CHAPTER 6

THE PREPARATION AND SEPARATION OF PHYTOACTIVE COMPOUNDS

Abstract

This Chapter deals with the materials and methods employed for the preparation of crude extract of flower pigments of the plant Cassia Fistula Linn. and chromatographic techniques employed for the separation of phytocompounds in it. The classical chemical procedure for obtaining organic constituents from dried plant tissues was employed for this. TLC and column chromatographic techniques were then used to separate some fractions of the crude extract.
6.1. **Introduction**

The main objective of the present investigation is to study the inhibitory effect of various factors on cholesterol growth. The author took efforts to extract and characterize the different flower pigments of Cassia Fistula Linn. and then study their effect on the cholesterol crystal growth in gel medium. To achieve this aim, the most suitable experimental strategy was adopted. The details regarding apparatus, chemicals and analytical techniques for the extraction and separation of phytocompounds in the flowers of cassia fistula are presented in this chapter.

First we consider the experimental set up for the preparation of crude extract of the flower pigments of the plant chosen. The classical chemical procedure for obtaining organic constituents from dried plant tissues was employed for this. Thin Layer Chromatography and Column chromatography were the techniques then used to separate some fractions of the crude extract.

The components separated were identified and were set aside to use, along with the crude extract, as additive material in cholesterol growth studies.

6.2 **Experimental set up**

The important apparatus used for the extraction and separation of phytocompounds in the flower pigments of Cassia Fistula Linn. are listed below.

- Soxhlet apparatus
- Conical flask
- Beakers
- Chromatographic chamber
- Homogenizer
- Measuring Jar
- Separating Funnel
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- Micropipette
- Slide and scissors
- Test tubes
- Watch glass
- Weighing balance
- Water bath
- UV trans-illuminator.
- Glass rods
- Chromatographic Tanks
- Chromatographic plates
- Iodine chamber
- Adsorption column

6.3. Materials used for extraction procedure

- Dried powered flowers of Cassia Fistula Linn
- Pure ethanol
- Petroleum Ether
- Acetone (AG)
- Methanol (AG)
- Silica Gel (AG)
- Chloroform (AG)
- Acetic acid
- Iodine globules
- Amyl alcohol
- Ammonium hydroxide
- Diethyl ether
- Ethyl acetate
- Ethyl alcohol
- Hydrochloric acid
- Sodium hydroxide
6.4. **Extraction methods**

For obtaining organic constituents from the dried plant tissues Soxhlet Apparatus was used, which is an extraction equipment popularly used in chemical and pharmaceutical laboratories. The extract of any organic material in suitable solvents can be taken without much loss of volatile solvent. The extraction unit consists of a round bottomed flask (borosil) in which the plant material and solvent are taken. After continuously heating the flask in water bath for about five hours a crude extract is obtained. The important steps involved in the extraction of Cassia Fistula Linn. flowers pigments and separation of the compounds in it are given below.

6.4.1. **Pre-treatment of flowers.**

The fresh flowers and flower buds of CF were collected during the month March to April. The flower petals were separated and washed to remove the dust particles and shadow-dried.

6.4.2. **Preparation of the extract of flower pigments**

Standardized protocols developed by Harborne (1973) and Daniel (1991) were employed for the extraction of flower pigments. The solution was separated into two layers and they were collected in separate fraction collectors. The upper layer which contains petroleum ether was discarded. The lower layer was then mixed with 25 ml ethyl acetate in another separating funnel. Two layers were...
formed. The lower layer was collected in a fraction collector. The upper layer was re-extracted with amyl alcohol. The ethyl acetate extract and amyl alcohol extract were used for further analysis.

