5.1) ANALYTICAL METHOD DEVELOPMENT AND VALIDATION

The chemical standardization of herbal drugs requires different analytical methods depending on the number of markers and instruments available. This section of the study deals with the development and validation of some analytical methods for the markers of the drugs used in the study as the marker available play a major role in monitoring the therapeutic potential of the drug and at the same time are responsible for the narrow therapeutic index.

Different analytical methods have been reported for the determination of Glycyrrhizin [1-21], Quercetin [22-32], Rosmarinic acid [33-43], Betulinic acid [44-57], which involved methods using, spectrophotometry, RP-HPLC with UV detection, HPLC with DAD, Ion pair HPLC, HPLC with tandem MS GC-MS etc. Some groups have described HPLC methods for the analysis of four markers individually in biological fluids also.

The following is a brief and condensed description of the methods developed which can offer new analytical possibilities for the determination of content and identity of drug in their combination dosage form.

The spectrophotometric, spectrofluorimetric, HPLC and HPTLC methods were developed for analyzing the biomarkers in the formulations. Apparatus and materials requirement for all the methods were similar and are described in the following section.

5.1.1) MATERIALS AND METHODS

5.1.1.1) Instrumentation

5.1.1.1.1) Apparatus and software for HPLC measurement (Isocratic)

The chromatographic system (Shimadzu, Kyoto, Japan) consisted of Shimadzu LC-20 AT Prominence solvent delivery module, a manual Rheodyne injector with a 20 µl fixed loop and a SPD-M20A Prominence Photo Diode Array (PDA) detector. The separation was performed on a Phenomenex C_8 column (particle size 5 µm; 250 mm X 4.6 mm ID; Phenomenex Torrance, USA) preceded by an ODS guard column (10 µm, 10 mm × 5 mm ID) at an ambient temperature.

Chromatographic data were recorded and processed using a Spinchrom Chromatographic Station® CFR Version 2.4.0.193 (Spinchrom Pvt. Ltd., Chennai, India).

5.1.1.1.2) Apparatus and software for HPLC measurement (gradient)

A Waters Alliance HPLC system, which consisted of a solvent management system 2998, a photodiode array detector (PDA) 2998, a with a 20µl loop, was used. Control of the HPLC system (515 HPLC pump) and data processing were performed using the Empower®3 software (All Waters, Millford, MA, USA). A phenomenex C_{18} column (particle size 5 µm; 250mm × 4.6 mm ID; Phenomenex Torrannce, USA) preceded by an ODS guard column (10 µm, 10mm × 5mm i.d.) at an ambient temperature. Chromatographic data were recorded and processed using a Spinchrom chromatographic station ® CFR Version 2.4.0.195 (Spinchrom Pvt. Ltd., Chennai, India).

5.1.1.1.3) Apparatus and software for HPTLC measurements

A Camag microlitre sample (Hamilton, Bonaduz, Switzerland) syringe was used for sample application on pre-coated silica gel aluminium plate 60F-254, (20 cm x 10 cm with 0.2 mm thickness, (E. Merck, Darmstadt, Germany) using a Camag Linomat-V (Switzerland). The linear ascending development was carried out in 20 cm X 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). Dimensions: length X width X height =12 cm X 4.7 cm X 12.5 cm. Densitometric scanning was performed on Camag TLC scanner III in the reflectance-absorbance mode for all measurements and operated by CATS software (V1.4.3 Camag).

5.1.1.1.4) Apparatus for Spectrofluorimetric measurement

A Shimadzu spectrofluorometer (Tokyo, Japan Model RF-5301PC with RFPC software), and fused fluorescence free quartz square 10 mm cell having capacity of 5 ml was employed for all spectral and fluorescence measurements.

5.1.1.1.5) Apparatus and software for spectrophotometric measurement

Spectrophotometric measurements were carried out on a Shimadzu 1700 double beam UV Visible spectrophotometer with a fix slit width of 1 nm coupled HP7540 computer loaded with Shimadzu UV PC software of version 2.10. The spectral bandwidth was 1 nm and the wavelength scanning speed was 2800 nm/min Matched quartz cuvettes (1cm) were used for all the spectral measurements.

Ultrasonic bath (Ultrasonics Selec, Vetra, Italy) was used in the study wherever required.

The pH was measured using Lab India pH-meter (TICO+), Mumbai.

5.1.1.1.6) Markers and Formulation

Standard glycyrrhizin (95% by HPLC), quercetin (97%) and betulinic acid (90%) were all purchased from Sigma Aldrich Pvt. Ltd. (USA) whereas rosmarinic acid (95%) was purchased from Natural Remedies, Bangalore. **Polyherbal formulation (Form B) was developed which discussed in chapter 6.**

5.1.2) HPLC method for simultaneous estimation of Glycyrrhizin and Betulinic acid in Herbal formulation (Method I)

5.1.2.1) Chemicals and Reagents

Acetonitrile and methanol were of HPLC grade (Spectrochem, Mumbai). Potassium di-hydrogen phosphate (KH_2PO_4), Tri-ethylamine and 0.03% *ortho*-phosphoric acid (H_3PO_4) of analytical-reagent grade were also purchased from the same supplier. Triple distilled water was used throughout the study. All the other solvents and reagents used were of analytical grade and were filtered through a 0.2 μm Ultipor ® Nylon 66 membrane filter (Pall Life Sciences, USA) prior to use.

5.1.2.2) Preparation of Standard Solutions

Standard stock solutions of glycyrrhizin and betulinic acid (1000μg/ml) were prepared by dissolving 10 mg of pure drug in 10 ml methanol. Appropriate and accurate aliquots of the stock solutions were transferred to 10 ml calibrated flasks and diluted up to the volume with mobile phase in the range of 10-100μg/ml.

5.1.2.3) Preparation of sample solutions

Twenty tablets of the formulation was powdered and about 1gm of the formulation was accurately weighed and extracted in 25 ml of methanol by sonication for 20 minutes at room temperature and filtered with 0.2μm Ultipor Nylon 66 membrane filter paper and further dilutions were made with mobile phase.

5.1.2.4) Analytical conditions

The measurement of Glycyrrhizin and Betulinic acid were carried out on Oyster C_8 column (250 mm × 4.6 mm, 5μm) at a room temperature with a flow rate 1ml/min at 254 nm and 210 nm respectively. The mobile phase consisted of acetonitrile and 0.03% phosphoric acid. (90:10).

Analysis was isocratic at 1.0 ml/min flow rate with water (pH 3.1 was adjusted using OPA): acetonitrile (10:90 v/v) as mobile phase. The mobile phase was prepared freshly every day. The mobile phase was filtered through a 0.2 μm membrane filter to remove any particulate matter, mixed and degassed by sonication before use. The

absorbance of glycyrrhizin was good at 254 nm and betulinic acid was at 210nm but the common wavelength is 230nm and further it was free from any interference.

Hence, the eluted peak was detected at 230nm. The sensitivity of the detector was set at 0.01 AUFS. Prior to injecting solutions, the column was equilibrated for at least 60 min with the mobile phase flowing through the system. Each solution was injected in triplicate, and the relative standard deviation (RSD) was required to remain below 1.0% on peak area basis.

5.1.2.5) Optimization of Chromatographic conditions

Chromatographic separations are significantly affected by the mobile phase conditions, such as the type and composition of the organic modifiers. Therefore, before selecting the conditions for the optimization, a number of preliminary trials were conducted with different combinations of different organic solvents and buffers at various pH, mobile phase compositions, and flow rate to check the retention time, shape, resolution, and other system suitability parameters of all the peaks.

In order to achieve an optimum separation, following conditions were studied:

(i) Mobile phase pH varied at 3, 3.5 and 5 keeping the composition of water: Acetonitrile (50:50 v/v) and flow rate of 1.0 ml/min fixed. (ii) Mobile phase composition varied at 40:60, 30:70 and 10:90 (v/v) with pH and flow rate kept constant at 3.1 and 1.0 ml/min, respectively. (iii) Flow rate was varied (0.8, 1.0, and 1.2 ml/min) with mobile phase composition and pH maintained at 10:90 (v/v) and 3.1, respectively. The effects of all these three factors were systematically addressed on system suitability parameters such as resolution, theoretical plates, retention time, capacity factor, separation factor, asymmetry, and HETP etc.

All mobile phases used in optimization study were prepared by mixing the water/buffer with the organic solvent in the desired proportions.

5.1.2.6) Method Validation

In order to verify that the proposed method is applicable to formulation analysis, validation was performed as per the ICH Guidelines [58, 59].

a) *Calibration curve (linearity)*

Six different concentrations of glycyrrhizin and betulinic acid were analyzed and their calibration curve was constructed in the specified concentration range (10-100µg/ml). The calibration plots were generated by replicate analysis (n = 3) at all concentration

levels and the linear relationship was evaluated using the least square method within Microsoft Excel® program.

b) Repeatability, precision and stability

The injection repeatability was determined by the analysis of six continuous injections using the same sample, while the analysis repeatability was examined by the injection of six different samples prepared by the same procedure. The standard solution (10, 40, 100µg/ml) was used for the test of injection repeatability and analysis repeatability.

The instrument precision was examined by performing the intra-day and interday assays of six replicate injections of the standard solutions at three concentration levels (10, 40, 100µg/ml). The intra-day assay precision was performed at the interval of 4 h in 1 day, while the inter-day assay precision was performed over 6 days.

c) Limit of detection and limit of quantification

LOD and LOQ were determined by *kSD/s* where *k* is a constant (3 for LOD and 10 for LOQ), *SD* is the standard deviation of the analytical signal, and *s* is the slope of the concentration/response graph.

d) Specificity

Specificity is the ability of the analytical method to measure analyte response in the presence of interferences present in the sample matrix.

e) Robustness

Robustness of the proposed method was evaluated by keeping the chromatographic conditions constant, except for the followings changes:

1) Detection wavelength: Changed from 254nm to 252 nm and 256nm for Glycyrrhizin and 210nm to 208nm and 212nm

2) Column: Using another column (Hypersil ODS, particle size 5 µm; 250 mm X 4.6 mm ID)

3) Solvent Brand: Acetonitrile supplied by Qualigen Pvt Ltd. (Mumbai, India) and Rankem (Mumbai, India).

