

10. ISOLATION AND CHARACTERIZATION OF MAJOR PHYTOCONSTITUENT

10.1 Introduction

Phytochemical investigation of a plant involves extraction of the plant material, separation and isolation of the constituent of interest, characterization of the compound and quantitative evaluations.

10.1.1 Chromatography

Chromatography is a technique for the separation of mixture of compounds by their continuous distribution between two phases one of which is moving past the other. The basis of separation of components of a mixture may be defined in term of adsorption, partition, ion exchange or gel chromatography [Beckett AH, 1997].

10.1.2 Column chromatography

Column chromatography is one of the oldest and most popularly used chromatographic techniques for use in analytical and preparative separation of components from plant extracts/mixtures. In column chromatography the separation of compound is based on adsorption at the solid liquid interface. For successful separation, the compounds of a mixture must show different degrees of affinity for a solid support (adsorbent) and the interaction between adsorbent and component must be reversible. As the adsorbent is washed with fresh solvent the various components will therefore move down the column until ultimately they are arranged in order of their affinity for the adsorbent. Those with least affinity move down the column at a
faster rate than and are eluted from the end of the column before, those with greatest affinity for the adsorbent.

10.1.3 Thin layer chromatography

Thin layer chromatography (TLC) is a technique in which substances are separated by the differential migration that occurs when the solvent flows along a thin layer of fine powder spread on a glass or plastic plate. TLC offers a faster and efficient separation. The substances most frequently used as coating materials are silica gel or alumina. The coating material may also contain an inorganic fluorescent indicator, which fluoresces when irradiated at a suitable wavelength. The wavelength of irradiation is specified by a subscript (Silica gel GF254). Silica gel and alumina are available with different specific surface areas and these grades are identified by a number (eg. Silica gel 60).

10.1.4 Mass Spectroscopy

Mass Spectrometry (MS) has proved to be one of the most effective techniques in biomedical research, particularly when complex matrices of biological samples must be analyzed. It is an accurate method to determine the molecular mass of the compound. The main advantages of MS are its high sensitivity, which allows analysis of compounds present in the microgram scale, and high specificity, as it is able to separate molecules of the same molecular weight but different atom composition, and sometimes even to differentiate stereoisomeric compounds. MS uses the electric and magnetic fields to produce electrically charged ions of chemical substance under analysis. Electro Spray Ionization (ESI) is a technique in which ions are generated by solvent evaporation under a high voltage potential, and can be applied directly, by infusion of the sample with a flow-controlled syringe, or coupled to separation techniques such as Liquid Chromatography (LC) or capillary
electrophoresis. In both cases, a steady liquid stream enters the system, allowing multiple analyses to be performed over a relatively large period of time. ESI interfaces are mostly coupled to quadrupole mass spectrometers; both are simple and robust equipments, able to produce either positive or negative ions, and their main limitation is the relatively limited $m/z$ range, usually below 2 kDa [Fossen T. 2005].

10.1.5 UV - Visible spectroscopy

The most important characteristics of spectrophotometric methods are their wide applicability, high sensitivity, moderate to high selectivity, good accuracy and ease of convenience. Ultra violet- Visible spectrophotometric analysis for any organic compound containing one or more of the chromophoric groups are potentially feasible, since a large number of organic molecule absorb radiation in the ultraviolet and visible region. The molecules having high molecular absorptivity may be directly determined. Those that do not, can be converted chemically into derivatives which have high molar absorptivity by suitable chemical reactions and can be determined colorimetrically [Sharma YR 2001].

10.1.6 Infra red spectroscopic analysis

IR spectrometry is a versatile tool applied to the qualitative and quantitative determination of molecular species of all types. The IR region of the electromagnetic spectrum extends from 12,800-10 cm$^{-1}$ and is sub divided into near IR (12,500-4000 cm$^{-1}$), middle IR (4000-400 cm$^{-1}$) and far IR (600-50 cm$^{-1}$). The fundamental region between 4000-600 cm$^{-1}$ provides the greatest information for the elucidation of molecular structure and most IR spectrophotometers are limited to measurements in this region.
10.1.7 Nuclear magnetic resonance spectroscopic analysis

Based on the measurement of absorption of electromagnetic radiation in the radio-frequency region of roughly 4-900 MHz, NMR spectroscopy is one of the most powerful tools available to the chemist and biochemist for elucidating the structure of a chemical species. It permits exploration of a molecule at the level of the individual atom and affords information concerning the environment of that atom. Only about one half of the known element isotopes, when placed in a magnetic field, absorb energy from the radio frequency range of the electromagnetic spectrum. Of these isotopes, $^1$H and $^{13}$C are the most important from the viewpoint of the organic and pharmaceutical chemist. The precise frequency from which energy is absorbed gives an indication of how an atom is bound to or located spatially with respect to other atoms. Proton NMR provides valuable information about the relative number of hydrogen and also their type, by comparison of the recorded chemical shifts with compiled data. $^{13}$C NMR data is used to complement $^1$H NMR data, and is particularly useful at establishing the type of groups present in the sample molecules by comparison with compiled data. Thus NMR offers an excellent physical means of investigating molecular structure and interactions.