6.4.3. Separation of pigments by paper chromatography

The ethyl acetate extract was concentrated and dissolved in 10 ml ethanol. Similarly the amyl alcohol extract was concentrated and dissolved in 10 ml of 1% methanolic HCl. A few drops of both the fractions were loaded on a strip of chromatographic paper (Wattman No. 1). The chromatograms were developed on forestall (acetic acid: conc. HCl: water- 10:3:30). The developed chromatogram was allowed to dry and was viewed in UV, NH₄OH + UV and in iodine. The distance traveled by the different solute and solvent fronts were measured and their R_f values were determined with respect to the bands and spots separated on paper chromatogram. Various components separated by solvent extraction were used for UV- spectral analysis. The spectra of each were recorded in Shimadzu UV -160A spectrophotometer.

6.4.4. Qualitative test for the phytocompounds present in flower extract

Preliminary qualitative test for the identification of flavonoid pigment (Harborne, 1973) was conducted with respect to both fractions in ethyl acetate and amyl alcohol. The test solutions of both fractions were tested with the chemicals

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2N sodium hydroxide and 2% hydrochloric acid. The observations are presented in Table.6.1.

Table 6.1. Qualitative test for the identification of flavonoid pigment

<table>
<thead>
<tr>
<th>Test</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test I</td>
<td>To 1ml of ethyl acetate fraction 1ml of 2N NaOH was added. Yellow color observed conformed the presence of the flavanoid.</td>
</tr>
<tr>
<td>Test II</td>
<td>When 1ml of 2% hydrochloric acid was added to the above solution obtained in test I, it turned colourless. This test again confirmed the presence of flavanoid.</td>
</tr>
<tr>
<td>Test III</td>
<td>To 1ml of amyl alcohol solution, 1ml of 2N NaOH was added. Here too the observed color in the test solution shows the presence flavanoid.</td>
</tr>
<tr>
<td>Test IV</td>
<td>Again to the solution resulting in Test III, 1ml of 2% HCl was added. This too resulted in turning the test solution colorless confirming the presence of flavanoid</td>
</tr>
</tbody>
</table>

6.4.5. Conformation tests for the phytocompounds present in flower Extract.

Petroleum ether (hbp) of CF flower was tested for Liebermann – Burchard Reaction test. A play of colors from green to red, brown and then violet was seen, which indicate the presence of plant steroids. The suspected steroid is β-sistasterol,
which is not yet published in literature. The characteristic test for the content carotenoid was also found to be positive. Thus the flower extract was confirmed for the presence of steroids and carotenoids. This result is not yet found to be reported in literature.

6.5. **Identification of subcomponents of flavanoid pigment by Paper Chromatography**

The two fractions, that is, Ethyl acetate fraction and Amyl alcohol fraction obtained by solvent extraction were subjected to paper chromatography. Two-dimensional paper chromatography was successfully employed for the separation and characterization of flower pigments. The developed chromatogram was viewed in UV (360nm), UV+NH₄OH, and iodine. The results of the experiment conducted with respect to two fractions are presented in the following table-2. The ethyl alcohol fraction yielded 3 components and the other fraction 9 components.

Thus the Chromatographic study conducted with respect to the flower pigments of CF during this study revealed the presence of 12 subcomponents of the flavanoid pigments. The observations on the compounds identified are listed in the table. 6.2.
The classical chemical procedure for obtaining organic constituent from dried plant tissues was employed. The Soxhlet Apparatus was employed as this
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extraction equipment popularly used in chemical and pharmaceutical laboratories. The extract of any organic material in suitable solvent can be taken without any loss of viable solvent is possible in this.

6.7. Result and Discussion of Phytochemical Analysis

The standard Rf values of different components of flavanoid presented by Daniel (1991) were used for comparison and hence identification of the components in the flower extract of Cassia. The present study revealed the presence of 11 major bioactive compounds in the flowers of Cassia fistula Linn. The main components identified were flavanoids, caratenoids, plant steroids and lipid fractions.

6.8. Bibliography

7. Wong, E. “Biosynthesis of flavanoids”, pp.464-524 (Goodwin, T.W.eds.).


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