Standard solution was injected 6 times for each change. System suitability parameters like resolution, peak asymmetry, theoretical plates, capacity factor, and RSD were

calculated for each peak. Recoveries and % RSDs were calculated during each change.

f) Accuracy

The accuracy of the method was determined by calculating the recoveries of glycyrrhizin and betulinic acid by the method of standard additions. Known amount of standard (80%, 100% and 120 %) was added to the pre-analyzed sample solution, and the amounts of the standard was estimated by measuring the peak area and by fitting the value to the straight-line equation of calibration curve.

5.1.3) HPLC method for simultaneous estimation of glycyrrhizin, quercetin, rosmarinic acid and betulinic acid in Herbal formulation (Method II)

5.1.3.1) Preparation of Standard solution

Approximately 5 mg of standard glycyrrhizin, 5 mg of standard quercetin, 5 mg of standard rosmarinic acid and betulinic acid were weighed precisely and dissolved in 5 ml of methanol obtaining stock concentrations of 1000 µg/ml. The curves were found to obey Beer's law in the concentration range of 20-80 µg/ml for glycyrrhizin, quercetin, betulinic acid and 5-20µg/ml for rosmarinic acid. The stock solutions were stable for few weeks in refrigerator.

5.1.3.2) Preparation of sample solution

About 1gm of the formulation was powdered and then extracted in 25 ml of methanol by sonication for 20 minutes at ambient temperature and further dilutions were made in mobile phase.

5.1.3.3) Analytical conditions

HPLC separation of four markers was achieved using a reversed phase Phenomenex-C_{18} column (250mm×4.6mm i.d., 5µ particle size). A gradient mobile phase system consisting of (A) water (pH 3.1 adjusted with 0.03% ortho phosphoric acid) and (B) acetonitrile was used. The injection volume was 10 µl and the detector was set at a wavelength range of 200–400nm with a spectral resolution of 1.2 nm, a sampling rate of 10 points/s and fifth level spectral smoothing. The purity parameters included a 100% active peak region and an auto-threshold. The mobile phase was filtered by a 0.2 µm membrane filter to remove any particulate matter, mixed and degassed by sonication before use. Analysis was performed using PDA-detection at 239 nm for rosmarinic acid, quercetin and glycyrhhizin and betulinc acid. The flow rate is 1.0

ml/min. Each solution was injected in triplicate. The relative standard deviation (RSD) was required to remain less than 1.0% on peak area basis.

The chromatograph is programmed as follows:

Time	Solution A (%)	Solution B (%)	Elution
0.00	80	20	Isocratic
4.0	60	40	Linear gradient
8.8	40	60	Linear gradient
11.0	20	80	Linear gradient
12.0	10	90	Linear gradient
20.0	10	90	Linear gradient
22.0	40	60	Linear gradient
24.0	60	40	Linear gradient
26.0	80	20	Equilibration

5.1.3.4) Optimization of Chromatographic conditions

The different mobile phase conditions such as the type and composition of the organic modifiers are significantly affected the chromatographic separations. So, before selecting the conditions for the optimization, A number of preliminary trials were taken for the optimization of chromatographic conditions using different combinations of different organic solvents and buffers at wide range of pH, mobile phase compositions and also flow rate to check the shape, resolution, retention time and other system suitability parameters of all the peaks.

The following conditions were studied to achieve an optimum separation:

(i) Mobile phase pH varied at 3, 3.5 and 5 keeping the composition of acetonitrile-water (v/v) and flow rate of 1.0 ml/min fixed. (ii) Mobile phase composition changed at different gradient conditions with pH and flow rate kept constant at 1.0 ml/min (iii) Flow rate was varied (0.8, 1.0 and 1.2 ml/min) with mobile phase composition and pH maintained at 3.1. The effects of all these factors were systematically addressed on system suitability parameters such as theoretical plates, resolution, retention time, separation factor, capacity factor, HETP, asymmetry etc.

5.1.3.5) Validation

In order to confirm that the proposed method is applicable to the analysis of herbal formulation, validation was performed according to the ICH Guidelines.

a) Calibration curve (linearity)

Six different concentrations of glycyrrhizin, quercetin and betulinic acid were analyzed in and their calibration curve was constructed in the specified concentration range of 20 - 60µg/ml and rosmarinic acid in concentration range of 5-15µg/ml. The calibration plots were generated by replicate analysis (n = 3) at all concentration levels and the linear relationship was evaluated using the least square method using Microsoft Excel® program.

b) Repeatability, precision and stability

The analysis repeatability was examined by the injection of 6 different samples prepared by the same procedure while the injection repeatability was determined by the analysis of 6 continuous injections using the same sample. The mixture of standard solutions (40 µg/ml) was used for the test of analysis repeatability and injection repeatability.

The instrument precision was examined by performing the intra - day and inter - day assays of six replicate injections of the mixture of standard solutions at three concentration levels (10, 40 and 100µg/ml). The intra-day assay precision was performed with the interval of 4 h in one day, while the inter - day assay precision was performed over six days.

c) Limit of detection and limit of quantitation

LOD and LOQ were determined using the formula kSD/s where k is a constant (3 for LOD and 10 for LOQ), SD is the standard deviation of the analytical signal, and s is the slope of the concentration/response graph.

d) Specificity

Specificity is defined as the ability of the analytical method to measure analyte response in the presence of interferences present in the sample matrix. It was checked by determining the standards in laboratory prepared ternary mixtures. Moreover, the proposed method was also applied to the proprietary formulation.

e) Robustness

Robustness of the proposed method was evaluated by keeping the chromatographic conditions constant, except for the followings changes:

1) Detection wavelength: Changed from 239 nm to 234 nm and 244

2) Column: Using another column (Hypersil ODS, particle size 5 μm; 250 mm X 4.6 mm ID)

3) Solvent Brand: Acetonitrile and methanol supplied by Qualigen Pvt Ltd. (Mumbai, India) and Rankem (Mumbai, India).

Standard solution was injected 6 times for each change. The system suitability parameters like peak asymmetry, resolution, capacity factor, theoretical plates and Relative Standard Deviation were calculated for each peak. Recoveries and percentage RSDs were calculated for each and every component during each change.

f) Accuracy

The accuracy of the method was determined by calculating the recoveries of Glycyrrhizin, quercetin, rosmarinic acid and betulinic acid by the method of standard additions. Known amounts of these standards (80%, 100% and 120 %) were added to the preanalyzed sample solution, and the amounts of these standards were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve.

5.1.4) HPTLC method for estimation of quercetin in *N. nucifera* extract (method III)

5.1.4.1) Chemicals and Reagents

Chloroform, toluene, ethyl acetate, glacial acetic acid of analytical grade were purchased from Merck (Mumbai). 10 % Aluminium chloride reagent was prepared as per reported method (Wagner).

5.1.4.2) Preparation of standard solutions

A stock solution of standard quercetin (100μg/ml) was prepared by transferring 1 mg of quercetin, accurately weighed, into a 10 ml volumetric flask, dissolving in methanol.

5.1.4.3) Preparation of sample solutions

Accurately weighed 100 mg of dried hydro alcoholic extract of *N. nucifera* was transferred to a 100 ml volumetric flask, initially dissolving in 80 ml of methanol. It

was then sonicated for 10 minutes and the contents of the flask were filtered through whatman No. 1 paper (Merck, Mumbai, India). The final volume of the solution was made up to 100ml with methanol to get stock solution containing 1.0 mg/ml.

5.1.4.4) Application of test samples

The samples were spotted in the form of bands of width 6 mm on the pre-coated silica gel G plates. The plates were pre-washed by methanol and activated at 110° C for 5 min prior to chromatography. A constant application rate of 0.1µl/s was employed and space between two bands was 6mm.

5.1.4.5) Calibration curve of quercetin

A stock solution of standard quercetin (100µg/ml) was prepared in methanol. Different volume of stock solution 1, 5, 10, 15, 20 and 25µl, were spotted on to TLC plate to obtain concentration 100, 500, 1000, 1500, 2000 and 2500ng/spot of quercetin, respectively. The data of peak areas plotted against the corresponding concentrations.

5.1.4.6) Development

The mobile phase consisted of toluene: methanol: ethyl acetate: formic acid, 5: 1: 4: 0.2 (v/v/v). Linear ascending development was carried out in trough chamber saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 15mins at room temperature. The length of chromatogram run was 85 mm. Subsequent to the development; TLC plates were dried in a current of air with the help of an air-dryer.

5.1.4.7) Detection

The deuterium lamp was used as a source of radiation. It was continuously emitting UV spectrum between 190 and 400 nm. From intensity of diffusely reflected light, the concentrations of the compound chromatographed were determined. The slit dimension was kept at 5 mm x 0.45 mm and 10 mm/s scanning speed was employed. The monochromator bandwidth was set at 20 nm with K 320 cut off filter, each track was scanned thrice and baseline correction was used. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. The plate was scanned and quantified at 366 nm. The plate was sprayed with 10% $AlCl_3$ immediately scanned and quantified at 366 nm using the Camag TLC Scanner-3. Data of peak area of each band were recorded. A calibration curve was obtained by plotting

peak area Vs concentration and peak height Vs concentration of quercetin. Spectra of the samples and standard quercetin were matched.