10.2 Materials and Methods

Column chromatography was performed in a 54 x 4.5 cm glass column using Silica gel G (60-120 mesh) for column chromatography (SISCO research, Mumbai). For TLC analysis, Silica gel 60 F254 pre coated TLC plates of layer thickness – 0.2 mm were used (E Merck, Mumbai). For spot visualization, resublimed iodine and potassium permanganate were used. All melting points were determined in open capillaries. All solvents and chemicals used were of analytical grade. 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. Deuterated Dimethyl sulfoxide was used as the solvent. Mass spectra (GC/MS) were recorded on Agilent MSD VL mass spectrometer. An IR spectrum was recorded on a Jasco FT-IR spectrophotometer.

10.2.1 Column chromatographic isolation of major compound

The ethanol extract was subjected to Silica gel column chromatography for the isolation of the phytoconstituents.

10.2.2 Adsorption of the extract

About 10 g of the ethanol extract was dissolved completely in 100 ml of ethanol. To this added around 100 g of silica gel 60-120 mesh with proper mixing. This was kept in an oven at 37° C for overnight.

10.2.3 Charging of column

An appropriate column of 540 mm length and 45 mm diameter was used for the separation. The column was washed with water, rinsed with acetone and then dried completely. A cotton plug was placed at the bottom of the column with the help of a big glass rod, wetted using hexane. With the help of a funnel, silica gel 60-120 mesh was packed in the column up to about 25 cm. Above this the free flowing powder of adsorbed extract was packed for about 10 cm. Over this kept a layer of silica gel for about 3 cm followed by a cotton plug to prevent disturbance of the column while pouring the solvent. Hexane was run into the column until the bed become wet. The knob at the bottom was slowly opened to release the solvent. A small quantity of solvent was allowed to remain at the top of the column (about 4 cm) in order to prevent the drying and possible cracking of the packed column.
**10.2.4 Elution**

Elution of the charged column was done with solvents of varying polarity. At first 100 % hexane was used, then it is mixed with 10, 20, 30, 40, 50, 60, 70, 80, 90 % toluene, 100 % toluene, 10, 20, 30, 40, 50, 60, 70, 80, 90 % ethyl acetate, 100 % ethyl acetate, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 % chloroform, 10, 20, 30, 40, 50, 60, 70, 80, 90 100% methanol. Around 100 fractions were obtained.

**10.2.5 Column requirement**

Stationary phase : Silica gel 60-120 mesh

Charged material : Ethanol extract

Mobile phase : Hexane, Toluene, ethyl acetate, chloroform, methanol.

Rate of elution : 20 drops/ min Volume of each Fraction : 100 ml.

**10.2.6 Thin layer chromatography (TLC)**

As soon as the fractions were eluted, it was analyzed by using silica gel 60 F254 pre coated TLC plates with suitable developing solvent according to the polarity of elute. The developed chromatogram was observed under UV, kept in iodine chamber and heated on a hotplate for the observation of spots. Similar fractions were pooled based on TLC profiles [Alagarmalai Jeyasankar, 2011: Arisawa, M 1995].
10.2.7 Isolation of compound

The ethanolic extract of *Glycosmis pentaphylla* was subjected to column chromatographic separation to isolate their phytoconstituents. The concentrated extract was run in various combinations of solvent systems for thin layer chromatography. The solvent system which gave maximum separation of spots was chosen as the appropriate mobile phase to be used for column chromatography for the purpose of separation. The column was filled with a uniform slurry of silica (stationary phase) of 60-120 mesh size and the concentrated extract was loaded. For starting the separation process, the ethanolic extract of *Glycosmis pentaphylla* was chromatographed separately over silica gel. Elution was carried out with solvents and solvent mixtures of increasing polarity. Fractions were collected and monitored by TLC. The fractions showing similar spots were combined. All the major fractions were rechromatographed over silica gel or recrystallized to isolate the pure compounds.


