3. EXPERIMENTAL

3.1 Plants selected for Quantification:

- *Colchicum luteum*
- *Gloriosa superba*
- *Iphigenia indica*

3.2 Techniques used for comparative study:

- HPLC
- HPTLC

HPLC and HPTLC are the most efficient and sophisticated efficient techniques for the identification and quantification of components.

Colchicine was purchased from Sigma Aldrich, USA. All the reagents purchased were of analytical grade. Methanol was purchased from Hi media and is of HPLC grade.

All the other chemicals and solvents of analytical grade were used without any further purification. Analytical grade water was obtained using water purification systems ELIX 03 (MILLIPORE, USA).

3.3 Extraction procedure:

The crushed corms of *C. luteum, G. superba* and *I. indica* were defatted with petroleum ether by percolation and the extraction of the marc was done by percolation, sonication
and Soxhlation in triplicate using CHCl₃ as a solvent. 20 g of plant material was taken for each extraction (fig. 4).

**Figure 4**

**3.4 RP-HPLC method development**

**3.4.1 Instrumentation**

Analysis was performed using instruments such as HPLC system of WATERS (Milford, USA) composed of 515 series pumps combined with Waters 2707 autosampler along with Waters PDA 2998 series photodiode array detector set at wavelength range 190-800 nm with column from Waters Spherisorb® C₁₈ bonded with 5 µm (4.6 x 250 mm) coupled with EMPOWER-2 software recording and processing of chromatographic data. Ultrasonic cleaner (Steryl medi-equip systems) and water purification system ELIX 03 (MILLIPORE, USA).
3.4.2 Methods

3.4.2.1 Sample preparation

Stock solutions of colchicine (marker) and samples, 1mg/ml were prepared in methanol and stored at 2–8°C until used. Aliquots from each stock solution were diluted stepwise with methanol to obtain 100 µg/ml.

3.4.2.1 Optimization of chromatographic conditions

The effects of different chromatographic conditions on the instrument response create a situation where one has to compromise between different experimental variables in order to achieve the best chromatographic separation. Chromatographic separations are significantly affected by the mobile phase conditions, such as the type and composition of the organic modifiers (Kanji et al., 1998) and therefore before selecting the conditions for the optimization, a number of preliminary trials were conducted with combinations of different organic solvents, compositions, and flow rate to check the retention time, shape, resolution, and other chromatographic parameters. Among all tried experiments, the mobile phase combination of H2O, MeOH and formic acid in the ratio of (50: 50:0.1 v/v/v) with isocratic elution at flow rate of 1.0 ml/min was found to be most suitable.

Best resolution and sensitivity of the method was obtained for colchicine at 352 nm. Typical chromatogram with optimized condition gives sharp and symmetric peak with retention time of 3.82 min.
3.4.2.2 Validation of optimized method

After chromatographic method development and optimization, method was validated. Optimized method was validated according to ICH guidelines for linearity, sensitivity, precision and recovery studies have been carried out (ICH Guidelines, 1994; ICH Guidelines, 1996).

3.4.2.3 Calibration curve (linearity)

Linearity was determined by six different concentrations of colchicine in triplicate and calibration curve was plotted in range of 1-100 μg/ml of colchicine. Calibration curve was plotted by replicate analysis at all concentration levels and linear relationship was evaluated using the least square method with Microsoft® Excel program.

3.4.2.4 Precision

Intra-day precision and inter-day precision for the developed method was determined in terms of percent relative standard deviation (% RSD.). The experiments were repeated three times a day for intra-day precision and on three different days for inter-day precision. The concentration values for both intra-day precision and inter-day precision were calculated six times separately and % RSD were calculated.

3.4.2.5 Detection and quantification limits (LOD and LOQ)

Limits of detection and quantification were calculated by method based on standard deviation (σ) and slope (S) of calibration plot using formula LOD = 3.3σ/S and LOQ = 10σ/S.

3.4.2.6 HPTLC Densitometric Method Development and Validation

High performance thin layer chromatography (HPTLC) method was developed and optimized for colchicine (marker). Method development involves evaluation and
optimization of the various stages of sample preparation, chromatographic separation, detection and quantification. Optimization of various parameters were performed in order to develop a selective and sensitive method for analysis of colchicine on high performance thin layer chromatography (HPTLC) using photo diode array detector (PDA).

3.4.3 Instrumentation

Camag HPTLC system equipped with a sample applicator Linomat 5 TLC Scanner III, Reprostar and Wincats 4.02 integration software (Switzerland), twin trough glass development chamber, Ultrasonic cleaner (Steryl medi-equip systems) and water purification system ELIX 03 (MILLIPORE, USA) were used during study.

3.4.3.1 Standard stock solution

Stock solutions of colchicine (marker) and samples, 10mg/ml were prepared in methanol and stored at 2–8°C until used.

3.4.3.2 Sample application

Samples were spotted as a band of 8 mm width on pre-coated silica gel (10x20 mm) aluminium backed plate 60f-254 with 200 µm thickness. Application rate of 20 µl per sample was employed and space between bands was 10 mm. Linear ascending development was carried out in a twin trough glass chamber previously saturated with mobile phase for 20 min. Densitometric scanning was performed on Camag TLC scanner III in the absorbance/reflectance mode.
Chromatographic separations are significantly affected by mobile phase conditions, such as type and composition of organic modifiers (Kanji et al., 1998) and therefore before selecting proper chromatographic conditions, numbers of preliminary trials were conducted with different combinations of different organic solvents, compositions, to obtain satisfactory retention factor, resolution, and other chromatographic parameters. From those experiments mobile phase combination of ethyl acetate: ethanol: formic acid (9: 1: 0.01 v/v/v) was found to be most suitable. Best resolution and sensitivity of the method for colchicine was detected at 352 nm. Typical chromatogram with optimized condition gave sharp and resolved peak with retention factor at 0.15.

3.4.3.3 Validation of optimized method

After chromatographic method development and optimization, method was validated. Optimized method was validated according to ICH guidelines for linearity, precision, accuracy, specificity, sensitivity, recovery and robustness (ICH Guidelines, 1994; ICH Guidelines, 1996).

3.4.3.4 Calibration curve (linearity)

Linearity was determined by six different concentrations of colchicine in triplicate and calibration curve was plotted in specified range of 200-1000 μg/spot. Calibration curve was plotted by replicate analysis at all concentration levels and linear relationship was evaluated using the least square method with Microsoft Excel program.
3.4.3.5 Precision

Intra-day precision and inter-day precision for the developed method was determined in terms of percent relative standard deviation (% RSD.). The experiments were repeated three times a day for intra-day precision and on three different days for inter-day precision. The concentration values for both intra-day precision and inter-day precision were calculated six times separately and % RSD were calculated.

3.4.3.6 Detection and quantification limits (LOD and LOQ)

Limits of detection and quantification were calculated by method based on standard deviation (σ) and slope (S) of calibration plot using formula LOD = 3.3σ/S and LOQ = 10σ/S.