5.1.4.8) Method Validation

a) Calibration Curve of quercetin

A stock solution of standard quercetin (100µg/ml) was prepared in methanol. Different volume of stock solution 1, 5, 10, 15, 20 and 25µl, were spotted on to TLC plate to obtain concentration 100, 500, 1000, 1500, 2000 and 2500ng/spot of quercetin, respectively. The data of peak areas plotted against the corresponding concentrations were treated by linear least-square regression.

b) Precision

The intra-day and inter-day variation for the determination of quercetin was carried out at two different concentration levels 100 and 2500ng per spot.

c) Robustness of the method

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions were tried at two different concentration levels of 200 and 1000ng per spot.

d) Limit of detection and limit of quantitation

The blank methanol was spotted six times to estimate the limit of detection (LOD) and limit of quantitation (LOQ). The signal-to-noise ratio was determined and LOD was considered as 3:1 and LOQ as 10:1. The LOD and LOQ were practically checked by diluting known concentrations of quercetin until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

e) Recovery studies

The analyzed samples were spiked with extra 80, 100 and 120% of the standard quercetin and the mixtures were reanalyzed by the proposed method. The experiment was preformed three times. This was done to verify for the recovery of the drug at different levels in the formulations.

f) Spot Stability

Prior to chromatographic development, the sample is left to stand on the solvent. It can influence the stability of separated spots and also required to be investigated for validation. To find out any decomposition occurring during spotting and in development, two-dimensional chromatography was used. If decomposition occurs during development, peak(s) of decomposition product(s) shall be obtained for the analyte both in the first and second direction of the run.

5.1.5) HPTLC method for simultaneous estimation of glycyrrhizin, quercetin, rosmarinic acid and betulinic acid (Method IV)

5.1.5.1) Chemicals and Reagents

Ethyl acetate, toluene, methanol and formic acid of analytical grade were purchased from Merck (Mumbai). Anisaldehyde sulphuric acid reagent was prepared as per reported method. (Wagner)

5.1.5.2) Preparation of standard solutions

1 mg/ml stock solutions of each four markers were prepared in methanol. It was further diluted to 100µg/ml in methanol. Then each standard solution was mixed in equal proportion to make final working standard solution.

5.1.5.3) Preparation of sample solutions

A 10mg/ml solution of the formulation was dissolved in methanol. The methanol extract of the powdered formulation was prepared by sonication for 20 minutes. The extract was filtered through whatman paper no.1.

5.1.5.4) Application of test samples

The samples were spotted in the form of bands of width 6 mm on the pre-coated silica gel G plates. Prior to chromatography, the plates were pre-washed with methanol and also activated at 110 °C for 5 min A constant application rate of 0.1µl/s was used and distance between two bands was 6 mm. Different volumes from 4-19µl of the solution were applied, which gave different concentration 400-1900ng per spot respectively.

5.1.5.5) Development

The mobile phase consisted of ethyl acetate: toluene: methanol: formic acid (7: 1: 0.5: 0.5 v/v). The mobile phase was saturated in trough chamber for linear ascending development. The mobile phase was saturated for 30 minutes at room temperature.

The chromatogram was run up to 14.5cm. Subsequent to the development, the TLC plates were dried by using an air-dryer.

5.1.5.6) Detection

The deuterium lamp was used as a source of radiation. It was continuously emitting UV spectrum between 190 and 400 nm. From intensity of diffusely reflected light, the concentrations of the compound chromatographed were determined. The slit dimension was kept at 5 mm x 0.45 mm and 10 mm/s scanning speed was employed. The monochromator bandwidth was set at 20 nm with K 320 cut off filter, each track was scanned thrice and baseline correction was used. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. The plate was scanned at 366nm. The plate was sprayed with AAS reagent immediately scanned and quantified at 546 nm using the Camag TLC Scanner-3. Data of peak area of each band were recorded. A Calibration curve was obtained by plotting peak area Vs. concentration of four markers. Spectra of the samples and standard were matched.

5.1.5.7) Method Validation

a) Linearity

A stock solution of standards (1000µg/ml) were prepared in methanol and mixed in equal volume. The bands in the range of 400-1900ng per spot were applied. The data of peak height/ area versus drug concentration were treated by linear least-square regression.

b) Precision

The intra-day and inter-day variation was carried out at two different concentration levels 400 and 1900ng per spot.

c) Robustness of the method

The effects on the results were examined by introducing small changes in the mobile phase composition. The different compositions of mobile phase were tried at two different concentration levels of 400 and 1600ng per spot.

d) Limit of detection and limit of quantitation

The blank methanol was spotted six times to estimate the limit of detection (LOD) and limit of quantitation (LOQ). The signal-to-noise ratio was determined and LOD was considered as 3:1 and LOQ as 10:1. The LOD and LOQ were practically checked

by diluting known concentrations of all four standards until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

e) Recovery studies

The analyzed samples were spiked with extra 80, 100 and 120 % of the standards and the mixtures were reanalyzed by the proposed method. The experiment was repeated three times. This was done to verify the recovery of the drug at different levels in the herbal formulations.

5.1.6) Spectrofluorimetric method for estimation of quercetin in *Nelumbo nucifera* extract and in Herbal formulation (Method V)

5.1.6.1) Preparation of standard solution

Standard stock solutions of quercetin (1000µg/ml) were prepared by dissolving 10 mg of pure drug in 10ml methanol. Appropriate and accurate aliquots of the stock solution was transferred to 10 ml calibrated flasks and diluted up to the volume with methanol in the range of 10-70ng/ml.

5.1.6.2) Preparation of sample solution

- 10 mg of each extract was dissolved in 10 ml methanol individually. The solutions were sonicated for 15 min and were filtered through Whatman filter paper No. 40. Further dilutions were made in methanol.
- 20 Tablets of herbal formulation were weighed and crushed in to powder form. About 1gm of the prepared formulation was powdered and extracted in 25 ml of methanol by sonication for 20 minutes at room temperature and further dilutions were made in mobile phase. The solution was filtered by whatman filter paper (No. 42) into 100 ml volumetric flask and then diluted to volume with methanol to get stock sample solution.

5.1.6.3) Instrumental Parameters:

Fluorescence measurements were performed on a spectrofluorimeter RF-5301 PC (Shimadzu, Japan), with non fluorescent quartz cell of 1 cm path length. Data acquisition was performed using RFPC software version 2.04. Fluorescence intensity of all the solutions was measured at excitation wavelength: 242nm, emission wavelength: 515nm with scan speed: fast and slit with: 5nm.

5.1.6.4) Procedure:

Suitable aliquots from the stock solutions were transferred into 5 ml flask in the range of 10-70ng/ml. Standard solution of quercetin was scanned in the range of 200-600 nm using spectrofluorimeter and the excitation wavelength was determined to be 242 nm. The excitation wavelength was kept constant and the emission wavelengths were scanned in the range of 450 to 600nm. Each time the solvent blank was also scanned.

5.1.6.5) Method Validation

The proposed method was validated according to ICH guidelines for linearity, accuracy, sensitivity, precision and robustness.

a) Linearity and range

The linearity of quercetin was evaluated by analyzing a series of different concentrations of quercetin. In present study, seven different concentrations of quercetin were selected within the linearity range, and each was repeated three times. A linear relationship was found between the fluorescence intensity and the concentration of the quercetin in the range 10-70ng/ml. The optical characteristics such as linearity range and regression equation (slope, intercept and correlation coefficient) were determined for the method.

b) Detection and Quantitation Limits

The LOD and LOQ values for quercetin were calculated from the calibration curves using the formula kSD/b where k=3 for LOD and 10 for LOQ, SD is the standard deviation of the intercept and b is the slope of the calibration curve.

c) Precision

For evaluation of the precision, within the day (intra-day) and between-day (inter-day) precision variability was performed at three concentration levels (10, 40 and 70ng/ml) for quercetin. The experiments were repeated six times a day for intra-day precision and on three different days for inter-day precision.

d) Accuracy

Recovery study was carried out by the standard addition technique to confirm the accuracy of proposed method. Three different levels (80,100 and 120%) of standards were added to pre-analyzed samples and also in hydro alcoholic extract of *Nelumbo nucifera* and each level were repeated three times.

e) Robustness

To determine the robustness of the method, examine the reflection of the slight changes to the results in methodological parameters. The solvent brand used in the analysis was purposely altered in order to determine the robustness of the method.

f) Stability

The standard and sample solution of quercetin in methanol was stored at ambient temperature for 72 hrs and 5ºC in refrigerator for 5 days. The solution was found to be stable and no changes in fluorescence were observed.

g) Specificity

As per the ICH guideline for specificity, the method is specific when the results are unaffected by presence of other constituents in the extract as well as in developed polyherbal formulation, which can be observed from the results of recovery of extracts and in formulation.

5.1.7) Spectrofluorimetric method for estimation of glycyrrhizin in Glycyrrhiza glabra extract and in polyherbal formulation (Method VI)

5.1.7.1) Preparation of standard solution

Standard stock solutions of glycyrrhizin (1000µg/ml) were prepared by dissolving 10 mg of drug in 10 ml methanol. Appropriate and accurate aliquots of the stock solution was transferred to 10 ml calibrated flasks and diluted up to the volume with methanol in the range of 100-600pg/ml.

5.1.7.2) Preparation of sample solution

- 10 mg of each extract was dissolved in 10 ml methanol individually. The solutions were sonicated for 15 min and were filtered by Whatman filter paper No. 40. Further dilutions were made in methanol.
- 20 tablets of herbal formulation were weighed and then crushed in to powder form. About 1gm each of the prepared formulations were powdered and extracted in 25 ml of methanol by sonication for 20 minutes at room temperature and further dilutions were made in mobile phase. The solution was filtered by whatman filter paper (No. 42) into 100 ml volumetric flask and then diluted to volume with methanol to get stock sample solution.