10.3.1 Mass Spectroscopy

Mass spectra (GC/MS) were recorded on Agilent MSD VL mass spectrometer. Sample was dissolved in deuterated chloroform (CDC13) and injected through direct probe inlet. Electron impact ionization method was used.

10.3.2 UV –Vis spectroscopy

The analysis was performed on Double beam Perkin – Elmer UV Visible spectrophotometer. The isolated compound was dissolved in methanol and scanned in the region between 200-400 nm.
10.3.3 Infra red spectroscopic analysis

Infrared spectra was recorded on a Jasco FT-IR spectrophotometer. Sample preparation was done by KBr pellet method and it was scanned between 600 to 4000 cm\(^{-1}\).

10.3.4 Nuclear magnetic resonance spectroscopic analysis

Nuclear magnetic resonance spectra were recorded on a Bruker 200 MHZ instrument using Tetramethyl silane as an internal reference for both \(^1\)H and \(^{13}\)C NMR experiments. Deuterated dimethyl sulphoxide was used as the solvent.

10.4 Results and Discussion

10.4.1 Column Chromatography of ethanolic extract of \textit{Glycosmis pentaphylla}

The ethanolic extract of \textit{Glycosmis pentaphylla} was chromatographed separately over silica gel. Elution was carried out with solvents and solvent mixtures of increasing polarity (Table10.1) Fractions were collected and monitored by TLC.

Selection of Fraction

The ethanolic extract of the roots of \textit{Glycosmis pentaphylla}, eluted with toluene : ethyl acetate (60:40) fractions number 7 gave a yellow colour crystalline powder which showed major individual spots in TLC. Thus the fractions were mixed together and rechromatographed with the solvent system. The homogeneity of the compound was checked with running the TLC of the samples in different mobile phases. After confirmation of the presence of individual spot, the compound was designated as GP-I.
### Table 10.1 Fractions of Column Chromatography of ethanolic extract of *Glycosmis pentaphylla*

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Solvent System</th>
<th>Ratio</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n-Hexane</td>
<td>100</td>
<td>Colourless</td>
</tr>
<tr>
<td>2</td>
<td>Toluene</td>
<td>100</td>
<td>Slight Yellow</td>
</tr>
<tr>
<td>3</td>
<td>Toluene : Ethyl acetate</td>
<td>100</td>
<td>Dark Yellow</td>
</tr>
<tr>
<td>4</td>
<td>Toluene : Ethyl acetate</td>
<td>90:10</td>
<td>Greenish Yellow</td>
</tr>
<tr>
<td>5</td>
<td>Toluene : Ethyl acetate</td>
<td>80:20</td>
<td>Yellowish Green</td>
</tr>
<tr>
<td>6</td>
<td>Toluene : Ethyl acetate</td>
<td>70 : 30</td>
<td>Yellow</td>
</tr>
<tr>
<td>7</td>
<td><strong>Toluene : Ethyl acetate</strong></td>
<td><strong>60 : 40</strong></td>
<td><strong>Yellow</strong></td>
</tr>
<tr>
<td>8</td>
<td>Toluene : Ethyl acetate</td>
<td>50 : 50</td>
<td>Yellow</td>
</tr>
<tr>
<td>9</td>
<td>Toluene : Ethyl acetate</td>
<td>40 : 60</td>
<td>Yellow</td>
</tr>
<tr>
<td>10</td>
<td>Toluene : Ethyl acetate</td>
<td>30 : 70</td>
<td>Yellow</td>
</tr>
<tr>
<td>11</td>
<td>Toluene : Ethyl acetate</td>
<td>20 : 80</td>
<td>Light Yellow</td>
</tr>
<tr>
<td>12</td>
<td>Toluene : Ethyl acetate</td>
<td>10 : 90</td>
<td>Yellow</td>
</tr>
<tr>
<td>13</td>
<td>Ethyl acetate</td>
<td>100</td>
<td>Light Yellow</td>
</tr>
<tr>
<td>14</td>
<td>Chloroform</td>
<td>100</td>
<td>Colourless</td>
</tr>
<tr>
<td>15</td>
<td>Chloroform : Methanol</td>
<td>90 : 10</td>
<td>Light Yellow</td>
</tr>
<tr>
<td>16</td>
<td>Chloroform : Methanol</td>
<td>80 : 20</td>
<td>Yellowish Brown</td>
</tr>
<tr>
<td>17</td>
<td>Chloroform : Methanol</td>
<td>70 : 30</td>
<td>Yellowish Brown</td>
</tr>
<tr>
<td>18</td>
<td>Chloroform : Methanol</td>
<td>60 : 40</td>
<td>Yellowish Brown</td>
</tr>
<tr>
<td>19</td>
<td>Chloroform : Methanol</td>
<td>50 : 50</td>
<td>Yellowish Brown</td>
</tr>
<tr>
<td>20</td>
<td>Chloroform : Methanol</td>
<td>40 : 60</td>
<td>Light Yellow</td>
</tr>
</tbody>
</table>

