due to retro-diel’s-alder rearrangement. These fragments confirmed that the aglycone was a Ursane-12-ene type$^8$.

On the basis of above facts conclusively indicated that aglycone is a pentacyclic triterpene of $\alpha$-amyrin series which contain hydroxyl and –COOH group/groups. The partial structure of aglycone thus can be represented as:
Position of hydroxyl group

In $^{13}$C NMR the C-4 $\delta$ value of aglycone appeared at $\delta$ 43.1. The signal of C-4 appeared at $\delta$ 38.7 in $\alpha$-amyrin and at $\delta$ 43.2 in rotungenic acid. This clearly suggested that the substitution at C-4 is different from the $\alpha$-amyrin but identical with rotungenic acid which contained methyl and hydroxymethyl group. In $^{13}$C NMR spectra it was also found that C-23 appeared as quartet at $\delta$ 23.7 and C-24 as a triplet at $\delta$ 64.2, C-24 appeared at high $\delta$ was due to the presence of $-\text{OH}$ group conclusively it confirmed that C-24 present in the form of hydroxy methyl at C-24 positions.

The orientation of methyl and hydroxymethyl group at C-4 was assigned on the basis of $^{13}$C NMR spectra. A signal at $\delta$ 23.7 corresponds for 4$\alpha$-methyl group because it appeared at $\delta$ 10 ppm higher than the 4$\beta$ methyl group (15.6). Therefore the hydroxy methyl group giving a signal at $\delta$ 64.6 must be in the form of $\beta$ at C-4 (when it would be in $\alpha$-form then the value would be $\delta$ 68.4).

The $^1$H NMR spectrum showed singlet at $\delta$ 2.60 ppm. This value corresponds to H-18 proton. Appearance of H-18 proton as singlet is due to presence of substituent on C-19. The H-18 proton resonance agreed the existence of a 19$\alpha$ hydroxyl group. Also the presence of the hydroxyl group was corroborated by a downfield effect on Me-29 due to deshielding effect. The orientation of $-\text{OH}$ group at C-19 in the $\alpha$-form confirmed by accountful effect on methyl group at 29$^{\text{th}}$ position which shifted its $\delta$ value 0.80 to 1.26 i.e.from lower $\delta$ to higher. It was further confirmed by comparing $^{13}$C NMR of the
compound with rotugenic acid the C-19 δ appeared at 72.8 in both. A double doublet at δ 2.90, J=11.5, J=4.5 cps correspond for the axial hydrogen present at C-3. The axial orientation of 3-H is only when the substituent –OH group present in the β form.

On the basis of above observations the partial structure of the aglycone can be represented as:

![Diagram of aglycone structure with -COOH group](image)

**Position of –COOH group**

$^{13}$C NMR spectra of the aglycone showed peak at δ 181.0 which correspond for –COOH group when aglycone was esterified it gave monomethyl ester m.p. 210°, M$^+$,502 indicated only one –COOH group present in aglycone. The position of –COOH group was confirmed by comparing the $^{13}$CNMR of aglycone and rotugenic acid which showed C-17 at δ 48.4 in both. Thus the –COOH group is present at C-17.
SCHEME

M* 488

m/z 223 + m/z 264

- H₂O

m/z 246

- COOH

m/z 219

- H₂O

m/z 201
The structure of the compound was further supported by its mass spectrum which showed characteristic fragment peaks at m/z, 488 [M⁺], 470,264, 246,223,219 and 201 [scheme-1]

On the basis of the above facts the structure of aglycone is 3-β, 19-α, 24-trihydroxy Urs-12-ene, 28-oic acid i.e. the Rotungenic acid.

![Chemical Structure](image)

**Glycoside**

1H NMR spectra of glycoside showed anomeric proton of D-xylose at δ 5.15 (d, 1H, J=7 cps) and showed multiplet at δ 3.5-4.8 for sugar protons. The molecular formula weight of the glycoside and aglycone suggested the presence of one mole of sugar per mole of glycoside.

Easy hydrolysis eliminated the possibility of C-C glycosidic linkage so that the linkage must be C-O-C type.