5.1.7.3) Instrumental Parameters

Fluorescence measurements were done on a spectrofluorimeter RF-5301 PC (Shimadzu, Japan), with non fluorescent quartz cell of 1 cm path length. Data acquisition was performed using RFPC software version 2.04. Fluorescence intensity of all the solutions was measured at excitation wavelength: 272nm, emission wavelength: 545nm with scan speed: fast and slit with: 5nm.

5.1.7.4) Procedure

Suitable aliquots from the stock solutions were transferred into 5 ml flask in the range of 100-600pg/ml. Standard solution of glycyrrhizin was scanned in the range of 220-770 nm using spectrofluorimeter and the excitation wavelength was determined to be 272 nm. The excitation wavelengths were kept constant and the emission wavelength were scanning in the range of and 220 to 770nm. Each time the solvent blank was also scanned.

5.1.7.5) Method Validation

The proposed method was validated according to ICH guidelines for accuracy, linearity, precision, sensitivity and robustness.

a) Linearity and range

The linearity of glycyrrhizin was evaluated by analyzing a series of different concentrations of it. In this study, six different concentrations of glycyrrhizin were chosen within the linearity range, and each was repeated three times. The fluorescence intensity and the concentration of the glycyrrhizin showed a linear relationship in the concentration range of 100-600pg/ml. The optical characteristics such as linearity range and regression equation (slope, intercept and correlation coefficient) were determined for the method.

b) Detection and Quantitation Limits

The LOD and LOQ values for glycyrrhizin were calculated from the calibration curves using the formula kSD/b where k=3 for LOD and 10 for LOQ. Where SD: the standard deviation of the intercept, b: slope of the calibration curve.

c) Precision

The intra-day and inter-day precision variability was performed at three different concentration levels (100, 300 and 600pg/ml) for glycyrrhizin. For intra-day and

inter-day precision, the experiments were repeated six times a day and on three different days respectively.

d) Accuracy

The standard addition technique for recovery study was performed to confirm the accuracy of developed method. To pre-analyzed tablet samples, three different levels (80,100 and 120%) of standards were added and also in water extract of *Glycyrrhiza glabra* and each level were repeated three times.

e) Robustness

To determine the robustness of the method, examine the reflection of the slight changes to the results in methodological parameters. The solvent brand used in the analysis was purposely altered in order to determine the robustness of the method.

f) Stability

The standard and sample solution of glycyrrhizin in methanol was stored at ambient temperature for 72 hrs and 5ºC in refrigerator for 5 days. The solution was found to be stable and no changes in fluorescence were observed.

g) Specificity

As per the ICH guideline for specificity, the method is specific when the results are unaffected by presence of other constituents in the extract as well as in developed polyherbal formulation, which can be observed from the results of recovery of extracts and in formulation.

5.2) RESULTS AND DISCUSSION

5.2.1) Simultaneous estimation of Glycyrrhizin and Betulinic acid in polyherbal formulation (Method I)

5.2.1.1) Optimization of Chromatographic conditions

Experimental conditions were chosen such that the obtained chromatograms had better resolution and short analysis time, specifically when several samples were to be analyzed. Initially numerous mixtures of methanol-water, 0.1% orthophosphoric acid and methanol-acetonitrile-water, 0.4% glacial acetic acid were tried as mobile phase but sufficient separation was not obtained. Later acetonitrile and water, 0.03% orthophosphoric acid was chosen as mobile phase because better separation of the

terpenoids (glycyrrhizin and betulinic acid) could be achieved. The common detection wavelength was selected to be 230nm, depending on its maximum absorbance in the scan range of 200nm - 400nm.

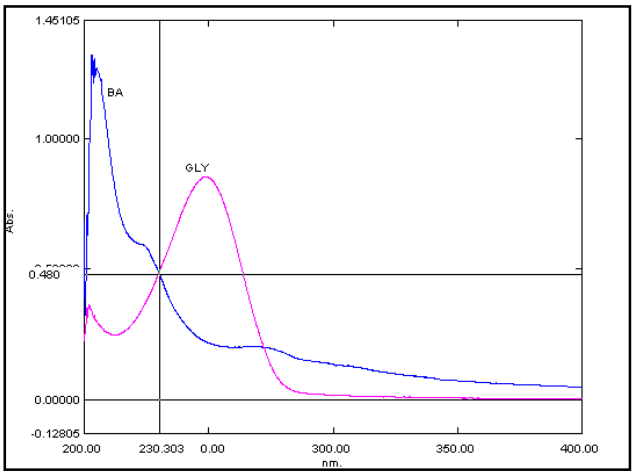

Fig. 5.1 UV spectra of Glycyrrhizin and Betulinic acid (100µg/ml)

5.2.1.2) Effect of pH value in the mobile phase on the retention time of HPLC

HPLC was performed at low or mid-range pH values in the mobile phase buffer. It was difficult to separate the components from each other and from other compounds in the polyherbal formulation. An alternative method was employed to separate them by using a mobile-phase pH which was below its pKa. Under such conditions, markers were in its free base form because its negative charge was diminished, resulting in the improvement of the peak shape. The pH dependence of the retention time was investigated within a range of 3-4 pH values by adjusting with 0.03% *ortho* phosphoric acid. This showed that along with increase of the pH values in the water, the retention time of HPLC was increasing due to the stronger interaction between this less-polar molecule and the hydrophobic bonded phase. The results of the chromatographic analysis indicated that the pH value has distinct effects, on the resolution. At the pH value 3.1, all the components in the samples were baseline separated from each other with resolution values above 2. Therefore, the analysis was carried out at a pH range of 3-4, where the water solution works effectively.

5.2.1.3) Effect of mobile phase composition

The effect of mobile phase composition (i.e. ratio of water and acetonitrile was studied at 40:60, 30:70 and 10:90 (v/v) levels with pH and flow rate of 3.1 and 1.0 ml/min, respectively. The resolution was > 3 at all ratio of mobile phase, but the peak shape is not good in 40:60 and 30:70 ratio. Also the retention time was shorter with

satisfactory asymmetry values. An adequate theoretical plate (~4000) is indicative of a good column performance. The asymmetry was >1.12 at 40:60 v/v indicating the tailing of peaks.

5.2.1.4) Effect of mobile phase flow rate

The number of theoretical plates was highest at flow rate of 1 ml/min. The change in flow rate had no significant effect on resolution and asymmetry while retention time decreased as the flow rate increased. The other system suitability parameters also indicated optimum flow rate of 1 ml/min.

Table 5.1 Effect of mobile phase pH, composition, and flow rate on various chromatographic parameters

Variable	Value	Retention time		Assymmetry		Resolution		Theoretical plates	
		GLY	BA	GLY	BA	GLY	BA	GLY	BA
pH	2.8	2.5	10.8	0.954	0.953	-----	19.75	3466	4302
	3.1	2.7	11.3	1.002	0.977	-----	21.46	3595	4500
	4	3.2	11.4	1.012	0.899	-----	21.12	3608	4496
M.P. Ratio v/v	40:60	3.4	12.8	1.108	1.202	-----	22.51	3417	4007
	30:70	2.9	12.5	1.114	1.260	-----	23.07	3484	4059
	90:10	2.7	11.3	1.002	0.977	-----	21.46	3595	4500
Flow rate (ml/min)	0.8	3	12.5	0.924	0.967	-----	22.35	3346	4283
	1	2.7	11.3	1.002	0.977	-----	21.46	3595	4500
	1.2	2.5	10.5	0.014	0.969	-----	19.28	3427	4467

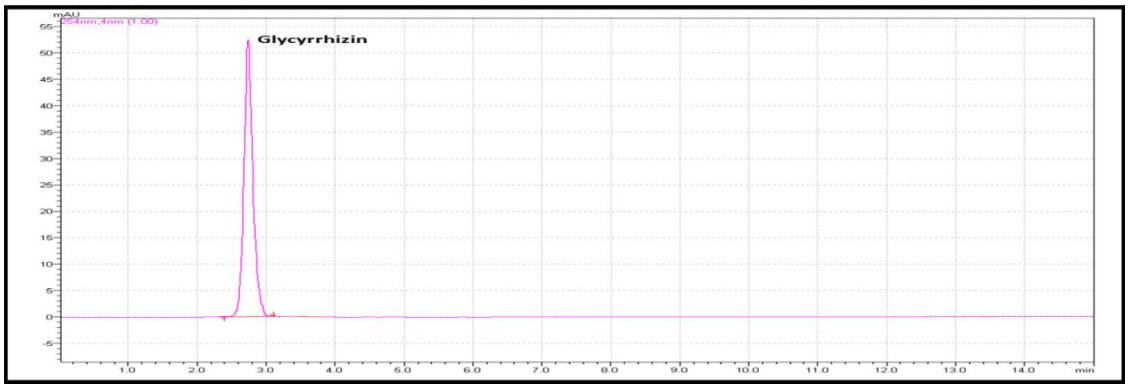

Fig. 5.2 Representative HPLC chromatogram of standard Glycyrrhizin

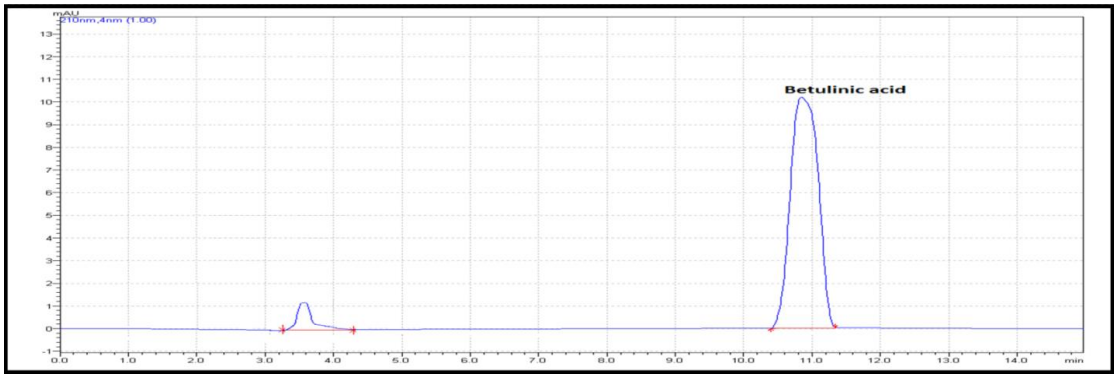

Fig. 5.3 Representative HPLC chromatogram of standard Betulinic acid (Extra peak shown because of impurity)

