CHAPTER 10

ISOLATION OF PHYTOCONSTITUENTS

10.1 COLUMN CHROMATOGRAPHY

Column chromatography is one of the most useful methods for the separation and purification of plant constituents individually. It is another solid-liquid technique in which the two phases are a solid (stationary phase) and a liquid (mobile phase). The theory of column chromatography is analogous to that of thin-layer chromatography. The most common adsorbents - silica gel and alumina - are the same ones used in TLC. The sample is dissolved in a small quantity of solvent (the eluent) and applied to the top of the column. The eluent, instead of rising by capillary action up a thin layer, flows down through the column filled with the adsorbent. Just as in TLC, there is an equilibrium established between the solute adsorbed on the silica gel or alumina and the eluting solvent flowing down through the column. Column chromatography is generally used as a purification technique, it isolates desired compounds from a mixture.

10.1.1 Materials and Methods

NMR spectra were recorded on a Bruker 200 MHZ instrument using TMS as the internal reference for both $^1$H and $^{13}$C NMR experiments. CDCl$_3$ was used as the solvent. Chemical shifts are given in terms of parts per million (δ scale). MS analyses were recorded on a Shimadzu QP 5000 instrument. IR spectra (cm$^{-1}$) were recorded on a Perkin –
Elmer RX1 FT-IR spectrophotometer. All the Spectral analyses were done at Sophisticated Analytical Instrument Facility (SAIF) centre at IIT, Chennai.

Precoated thin layer chromatography plates (E-Merck, Darmastdt, Germany; Silica gel 60 F254, 0.2 mm thickness, coated on aluminium sheets) were used. Column chromatography was performed using silica gel (70-325 mesh).

Extract used : Methanolic extract
Method : Wet packing method
Packing material : Silica gel 70 – 325

10.1.2 Procedure

Isolation of phytoconstituents from the methanolic extract of *Limonia acidissima* was done by column chromatography. The lower end of the column was plugged with glass wool. The activated silica was mixed with hexane and the slurry formed was charged in to the column, in small portion, with gentle tapping after each addition in order to ensure uniform packing and to remove air bubbles, if any.

A small quantity of eluent was allowed to remain at the top of the column (about 4cms) in order to prevent the drying and possible cracking of the packed column. After the adsorbent had settled, a filter paper disc was placed, the methanolic extract of *Limonia acidissima* was then dissolved in the solvent system and adsorbed on silica gel and allowed to dry. This mixture in a powder form was loaded at top of the column and eluted with n-hexane, then with hexane-ethyl acetate mixture, followed by ethyl acetate - methanol mixture gradually. Several 50ml fractions were collected and each fraction was checked by TLC technique. Similar Rf value containing fractions were mixed together, allowed to evaporate to remove solvent. These fractions were
grouped together according to their homogeneity, judged from the TLC analysis.

Totally about 200 fractions were collected from column. Among all the fractions collected, the fractions from 51-100 and 150-200 gave a single compound with an $R_f$ value of 0.86 and 0.74 respectively and assigned as compound I and II.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Fraction No.</th>
<th>Number of Spots on TLC plate</th>
<th>$R_f$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-50</td>
<td>1</td>
<td>0.86 (I)</td>
</tr>
<tr>
<td>2</td>
<td>51-100</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>101-150</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>151-200</td>
<td>1</td>
<td>0.74 (II)</td>
</tr>
</tbody>
</table>

The isolated compounds I and II were subjected to spectroscopy techniques like IR, $^1$HNMR, $^{13}$CNMR and MS to determine the functional group and confirm the structure.

**10.2 RESULT**

**Physical and Chemical Properties of Compound 1**

- Physical state: White crystals
- Melting Point: 284°C
- Chemical Test: It gave pink to purple colour with Liebermann-Burchard’s test indicating terpenoidal nature.
- Molecular formula: $C_{25}H_{28}O_{12}$
- Molecular weight: 452
Spectral Data of Compound I

IR spectrum of compound I

a) The IR (KBr) $\nu_{\text{max}}$: 1740 (Aliphatic–CH bending), 1720 (aromatic - CH bending), 1030 (C - O- C stretching), 975 (C-C Stretching) cm$^{-1}$, 2926 (CH - stretching), 1686, (C=O stretching), 1602 (COO– asymmetry).