**Attachment of sugar to aglycone**

From structure of aglycone it was evident that four positions were present for the attachment of sugar moiety i.e. C-3 –OH, C-19 –OH, C-17 –COOH, C-4 –CH₃OH.

On the basis of 13CNMR spectra of the aglycone and glycoside showed shielding effect at C-3 position due to the attachment of sugar moiety. The δ value at C-3 of
aglycone and glycoside was found to be δ 80.3 and 78.8 respectively confirmed that sugar moiety attached at C-3.

From the foregoing evidences the structure of compound represented as, 19α, 24-dihydroxy Urs-12-ene, 28-oic acid-3-0-β-D-xylopyranoside.

![Chemical Structure]

**EXPERIMENTAL**

It was crystallized from ethylacetate as a white compound, m.p. 280°

**Solubility** : Benzene, Chloroform, Ethylacetate, Methanol.

**Rf** : 0.62 Benzene: Ethylacetate 9.5:0.5 v/v

**Elemental analysis**

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Spectral studies

\[ \text{IR } V_{\text{KBr}}^{\text{max}} \text{ cm}^{-1} \]
3600, 2600, 1695, 1645, 1610, 1050, 940

\[ {^1}\text{H NMR [CDCl}_3, 200 \text{ MHz}] \]
\[ \delta \ 0.86-1.27 \ (18\text{H, 6X CH}_3), 2.60 \ (1\text{H, brS}), 2.90 \]
\[ (d, 1\text{H, J}=11.5, J=4.5), 3.35 \ (1\text{H, d, J}=11 \text{ cps}), 4.20 \]
\[ (1\text{H, d, J}=11 \text{ cps}), 5.15(1\text{H, d, J}=7 \text{ cps anomic proton of D-xylose}), 3.2-4.0 \ (\text{multiplet for sugar protons}) \text{ ppm} \]

\[ {^{13}}\text{C NMR [CDCl}_3] \]
\[ \delta \ 38.8, 28.5, 78.8, 41.1, 56.5, 19.3, 34.0, 40.5, 48.0, \]
\[ 37.4, 24.3, 128.0, 140.00, 42, 29, 26.6, 48.4, 54.7, \]
\[ 72.8, 42.4, 27.0, 38.6, 23.7, 64.6, 17.2, 16.8, 24.7, \]
\[ 181.0, 27.4, 16.1, 106.6, 74.8, 79.5, 71.2, 66.8 \]

**Acid hydrolysis of Glycoside**

The glycoside (0.05 gm) was refluxed with 50 ml of 7% ethanolic sulphuric acid on water bath for 4 hrs. The reaction mixture was taken in ether, concentrated and crystallized from ethylacetate: petroleum ether (1:3)

Remaining aq. Solution was neutralized with barium carbonate and filtrate was concentrated in a rotatory evaporator. The concentrate gave positive Molisch’s test and reduces Fehling’s solution.

**Aglycone**

m.p. 300°

\[ \text{UV } \lambda_{\text{max}} \]
209 nm

\[ \text{IR } V_{\text{KBr}}^{\text{max}} \text{ cm}^{-1} \]
3600, 1110, 1050, 1000, 940, 1695, 1610
Mass Spectrum m/z

$M^+$ 488, 470, 452, 442, 264, 246, 223, 219, 218, 205, 201, 175, 146.

$^{13}$CNMR [$\text{CDCl}_3$]

$\delta$ 38.8, 28.5, 80.3, 43.2, 56.5, 19.3, 34.0, 40.0, 47.9, 37.2, 24.3, 127.9, 140.0, 42.1, 29.1, 26.5, 48.4, 54.7, 72.8, 42.4, 27.0, 38.6, 23.7, 64.6, 17.2, 16.8, 24.7, 180.8, 27.2, 16.1
REFERENCE

3) Narendra Kumar and Tiruvenkata R. Sheshadri, Phyto, 14, 521 (1975).
5) Munehiro Nakatani, Yuji Miyazaki, Takashi Iwashita, Hideo
8) Ruzicka and Liebigs, Ann. Chem. 25, 471 (1929)