Table 5.2 Peak Area of Glycyrrhizin and Betulinic acid

Concentration (µg/ml)	Peak Area	
	Glycyrrhizin	Betulinic acid
10	141494	98331
20	242759	180545
40	444207	321527
60	634853	467634
80	812789	607481
100	1002408	743741

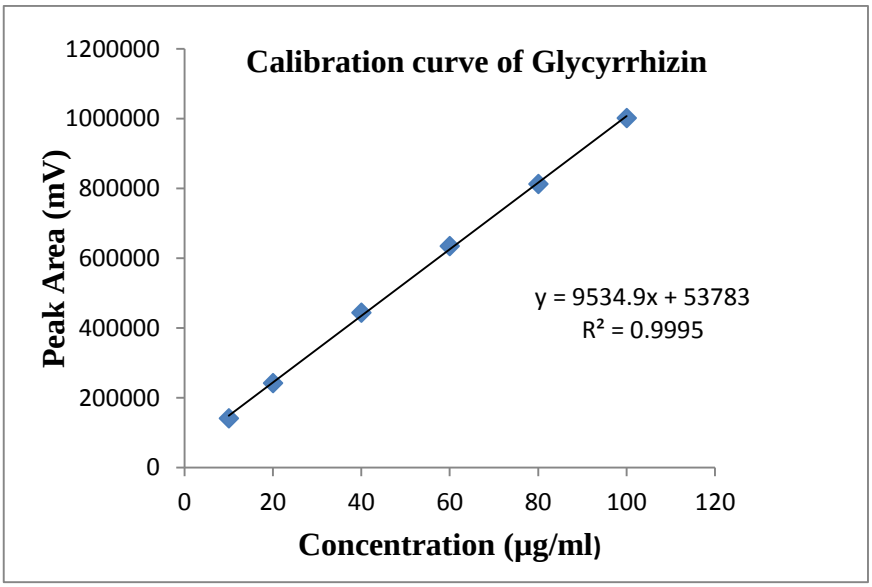

Fig. 5.4 Calibration curve of Glycyrrhizin

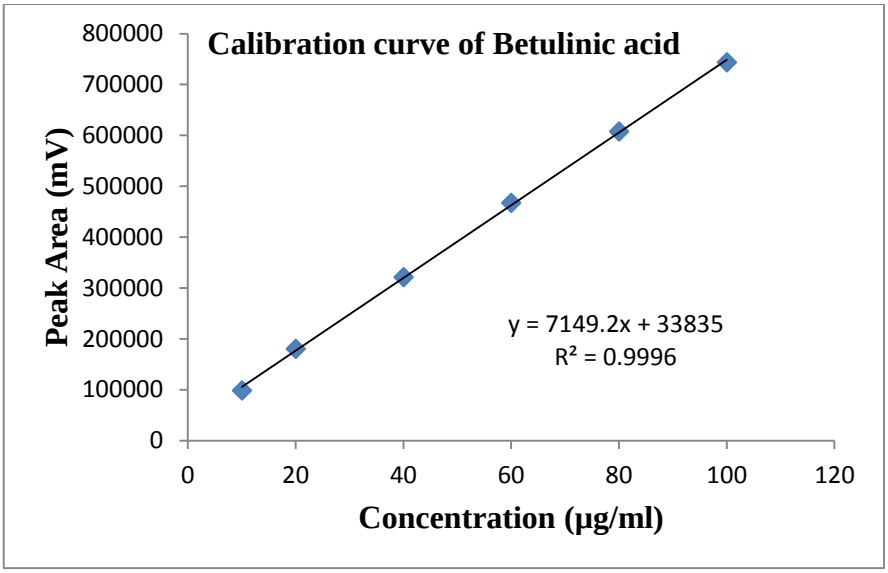

Fig. 5.5 Calibration curve of Betulinic acid

5.2.1.4) Validation

a) Calibration curve (linearity)

The calibration curves (n=3) constructed for glycyrrhizin and betulinic acid were linear over the concentration range of 10-100 µg/ml. Peak area of the marker was plotted versus the concentration and linear regression analysis performed on the resultant curve. The coefficients of determination were 0.9992 and 0.9976 for glycyrrhizin and betulinic acid respectively. The % RSD values ranging from 0.5 to 2% across the concentration range studied were obtained following linear regression analysis (Table 5.3).

Fig. 5.6 Simultaneous determination of standard glycyrrhizin and betulinic acid (Extra peak shown because of impurity of Betulinic acid)

Table 5.3 Linear Regression data for the calibration curves (n=3)

Parameters Values	Glycyrrhizin	Betulinic acid
Retention time, min.	2.7	10.8
Detection wavelength	230nm	230nm
Linearity Range	10-100 µg/ml	10-100 µg/ml
Coefficient of Determination (Area)	0.9995	0.9996
Regression equation (Area)	y = 9534.9x + 53783	y = 7149.2x + 33835
Interday Precision*	1.887	0.959
Intraday precision*	1.482	1.386
LOD (µg/ml)	0.83	0.68
LOQ (µg/ml)	2.1	1.76
% Recovery**	95.68 ± 1.01	97.09 ± 1.29
% RSD**	1.69 ± .21	1.65 ± .27

* mean of three determinants

**Mean ± SD

b) Repeatability, precision and stability

Injection repeatability- The calculated % RSDs of the peak areas was less than 2.0 % at each of the three concentration levels (Table 5.4).

Analysis repeatability- The % RSD values for analysis repeatability were less than 2.0 % both for retention time and peak area (Table 5.4).

Table 5.4 Repeatability of the developed method (n=6)

Components	Retention time (min)				Peak area (mVs)			
	Injection Repeatability		Analysis Repeatability		Injection Repeatability		Analysis repeatability	
	Mean	% RSD	Mean	% RSD	Mean	% RSD	Mean	% RSD
Glycyrrhizin	2.7	0.31	2.7	0.33	546418.33	0.52	544128.14	0.57

Betulinic acid	11.3	0.25	11.3	0.26	403209.83	0.94	402127.09	1.05

Table 5.5 Intra-day and inter-day precision of HPLC method (n=6)

Amount (µg/ml)	Intra-day precision				Inter-day precision			
	Glycyrrhizin		Betulinic acid		Glycyrrhizin		Betulinic acid	
	Mean	% RSD	Mean	% RSD	Mean	% RSD	Mean	% RSD
10	142478.00	0.66	98489.33	0.72	139794.00	1.25	97990.33	1.57
60	641254.33	0.95	460794.00	1.29	637853.00	1.57	466333.67	1.42
100	1003443.33	0.12	741950.00	1.01	1006973.67	0.61	744835.00	1.44

Instrument precision-The precision result of the solution at medium concentration is presented in Table 5.5, and it was shown that the % RSD values of retention time were less than 1%, while the % RSD values of peak area were less than 2 % both for intra-day assay and inter-day assay precision (Intra 4 h six injections, inter 6 days).

For the stability test, the same sample was analyzed within 24 h at the room temperature, and the solution was found to be stable (%RSD values of the retention time and peak area were both less than 3%).

c) Limit of detection and limit of quantification

The LOD and LOQ were found to be 0.83 and 2.1µg/ml for glycyrrhizin and 0.68 and 1.76µg/ml for betulinic acid.

d) Specificity

The specificity was complying according to USP. Resolution was found to be 21.46 which was greater than 2. The excipients and other components did not interfere in the estimation of glycyrrhizin and betulinic acid. Thus good resolution and absence of interference showed the specificity of HPLC method.

e) Robustness

Table 5.6 shows the mean obtained (n=6) for each factor studied, indicating that the selected factors remained unaffected by small variations of these parameters. The recovery obtained individually and the mean values were between 98 % and 102 % of glycyrrhizin and betulinic acid. Therefore, it can be concluded that the method is consistent for detection wavelength, selected column and solvent brand.

Table 5.6 Robustness of the method

[b] Factor	Level	Retention time (t_R) of GLY (min.)	Retention time (t_R) of BA (min.)	Asymmetric factor of GLY peak	Asymmetric factor of BA peak
A: flow rate (ml/min)					
0.95	-1	2.72	10.92	1.118	1.008
1	0	2.7	10.8	1.11	1.009
1.05	1	2.65	10.74	1.115	1.007
Mean ± S.D.		2.69 ± 0.0360	10.82 ± 0.0916	1.114 ± 0.004	1.008 ± 0.001
B: percentage of acetonitrile in mobile phase					
88	-1	2.723	10.862	1.109	1.012
90	0	2.7	10.8	1.118	1.009
92	1	2.67	10.754	1.112	1.005
Mean ± S.D.		2.697 ± 0.02658	10.805 ± 0.0542	1.113 ± 0.004	1.008 ± 0.00351
C: solvents of different Lots					
First lot		2.725	10.82	1.118	1.007
Second lot		2.7	10.8	1.12	1.009
Mean ± S.D.		2.7125 ± 0.01768	10.81 ± 0.01414	1.119 ± 0.001	1.008 ± 0.00141

a Average of three concentration (10, 60, 100 µg/ml), three replicates each.
b Factors were slightly changed at three levels (1, 0, -1); each time a factor was changed from level (0), the other factors remained at level (0)

f) System suitability

A system suitability test was performed to evaluate the chromatographic parameters (capacity factor, separation factor, column efficiency, number of theoretical plates, HETP asymmetry of the peaks and resolution between two consecutive peaks) before the validation runs (Table.5.7). Three replicate injections of the standard solution and three injections of the solution prepared for the specificity procedure were used.

