CHAPTER II

ISOLATION AND CHEMICAL EXAMINATION OF
LEUCOCYANIDIN FROM THE ROOTS OF
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INTRODUCTION

The air-dried roots of Terminalia tomentosa were powdered and extracted with petroleum ether (60-80°), ether and methanol. The petroleum ether extract was worked up for triterpenoids and sterols (Chapter III). The ether extract was investigated for triterpenoids and 3-O-acetyloleanolic acid was isolated (Chapter III). The methanol extract was concentrated, fractionated with petroleum ether and ethyl acetate. The ethyl acetate fraction on concentration and on keeping for 24 h, deposited a solid which was further shaken with ethyl acetate. Some amount of the solid was soluble in ethyl acetate and a yellow solid was left, which on repeated crystallisations with pyridine gave a pale-yellow crystalline solid, m.p.360°. It was characterised as ellagic acid (Chapter IV). To the portion soluble in ethyl acetate, petroleum ether was added, when a buff-coloured solid was thrown out. It was repeatedly crystallised with petroleum ether and ethyl acetate, when a creamish-white solid (900 mg) was obtained (Solid A).
The solid (A) showed $\lambda_{\text{max}}$ at 278 nm. All the colour reactions of leucoanthocyanidin were given by the compound. On hydrolysis with 6%-methanolic hydrochloric acid, the compound (A) gave anthocyanidin, which showed colour reactions of cyanidin. The compound was confirmed as leucocyanidin.

**EXPERIMENTAL**

Dried and powdered roots (2 kg) of *Terminalia tomentosa* were extracted exhaustively with petroleum ether and methanol. The methanol extract was concentrated under reduced pressure. Half of the concentrated methanolic extract was dried and extracted with solvents of increasing polarity (petroleum ether and ethyl acetate). The ethyl acetate fraction was subjected to T.L.C.

To the remaining half methanolic portion, water was added, filtered and the clear aqueous extract shaken with ethyl acetate and subjected to T.L.C.

Both the fractions showed the same substance and so the two ethyl acetate fractions were mixed.
The ethyl acetate fraction on concentration deposited a solid which was again shaken with ethyl acetate. A part of the solid was found to be soluble in ethyl acetate. Petroleum ether was added to the soluble part, when a buff-coloured solid precipitated. It was crystallised with ethyl acetate:petroleum ether (1:1), when a creamish-white solid (Solid 'A') was obtained (900 mg).

**Chromatography of the Compound 'A'**

**TLC** : The homogeneity of the compound was established by Thin layer chromatography. The plates were coated with silica gel G, impregnated with 0.3 M-sodium acetate and developed with a mixture of toluene:ethyl formate:formic acid (5:4:1, v/v) (46).

Thin layer chromatography was also done on silica gel G plates, using the solvent, n-butanol: acetic acid:water (4:1:5, v/v, organic phase).

In both the cases, 20% ethanolic p-toluene sulphonyl acid was used as a spray reagent.

The reagent p-toluene sulphonyl acid was prepared by dissolving 3-gm of p-toluene sulphonyl acid in 100 ml of absolute ethanol. On heating the sprayed plates at 100° for 5 min, a red spot appeared.
Paper Chromatography: The purity of the compound was further established by paper chromatography on Whatman No.1 chromatographic paper, using the following solvents as developer (47-49):

(i) 6%-acetic acid
(ii) n-butanol:acetic acid:water (4:1:5, v/v, organic phase)

The spray reagent used was Vanillin-hydrochloric acid. It was prepared by dissolving 1-g vanillin in 100 ml of hydrochloric acid. The chromatogram was sprayed with the reagent.

In both the developing solvents, single reddish spot appeared. The Rf values were 0.86 (solvent ii) and 0.45 (solvent i).

A separate paper chromatogram was run in the solvent 2%-acetic acid, and sprayed with a reagent made by mixture of equal volumes of 0.3%-aqueous ferric chloride and 0.3%-aqueous potassium ferri-cyanide. A blue spot was observed (50).
Reactions of the Compound 'A'

The compound produced a deep-red solution on heating with ethanolic hydrochloric acid, and the ethanolic solution of the compound developed green colour with ethanolic ferric chloride and dark pink colour with vanillin-hydrochloride reagent.