### 10.4.2 Physical and chemical properties of isolated compound

- **Appearance**: Yellow amorphous powder
- **Percentage yield**: 0.7 %
Solubility: Soluble in ethyl acetate, alcohol, and dimethyl sulphoxide, insoluble in water

Melting point: 185-192 °C

Rf Value: 0.51

Molecular formula: C_{14}H_{14}O_{4}

Molecular weight: 246

Chemical Test: Evaporate 5 ml of ethanolic solution, dissolve the residue in 1-2 ml of hot distilled water and divide the volume into two parts. Take half the volume as a witness and to add another volume of 0.5 ml 10% NH_{4}OH. Put two spots on filter paper and examined under UV light. Intense fluorescence indicates the presence of furanocoumarins.

10.4.2 Spectral data of isolated compound

10.4.2.1 Mass spectrum of isolated compound

Electrospray ionization (ESI) is considered to be a soft ionization process, since the internal energy of the formed ions is low, little or no fragmentation takes place. This feature is very interesting for identification of the molecular weight or molecular formula. However, it represents an inconvenience for structural elucidation studies, which are based on fragment ions. The molecular formula, C_{14}H_{14}O_{4} was established by MS (EI+)M+1 m/z = 246.09. The compound with molecular weight exhibit m/z peak at 247 for [M-H]_{+} ion in its mass spectrum. The mass spectrum of the compound was given in figure 10.1
10.4.2.2 UV visible spectrum of isolated compound

In order to investigate the solution optical property of the isolated compound, its UV spectrum was performed in the methanol solution. The spectrum displayed two major absorption bands in the region of 400~200 nm, appearing at 335 nm which are associated with the Furanocoumarin derivative in the molecule, respectively [Wang Yong 2014]. The absorption spectrum of the extract is shown in (Figure 10.2). The \( \lambda \) max of ethanolic extract was found to be 1.4799 at 335 nm.
10.4.2.3 IR spectrum of isolated compound

In its IR (KBr) cm\(^{-1}\) spectrum it exhibits a broad band at 3279 cm\(^{-1}\) for the hydroxyl group, band at 1631.18 cm\(^{-1}\) for C=O group, band at 1568 cm\(^{-1}\) and 1178 cm\(^{-1}\) for C-O-C group. [Gulten Kavik 2009]. The IR spectrum is presented in Figure 10.3.

10.4.2.4 \(^1\)H NMR spectrum of isolated compound

In its \(^1\)H NMR (CDCl\(_3\)) spectrum it exhibits peak at \(\delta\) 1.595 (6H, s) for two methyl groups, peak at \(\delta\) 2.043 (1H, s) for OH group, peak at \(\delta\) 4.244 (2H, t) for CH group present in furan nucleus, peak at \(\delta\) 6.9, 7.2, 9.0 are present in the benzopyran nucleus. The \(^1\)H NMR spectrum of the isolated compound is given in Figure 10.4.
Figure 10.3 IR spectrum of the isolated Compound GP-I
Figure 10.4 $^1$HNMR spectrum of the isolated compound GP-1
Figure 10.5 $^{13}$CNMR spectrum of the isolated compound GP-1
Figure 10.6 $^{13}$CNMR spectrum of the isolated compound GP-1
10.4.2.5  $^{13}$C NMR Spectrum of isolated compound

In its $^{13}$C NMR it exhibits peak at $\delta$ 26.955 for methyl groups, peak at $\delta$ 79.558 for tertiary alcohol, peak at $\delta$ 160.269 for C-O group in the furan/benzopyran nucleus, peak at $\delta$ 97.43, 100.75, 113.96, 117.7, 124.397, 136.40, and 140.79 for carbon present in the furan/benzopyran nucleus. The $^{13}$C NMR spectrum of the isolated compound is given in Figure 10.5.

Based on the characterization the proposed structures is

![Chemical structure](image)

**Figure 10.7** Structure of 7H-Furo (3, 2-G) (1) Benzopyran-7-one, 2, 3 – dihydro – 2 - (1-Hydroxy-1methylethyl) - (s) - or Marmesin

Molecular formula: $\text{C}_{14}\text{H}_{14}\text{O}_4$
10.4 References


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