Table 5.7 System Suitability Parameters

Parameters	Glycyrrhizin (Mean ± SD)	Betulinic acid (Mean ± SD)
Retention time (min)	2.7	10.8
Capacity factor (k')	2.901	2.86
Asymmetry	1.05	1.001
Theoretical plates	4113.887	4214.167
HETP	0.05	0.47
% RSD of area	0.74	1.02
Resolution	-----	18.19

g) Accuracy

As shown in Table 5.8, the recovery of the investigated components ranged from 97 % to 102 %, and their %RSD values were all less than 2 %. It was known from recovery tests that the developed method manifested the reliability and accuracy for the measurement of these components.

Table 5.8 Recovery Test (n=3)

Sample	Quantity added (%)	Theoretical content	% Recovery	% RSD
GLY	0	45.42	98.04	1.07
	80	82.37	99.86	0.94
	100	90.84	100.49	1.15
	120	101.55	100.23	0.97
BA	0	24.62	99.07	0.94
	80	48.59	100.66	1.12
	100	52.25	101.03	1.06
	120	59.09	99.87	1.14

5.2.1.5) Applicability of the developed method in formulations

The developed HPLC method was applied to the determination of glycyrrhizin and betulinic acid in the developed polyherbal formulation. It was observed that the content of glycyrrhizin and betulinic acid were 23.70 ± 0.03 %w/w and 12.21 ±0.025 %w/w.

5.2.2) HPLC method development for simultaneous estimation glycyrrhizin, quercetin, rosmarinic acid and betulinic acid (Method II)

5.2.2.1) Optimization of HPLC chromatographic conditions

A systematic study of the effect of various factors on chromatographic separation of four markers was undertaken by varying one parameter at a time and keeping all other conditions constant. Method development required selecting the appropriate wavelength and choice of mobile phase with suitable flow rate. The analytical wavelength (239 nm) was selected as the wavelength at which all the analytes were showing measurable absorbance (Figure 5.7).

To optimize the HPLC parameters, several mobile phase compositions with different buffer and different pH were tried. Various buffers e.g. potassium phosphate buffer, acetate buffer at different pH with different organic solvents like methanol and actonitrile were tried. The studies suggested that a mobile phase at acidic pH value favoured the peak shape of four markers on column to achieve a reasonable retention and resolution. After trying various mobile phases finally, the mobile phase system constituted of solvent A (acetonitrile) and solvent B (water), pH 3.1 (adjusted with ortho - phosphoric acid 0.03%), was used, at gradient conditions, at a flow rate of 1.0 ml/min was selected because it was found to ideally resolve the peaks. In the blank chromatogram at 239nm, the ghost peak was seen at 14.6min due to Acetonitrile (Figure 5.8).

The optimized chromatograph is programmed as follows:

Time (min)	Solution A (%)	Solution B (%)	Elution
0.00	80	20	Isocratic
4.0	60	40	Linear gradient
8.8	40	60	Linear gradient

11.0	20	80	Linear gradient
12.0	10	90	Linear gradient
20.0	10	90	Linear gradient
22.0	40	60	Linear gradient
24.0	60	40	Linear gradient
26.0	80	20	Equilibration

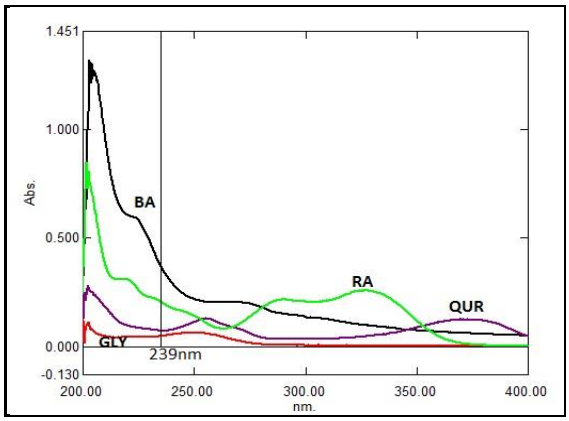

Fig. 5.7 UV spectra of Glycyrrhizin (GLY), Quercetin (QUR), Rosmarinic acid (RA) and Betulinic acid (BA)

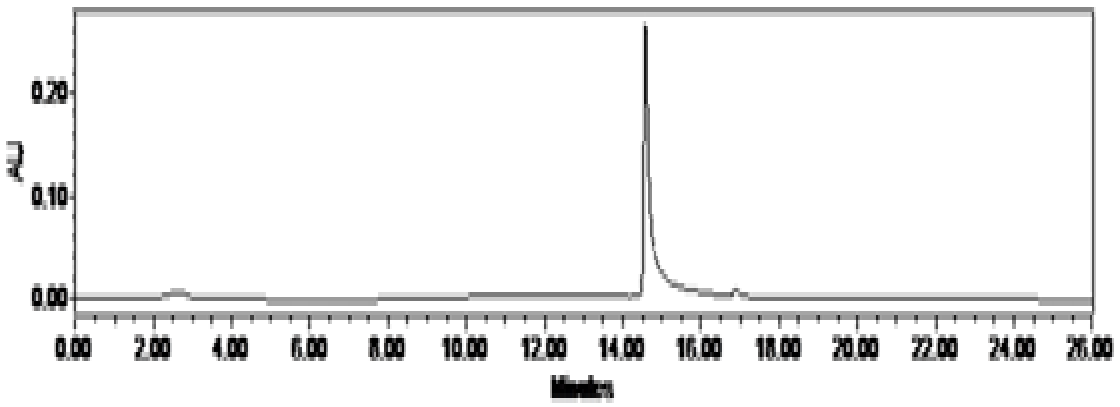

Fig. 5.8 Blank chromatogram at 239nm

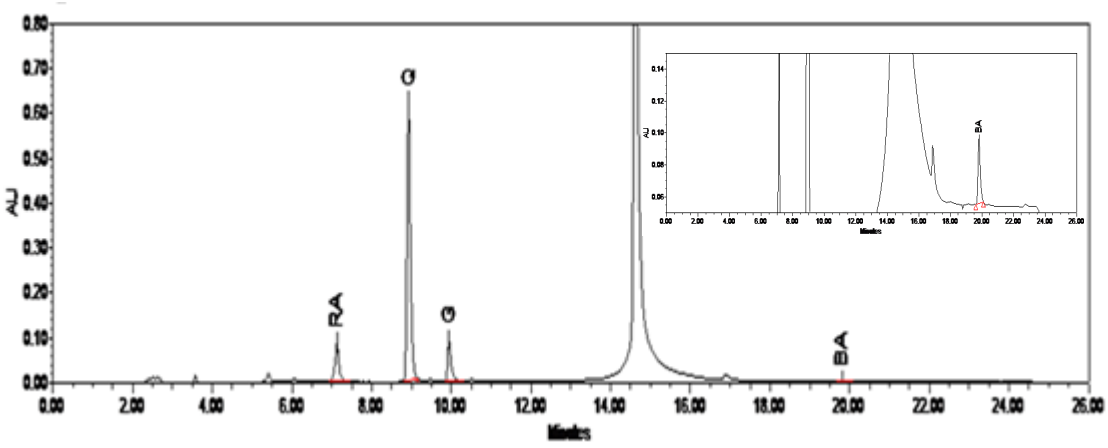

Fig. 5.9 Simultaneous determination of Rosmarinic acid (RA), Quercetin (Q), Glycyrrhizin (G) and betulinic acid (BA)

5.2.2.2) Validation

a) Calibration curve (linearity)

The calibration curves (n=3) constructed for the markers were linear over the concentration range of 20 - 60µg/ml for glycyrrhizin, quercetin and betulinic acid and concentration range of 5-15µg/ml for rosmarinic acid. Peak areas of the markers were plotted versus the concentration and linear regression analysis performed on the resultant curve. The coefficients of determination 0.9973, 0.9992, 0.9981 and 0.9992 for glycyrrhizin, quercetin, betulinic acid and rosmarinic acid respectively with % RSD values ranging from 0.5 to 2% across the concentration range studied were obtained following linear regression analysis (Table 5.9).