Infrared spectrum of Compound 'A'

The prominent peaks in the infrared spectrum of the compound (in KBr pellets) were:

<table>
<thead>
<tr>
<th>Absorption peak (cm⁻¹)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3200</td>
<td>O-H stretching</td>
</tr>
<tr>
<td>1610</td>
<td>C=C aromatic skeleton (51)</td>
</tr>
<tr>
<td>1580</td>
<td>aromatic nature</td>
</tr>
<tr>
<td>1440</td>
<td>aromatic nature</td>
</tr>
<tr>
<td>1300-1200</td>
<td>O-H stretching and C-O stretching (51)</td>
</tr>
<tr>
<td>1110-1050</td>
<td>O-H bending and C-O stretching (51)</td>
</tr>
</tbody>
</table>
Ultraviolet Spectrum of the Compound 'A'

The ultraviolet spectrum showed $\lambda_{\text{max}}$ at 278 nm (methanol).

Hydrolysis of the Compound 'A' and Identification of the Anthocyanidin

The compound (20 mg) was refluxed with 6%-ethanolic hydrochloric acid (10 ml) for 2 h. The deep red solution, so produced, was diluted with water and filtered. The filtrate was extracted several times with small amounts of amyl alcohol. The amyl alcohol layer was washed with water and then with 1%-hydrochloric acid. An excess of benzene (5-6 times the volume of amyl alcohol) was added, and the anthocyanidin was extracted with small portions of 1%-hydrochloric acid.

The anthocyanidin solution was again extracted with amyl alcohol, and the process repeated. The aqueous solution was finally washed with benzene to remove traces of amyl alcohol. The anthocyanidin, thus obtained, gave the following tests (52, 53):

(1) Sodium acetate was added to the amyl alcohol extract, which developed reddish-violet colour, changing to blue, on addition of a few drops of ferric chloride solution.
(ii) On shaking equal volumes of the solution and cyanidin reagent (cyclohexanol:toluene, 1:5, v/v), a rose-red colour developed.

(iii) A portion of the solution was shaken in air, and to its half of the volume was added aqueous sodium hydroxide (10%). The colour disappeared. This was followed by the addition of concentrated hydrochloric acid and amyl alcohol successively. The anthocyanidin, thus recovered, indicates its stability to the oxidation tests.

(iv) The amyl alcohol layer, containing the anthocyanidin, became purple on the addition of sodium bicarbonate. It turned red on acidification.

(v) The anthocyanidin was found to have absorption maximum (ethanol-hydrochloric acid) at 540 nm and bathochromic shift of 18 nm with 1%-ethanolci aluminium chloride solution (54).

(vi) The solution gave green colour with ferric chloride solution.
All these results clearly indicate that the anthocyanidin hydrolysed is cyanidin. This was further confirmed by cochromatography with an authentic sample of cyanidin (obtained from fresh rose petal hydrolysate) in Forestal (AcOH:H₂O:conc.HCl, 30:10:3).

Both the spots were found to run identically and had Rf value of 0.5.

**Acetylation of Compound 'A'**

The compound (50 mg) was dissolved in a mixture of acetic anhydride (5 ml) and pyridine (3 ml), and kept at room temperature for 48 h. The reaction mixture was then poured over crushed ice and left overnight. The solid so obtained, was filtered and washed well with water. It was crystallised from ethyl acetate-petroleum ether mixture. The acetylated compound had m.p. 147°-48°, with slight sintering at 113°. It gave red colour on heating with ethanolic hydrochloric acid but no green colour produced; when it was treated with ethanolic ferric chloride.

**Methylation of Compound 'A'**

The compound (30 mg) was refluxed with dimethylsulphate (0.25 ml) and potassium carbonate (1 g) in dry acetone (15 ml) for 8 h. After cooling,
the potassium salt was filtered off and washed with acetone. The combined filterates and washings were concentrated, poured over crushed ice, and allowed to stand. The solid, so obtained, was filtered and washed with water. It was crystallised from ethyl acetate: petroleum ether mixture, m.p. 131°.

RESULTS AND DISCUSSION

Negative Molisch test indicates that the leucoanthocyanidin isolated is free from sugar.