b) MASS Spectrum of Compound I

GC-MS : m/z 453.1551 [M+1], 411, 397, 381, 345, 241, 229, 201, 193, 175, 174, 135, 123, 121, 119, 115, 109, 107, 105, 95, 91, 77, 69, 59 and 43.

c) $^1$H NMR Spectrum of Compound I

$\delta$ 7.41(1H, s, H-21), 7.40(1H, s, H-23), 6.53(1H, d, H-1), 6.32(1H, s, H-22), 6.13(1H, s, H-2) 6.06(1H, s, H-19) 5.53 (1H, s, H-17), 3.99 (1H, s, H-15), 2.60-2.75 (4H, m, H-6, H-9, H-5), 1.50-1.87 (4H, s, H-11, H-12), 1.36 (3H, s, H-30), 1.28 (6H, s, H-18, H-29) and 1.16 (3H, s, H-28).

d) $^{13}$C NMR Spectrum of Compound I

Position Number and $^{13}$C NMR $\delta$ values (ppm): 150.5(C$_1$), 118.8(C$_2$), 160.1(C$_3$), 86.2(C$_4$), 53.1(C$_5$),37.5(C$_6$), 208.7(C$_7$), 49.2(C$_8$),41.7(C$_9$), 49.1(C$_{10}$), 19.0(C$_{11}$), 29.0(C$_{12}$), 38.3(C$_{13}$), 67.1(C$_{14}$), 54.1(C$_{15}$), 166.5(C$_{16}$), 77.7(C$_{17}$), 24.8(C$_{18}$), 103.8(C$_{19}$), 119.7(C$_{20}$), 141.0(C$_{21}$), 109.5(C$_{22}$), 143.1(C$_{23}$), 20.1(C$_{28}$) and 31.5(C$_{30}$).

The $^{13}$C and $^1$H NMR Spectra of compound I gave some characteristic features of glycoside type of triterpenoids.
e) Structure for Compound-1

ACIDISSIMIN (Limonoid) – A Triterpenoid Glycoside

\[
\begin{align*}
\text{O} & \text{O} \\
\text{O} & \text{O} \\
\text{A} & \text{C D} \\
\text{O} & 
\end{align*}
\]

Physical and Chemical Properties of Compound II

Physical state : Brown semisolid

Melting Point : 104º

Chemical Test : It gave brown colour with Molisch reagent indicating Carbohydrate nature.

Molecular formula : \(C_{14}H_{20}O_{12}\)

Molecular weight : 380

Spectral Data of Compound II

a) IR spectrum

\[\text{IR Cm}^{-1} : 3446(\text{OH}), 2988(\text{CH}), 1728(\text{C=O}), 1600 (\text{C=C}), 1394 (\text{C-O}), 1075 (\text{C-O}), 668 (\text{CH}).\]
Figure 10.1 IR Spectrum of Compound I
Figure 10.2 $^1$HNMR spectra of compound I
Figure 10.3 $^{13}$CNMR spectra of compound 1
Figure 10.4 $^{13}$CNMR Spectrum of Compound I
Figure 10.5  Mass Spectrum of Compound I
Figure 10.6  IR Spectrum of Compound II
Figure 10.7 $^1$HNMR Spectrum of Compound II
Figure 10.8 $^{13}$CNMR Spectrum of Compound II
Figure 10.9 Mass Spectrum of Compound II
c) $^{13}$C - NMR spectra

168.80 and 174.99 (C$_1$ and C$_1'$), 144.74 and 70.44 (C$_2$ and C$_2'$), 127.64 and 101.28 (C$_3$ and C$_3'$), 70.29 and 74.35 (C$_4$ and C$_4'$), 71.64 and 62.50 (C$_5$ and C$_5'$) 72.18 and 35.63 (C$_6$ and C$_6'$) and 71.13 and 66.83(C$_7$ and C$_7'$).

d) Mass Spectrum

(m/z)382(m+z) 382(m+2)  340 ,324, 310, 294, 263, 234, 217, 183, 152 , 120, 82.

e) Structure of Compound II

Unsaturated Di galacturonic acid

10.3 RESULT AND DISCUSSION

The methanolic extract of L.acidissima was subjected to column chromatography. The preliminary phytochemical investigation of MELA revealed the presence of various constituents like coumarins, triterpenoids, tannins, flavonoids, glycosides and carbohydrates. In the current study a crystalline compound, acidissimin was isolated which yielded a molecular ion peak at 453.15 consistent with the limonoid skeleton.