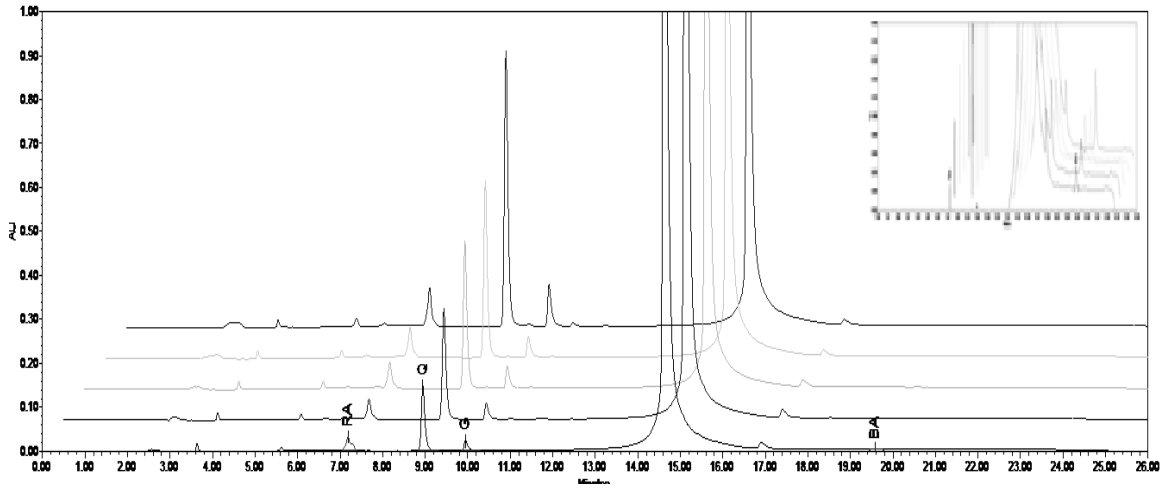

Fig. 5.10 Representative chromatograms of quarterly mixture for linearity study

Table 5.9 Linear regression data for the calibration curves (n=3)

Parameters	RA	GLY	QUR	BA
R.T., min	7.1 ± 0.04	8.8 ± 0.09	9.9 ± 0.05	19.9 ± 0.03
Detection wavelength	239 nm	239 nm	239 nm	239 nm
LOD, µg/ml	0.025	1.18	0.16	1.5
LOQ, µg/ml	0.076	3.6	0.49	4.6
Linearity range, µg/ml	5-20 µg/ml	20-60 µg/ml	20-60 µg/ml	20-60 µg/ml
Correlation coefficient (area)	0.9981	0.9973	0.9992	0.9992
Regression equation	y = 31530x + 93327	y = 9857x-46226	y = 65521x-44665	y = 5614x-22418
Slope	31530	9857	65521	5614
Intercept	93327	46226	44665	22418
Intra-day precision (%RSD)	1.25	1.03	0.89	1.24
Inter-day precision (%RSD)	1.64	1.31	1.74	1.88

b) Repeatability, precision and stability

Injection repeatability- The calculated % RSDs of the peak areas for all four compounds was less than 2.0 % at each of the three concentration levels (Table 5.10).

Analysis repeatability- The % RSD values for analysis repeatability were less than 2.0 % both for retention time and peak area (Table 5.10).

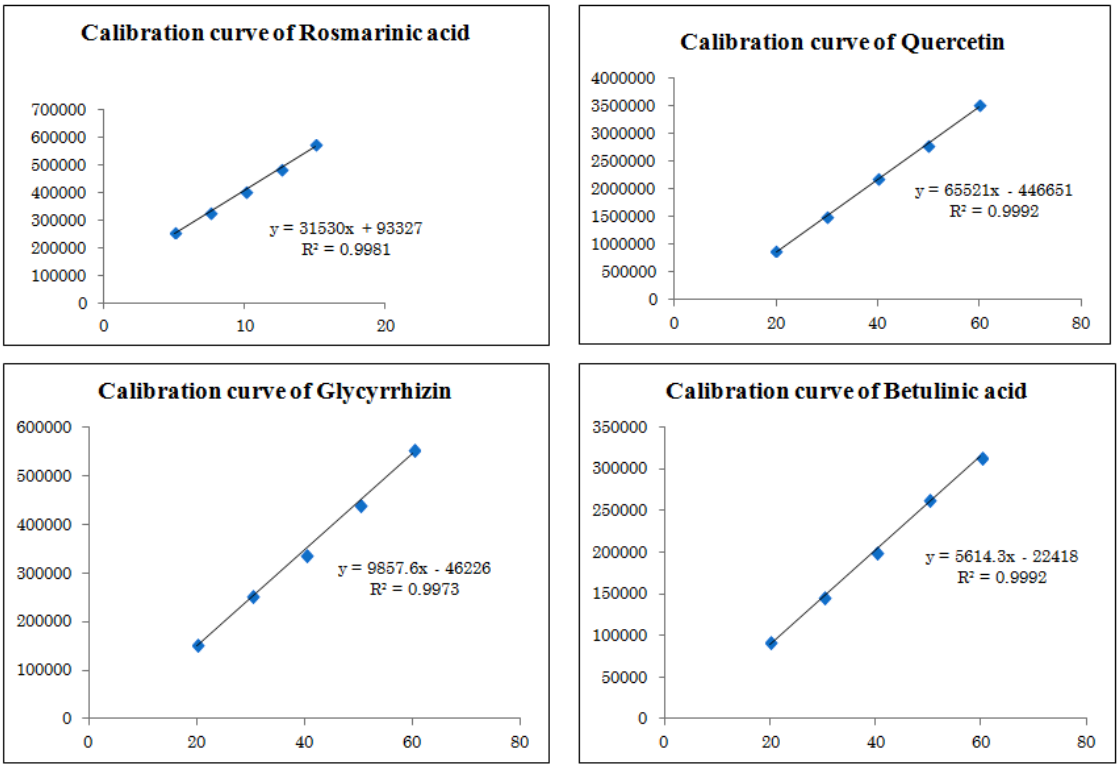

Fig.5.11 Calibration curves of Rosmarinic acid, quercetin, glycyrrhizin and betulinic acid

Table 5.10 Repeatability of the developed method (n=6)

Components	Retention time (min)				Peak area (mVs)			
	Injection Repeatability		Analysis Repeatability		Injection Repeatability		Analysis repeatability	
	Mean	% RSD	Mean	% RSD	Mean	% RSD	Mean	% RSD
RA	7.17	0.91	7.17	0.85	408631.4	0.82	407412.4	1.02
GLY	9.94	0.86	9.94	0.96	348076.2	0.91	348246.2	0.98
QUR	8.93	1.07	8.93	0.98	2174180.6	1.06	2176237.6	0.96
BA	19.87	0.88	19.87	1.13	202152.4	1.1	202152.4	1.07

Instrument precision- The precision result of the solution at medium concentration is presented in Table 5.11, and it was shown that the %RSD values of retention time were less than 1%, while the %RSD values of peak area were less than 2 % both for intra-day assay and inter-day assay precision(Intra 4 h six injections, inter 6 days).

Table 5.11 Intra-day and Inter-day precision of the developed method (n=6)

Components	Conc. (µg/ml)	Intra-day		Inter-day	
		Retention Time (mean)	%RSD	Retention Time (mean)	%RSD
RA	5	7.18	0.86	7.09	1.17
	10	7.17	1.23	7.19	1.55
	20	7.15	0.79	7.27	0.97
GLY	20	9.94	0.78	10.05	1.08
	40	9.94	0.82	9.96	1.1
	60	9.93	1.05	10.04	1.41
QUR	20	8.94	0.85	8.95	1.09
	40	8.94	0.96	9.01	1.24
	60	8.92	1.12	8.96	1.38
BA	20	19.86	0.94	19.81	1.27
	40	19.88	1.08	19.97	1.34
	60	19.84	1.03	19.82	1.36

For the stability test, the same sample was analyzed within 24 h at the room temperature, and the solution was found to be stable (%RSD values of the retention time and peak area were both less than 3%).

c) Limit of detection and limit of quantification

The LOD and LOQ were found to be 0.025 and 0.076µg/ml, respectively for rosmarinic acid, 1.18 and 3.6µg/ml, respectively for glycyrrhizin and 0.16 and 0.49µg/ml for quercetin, 1.5 and 4.6µg/ml, respectively for betulinic acid.

d) Specificity

Satisfactory results were obtained, indicating the high specificity of the proposed method for the determination of the markers in quarterly mixture and formulations. No interferences were observed as shown in overlaid chromatograms of standard solution containing all 4 compounds. Good resolution and absence of interferences between the drugs determined are shown in Fig.5.9

e) Robustness

Table 5.12 shows the mean obtained (n=6) for each factor studied, indicating that the selected factors remained unaffected by small variations of these parameters. The recovery obtained individually and the mean were between 98% and 102 % for all 4 compounds. Therefore it can be concluded that the method is consistent for detection wavelength, selected column and solvent brand.

Table 5.12 Robustness of the method (n=6)

Chromatographic change	**% Recovery Components**				
Factor	**Level**	**RA**	**GLY**	**QUR**	**BA**
Acetonitrile brand					
Spectrochem	1	98.55 ± 0.74	99.24 ± 0.71	99.67 ± 0.36	100.31 ± 0.48
Rankem	2	99.58 ± 0.65	99.12 ± 0.14	100.34 ± 0.81	101.19 ± 0.33
Qualigens	3	98.54 ± 0.74	100.78 ± 0.29	101.25 ± 0.69	99.87 ± 0.57
Column brand					
Hypersil	1	101.5 ± 0.84	99.56 ± 0.68	97.85 ± 0.49	99.08 ± 0.93
Phenomenex	2	99.14 ± 0.67	100.91 ± 0.56	101.24 ± 0.74	98.69 ± 0.78
Detection wavelength					
λmax-2nm	-1	99.12 ± 0.84	98.98 ± 0.84	99.15 ± 0.74	98.82 ± 0.96
Λmax	0	101.06 ± 0.61	99.26 ± 0.97	100.58 ± 0.62	101.02 ± 0.87
λmax + 2 nm	1	100.05 ± 0.44	101.03 ± 0.71	98.75 ± 0.87	98.95 ± 0.54

f) System suitability

A system suitability test was performed to evaluate the chromatographic parameters (capacity factor, number of theoretical plates, HETP, asymmetry of the peaks and resolution between two consecutive peaks) before the validation runs (Table 5.13). Three replicate injections of the standard solution and three injections of the solution prepared for the specificity procedure were used.