Identification of the Anthocyanidin by chemical tests:

The anthocyanidin gave the following tests:

(i) A portion of the solution was extracted with amyl alcohol and sodium acetate added. A reddish-violet colour was produced which changed to blue on the addition of a few drops of ethanolic ferric chloride.

(ii) When a portion of the solution was shaken with an equal volume of the 'cyanidin reagent', rose-red colour developed.

(iii) The sample was resistant to oxidation.
From the above reactions, it is apparent that the compound is a cyanidin derivative.

This is further supported by following facts:

(i) The anthocyanidin (in MeOH-HCl) has $\lambda_{\text{max}}$ at 540 nm. Which is in agreement with literature value.

(ii) When 1%-ethanolic aluminium chloride was added to the above solution, a bathochromic shift of 18 nm was observed. This indicates the presence of adjacent hydroxyl group. It has been pointed out by Greissman et al. (55-57) that addition of a few drops of aluminium chloride solution produces a bathochromic shift in the principal $\lambda_{\text{max}}$ of those anthocyanidin derivatives which contain adjacent hydroxyl groups, e.g., cyanidin.

The anthocyanidin was crystallised from methanolic HCl:ether. It was found to be homogenous as shown by PC with n-butanol:acetic acid:water (4:1:5, v/v). Its identity as cyanidin was finally confirmed by co-chromatography in Forestal (acetic acid: hydrochloric acid:water, 30:3:10, v/v) and n-butanol: acetic acid:water, 4:1:5,v/v).
Reactions of the Leucocyanidin

(i) When the original compound was treated with alcoholic hydrochloric acid, it gave a deep red solution with $\lambda_{\text{max}}$ 540 nm. The red coloured product, so obtained, was extracted out with amyl alcohol. The alcoholic extract gave reddish-violet colour with sodium-acetate, which is a characteristic reaction of naturally occurring anthocyanidins (58).

(ii) On addition of alcoholic ferric chloride solution to the alcoholic solution of compound, green colour was observed.

(iii) The compound gave dark pink colour with vanillin-hydrochloric acid reagent, indicating the presence of a dihydroxy system at position 5- and 7- (59).

(iv) $\lambda_{\text{max}}$ was 278 nm, which is in agreement with the data for leucoanthocyanins (60).

These colour reactions indicate the original compound to be a leucoanthocyanidin.

On heating with mineral acids, leucoanthocyanidins produce anthocyanidins. The reaction underlying the conversion is oxidation involving loss
of a hydrogen atom and dehydration (61).

\[
\begin{array}{c}
\text{Leucoanthocyanidin} & \text{Anthocyanidin} \\
\end{array}
\]

(v) Paper chromatogram of the compound was sprayed with p-toluene sulphonie acid and heated in oven. It gave a red spot. This test is specific for leuco-compounds, having a free hydroxyl group at position-5 (58,62).

(vi) The compound produced a deep red-brown colour, when treated with ammonium molybdate and acetic acid.

This colour reaction is specific for catechol unit, suggesting the presence of free OH at 3, and 4 (63).
The original compound gave negative Molish test, showing that the compound is free from sugar. These tests show the compound to be leucocyanidin. Therefore, the compound can be assigned the following structure:

![Leucocyanidin structure](image)

Leucocyanidin has been isolated from different plants by earlier workers, e.g., Subramanian and Nair have isolated it from seed coat of *Mangifera indica* (64). Austin and others isolated it from *Prunus cornuta* (65). Rangaswami and Venkateswarlu isolated it from leaves of *Rhododendron formosum* (54).
Biogenesis of Leucocyanidin

Sheshadri (66) has shown the biomechanistic path for the compound Leucoanthocyanidin. It is said that in the biogenesis, chalcone is first formed which undergoes cyclization to give dihydrafavanol. This compound on oxygenation produces the dihydroflavanol which undergoes reduction at position \( \text{M} \) to give Leucoanthocyanidin, as presented below:

\[
\begin{align*}
\text{Chalcone} & \rightarrow \text{Dihydroflavone} \\
\text{Flavan-3,4-diol} & \leftarrow \text{Dihydroflavonol}
\end{align*}
\]