The first compound was obtained as a white crystalline natured substance and exhibited a positive test to Liebermann burchard reagent.
The IR spectrum indicated that the compound I possessed carbonyl chromophore (1720 cm\(^{-1}\)) aromatic – CH bending (3030 cm\(^{-1}\)), aliphatic – CH bending (1740 cm\(^{-1}\)), C= C stretching 1680 cm\(^{-1}\), C- C stretching (975 cm\(^{-1}\)), C- O- C stretching (1030 cm\(^{-1}\)) groups.

The \(^1\)HNMR spectral displayed two pairs of typical H\(_1\) and H\(_2\) protons at \(\delta\)H 6.53, 6.11 (d 1H). The characteristic signals at H\(_5\), H\(_6\), H\(_9\), H\(_{11}\), and H\(_{12}\) exhibited multiplet which proves the existence of methylene and methine proton. Further signals for two exomethyl protons resonated a singlet peak at \(\delta\) value of 1.16, 1.28 and multiplet at 6.32 - 7.40 establish the aromatic proton.

The \(^{13}\)CNMR spectra composed of one ester carbonyl carbon 160.1 (C\(_3\)), two olefinic methines 150.5(C\(_1\)), 118.8 (C\(_2\)), two C-methyl groups 15.6 (C\(_{28}\)), 20.1 (C\(_{29}\)) one methane 103.8 (C\(_{19}\)) and one SP\(^3\) hybridized quaternary carbon attached to an oxygen atom 86.2 (C\(_4\)). The spectral details of \(^{13}\)CNMR substantiated the existence of the quaternary carbon, methane carbon 54.1 (C\(_{15}\)), 77.7(C\(_{17}\)), 103.8, (C\(_{19}\)) 141(C\(_{21}\)), 109.5 (C\(_{22}\)), 49.2 (C\(_8\)), 41.7(C\(_9\)), 49.1 (C\(_{10}\)), 38.3 (C\(_{13}\)), 119.7(C\(_{20}\)) methyl carbon. On comparison of complete spectral detail of the compound it was concluded that the proposed structure was identified as acidissimin (a tetra nor triterpene limonoid skeleton).

These results suggest that isolated compound I was reported from the fruit pulp source of \textit{Limonia acidissima} for the first time.

The second compound Unsaturated Digalacturonic acid was obtained as brown semisolid and exhibited a positive response to Molisch reagent. In the Mass spectrometry, it gave molecular ion peak at m/z 382 (M+2) \(^+\) corresponding to a molecular formula C\(_{14}\)H\(_{20}\)O\(_{12}\). The IR spectrum showed absorption bands at 3446,2988,1728,1600,1394,1075,and 668 cm\(^{-1}\).
which were in agreement with hydroxyl, carboxyl, olefinic and aliphatic groups. The $^1$H–NMR spectrum revealed the presence of two groups of equivalent proton signals at $\delta$ 9.02 and 85.34 resonated a singlet and multiplet respectively. The multiplet peaks at $\delta$ 3.58 – 3.68 depicted the presence of hydroxyl protons furthermore the $^{13}$CNMR spectrum displayed 14 carbon atoms corresponding to two galactopyranose rings joined by glycosidic linkage. Thus the compound II was identified for the first time as unsaturated Digalacturonic acid.

It has a molecular formula of $C_{14}H_{20}O_{12}$ based on the Mass spectrum exhibiting a molecular ion peak at m/z 382 (M+2)$^+$. The IR spectrum exhibited a strong absorption band corresponding to OH group (3446 cm$^{-1}$), CH stretching (2988 cm$^{-1}$), carbonyl stretching at 1728 cm$^{-1}$ and olefinic stretching at 1600 cm$^{-1}$.

The $^1$HNMR spectrum showed six hydroxyl protons at (9.02, 5.35, 5.58, 3.58, 3.68), 4.33. The $^{13}$CNMR indicated the presence of 14 carbons, including 2 carboxylic carbon (C-168.80 and 174.99), 5 oxy methene groups (C-101.28, 70.29, 74.35, 71.64, 62.50), 4 methene groups (C-127.64, 72.18, 35.63, 70.44), 2 methylene groups (at C-71.13, 66.83) and quarternary carbon at C-144.74. Therefore compound II was determined to be an Unsaturated Digalacturonic acid.