Table 5.13 System Suitability Parameters

Parameters	RA (Mean ± SEM)	GLY (Mean ± SEM)	QUR (Mean ± SEM)	BA (Mean ± SEM)
Retention Time (min)	7.1 ± 0.01	8.8 ± 0.005	9.9 ± 0.01	19.9 ± 0.058
Theoretical Plates	3600.81 ± 1.23	3772.68 ± 0.95	7847.76 ± 1.71	7248.06 ± 1.38
Asymmetry (As)	1.09 ± 0.04	1.12 ± 0.039	0.998 ± 0.033	1.108 ± 0.031
Resolution (Rs)	--	11.14 ± 0.17	4.86 ± 0.28	35.99 ± 0.15
Capacity factor (k')	2.912	4.546	5.713	7.963
HETP (mm)	0.050	0.048	0.047	0.049
Purity angle	0.232	0.185	0.415	0.932
Purity threshold	4.276	0.357	3.304	1.301

g) Accuracy

As shown in Table 5.14, the recovery of the investigated components ranged from 98.17 % to 100.69 %, and their %RSD values were all less than 2 %. It can be concluded from recovery tests that the developed method manifested the reliability and accuracy for the measurement of these components.

Table 5.14 Recovery Test (n=3)

Sample	Quantity added (%)	Total qty present	Amount qty found	Recovery	% RSD
RA	0	12.08	11.90	98.48	0.79
	80	21.74	21.54	99.09	0.94
	100	24.16	24.26	100.42	1.04
	120	26.57	26.28	98.91	1.11
GLY	0	50.68	49.69	98.04	0.88

	80	91.22	91.09	99.86	0.87
	100	101.36	101.85	100.49	1.05
	120	111.49	111.74	100.23	0.95
QUR	0	30.14	30.45	101.04	0.92
	80	54.25	54.33	100.15	1.02
	100	60.28	59.87	99.32	0.86
	120	66.31	66.25	99.91	1.07
BA	0	28.72	28.45	99.07	1.08
	80	51.69	52.03	100.66	1.03
	100	57.44	58.03	101.03	1.21
	120	63.18	63.10	99.87	0.99

5.2.2.3) Applicability of the developed method in formulation

The developed HPLC method was applied to the simultaneous determination of Rosmarinic acid, Glycyrrhizin, Quercetin and Betulinic acid in the laboratory polyherbal formulation. (Fig. 5.12) It was observed that the content of Rosmarinic acid, glycyrrhizin, quercetin and betulinic acid were 5.918 ± 0.02 %w/w, 24.07 ± 0.05%w/w, 14.62 ± 0.015 %w/w and 13.38 ± 0.01 %w/w respectively.

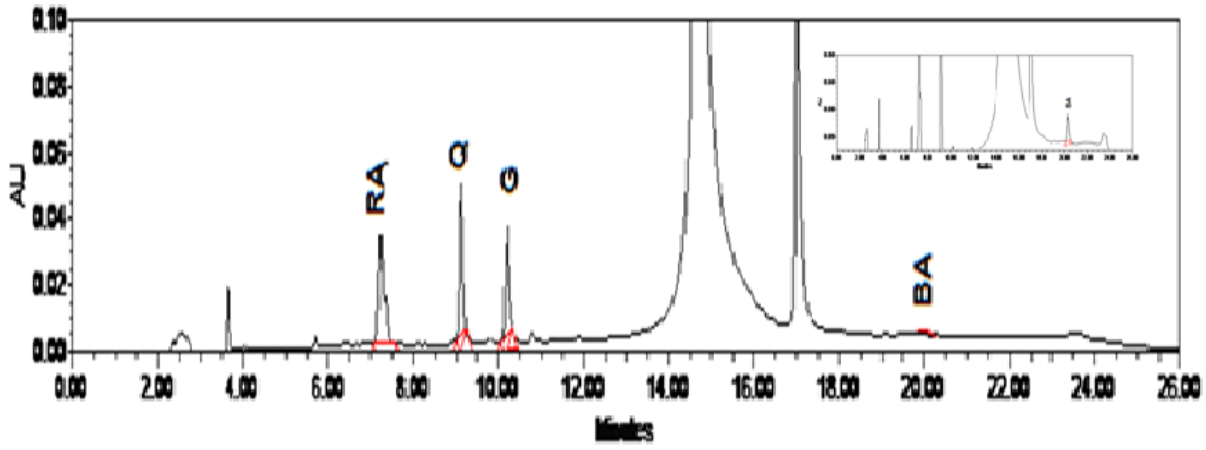

Fig. 5.12 Representative chromatogram of developed polyherbal formulation

5.2.3) HPTLC method development for quercetin estimation in *N. nucifera* extract (method III)

5.2.3.1) Development of the optimum mobile phase

The standard (quercetin) and the *N. nucifera* extract solution were spotted on the TLC plates and run in different solvent systems. Initially, toluene: ethyl acetate: methanol

in varying ratios was tried. The mobile phase toluene: ethyl acetate: methanol (5:4:1, v/v) gave good resolution but Rf value was less. Also, the typical peak nature was missing because the spot was slightly diffused. Addition of 0.2 ml of formic acid to the above mobile phase improved the spot characteristics and increased the *R*f value to 0.05 ± 0.02 when densitometric scanning was performed at 546nm after derivatisation with 10% $AlCl_3$. Finally, the mobile phase consisting of toluene: ethyl acetate: methanol: formic acid (5:4:1:0.2 v/v/v) gave a sharp and symmetrical peak. Resolution between spots of standard and other components appeared better when TLC plates (pretreated with methanol and activated at 100 °C for 5 min). Well-defined spots (compact dense spots) were obtained when the chamber was saturated with the mobile phase for 20 min at room temperature (Fig. 5.13).

5.2.3.2) Validation of the method

a) *Linearity*

Calibration graph was found to be linear that is adherence of the system to Kubelka Munk theory, which was found over the concentration range of 100-2500ng/spot (R^2=0.9994). Linearity was evaluated by determining the standard working solution containing 100 µg/ml of querectin in triplicate. Peak area and concentration were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficients. The regression data showed a good linear relationship over the low concentration range of 100–2500 ng/spot. The linearity of calibration graphs and adherence of the system to Kubelka Munk theory were validated by high value of correlation coefficient and the SD for intercept value was less than 2. No significant difference was observed in the slopes of standard plots.

Table 5.15 Linearity data of quercetin analysis by HPTLC

Track	Vial	Rf	Amount/ Fraction (ng)	Height	X (calc) (ng)	Area	X (calc) (ng)	Sample ID/Remark
1	1	0.44		221.77	460.88	808.73	490.74	*N.nucifera* ext
2	1	0.45		486.41	1455.08	23420.8	1266.03	*N.nucifera* ext
3	2							

4	2	0.45	500	123.43		328.02		
5	2	0.45	1000	310.54		8462.23		
6	2	0.46	1500	465.11		17193.6		
7	2	0.45	2000	614.79		25954.1		
8	2	0.45	2500	785.46		33512.14		

Table 5.16 Regression analysis for calibration plots (n=3)

Parameters	Results
Linearity Range	100– 2500 ng/spot
Coefficient of Determination (Height)	0.9985
Coefficient of Determination (Area)	0.9994
Regression equation (Height)	y = 0.3257x - 28.627
Regression equation (Area)	y = 16.772x – 8068
Slope (Height)	0.3257
Slope (Area)	16.772
Intercept (Height)	28.627
Intercept (Area)	8068

b) Precision

Six replicate determinations were performed and % RSD calculated was about 1.74 and 1.57 respectively for sample application and peak area, indicating repeatability of the analytical method. Also, intraday and interday precision of quercetin peak area at 500 and 2500 ng/spot was determined and the % RSD values are shown in Table 5.17.

Table 5.17 Intra-day and inter-day precision of HPTLC method (n=6)

Amount (ng/spot)	Intra-day precision		
	Mean area	S.D.	% RSD
500	329.51	9.90	1.04
2500	33526.33	18.38	1.21
Amount (ng/spot)	**Inter-day precision**		
	Mean area	**S.D.**	**% RSD**
500	331.2	22.48	1.28
2500	33487.15	54.10	1.52

c) Robustness of the method

As depicted in Table 5.18, standard deviation and % RSD of peak areas were calculated for each parameter. The % RSD value < 2% indicated robustness of the method.

Table 5.18 Robustness of the method

Amount (ng/spot)	Mobile phase composition (Area% RSD)	
	toluene: ethyl acetate: methanol: formic acid (5:4:1:0.2 v/v/v)	toluene: ethyl acetate: methanol: formic acid (4.5:4.5:1:0.2 v/v/v)
500	1.59	1.64
2500	1.75	1.98

d) LOD and LOQ

The calibration curve, plotted by amount of analyte vs average response (peak area), was subjected to regression analysis to derive regression equation and regression coefficient (0.9994). LOD and LOQ values were calculated as described earlier and came about 2.12 ng and 6.73 ng respectively indicating adequate sensitivity of the method.

e) Specificity

The specificity was complying according to USP. The other components of *N. nucifera* extract did not interfere in the estimation of quercetin. Thus good resolution and absence of interference showed the specificity of HPTLC method.

f) Accuracy

The pharmaceutical dosage form after spiking with 80, 100 and 120% of additional drug when subjected to extraction and subsequent estimation of quercetin afforded recovery of 98–100% as listed in Table 5.19. This proved the accuracy if the developed analytical method.

Table 5.19 Recovery studies (n=3)

Excess drug added to the analyte (%)	Theoretical content (ng)	Recovery (%)	RSD (%)
0	500	98.87	1.26
80	900	99.46	1.64
100	1000	98.39	1.47
120	1100	99.25	1.35

g) Spot stability

Decomposition was not observed during spotting and development.

5.2.3.3) Analysis of the N. nucifera extract

A compact spot with *R*f value of 0.45 was obtained in the HPTLC chromatogram of extracted drug samples (Fig. 5.15). Other components of the extract did not interfere in separation of quercetin. The % of quercetin about 15.02 ± 0.05 %w/w was found in *N. nucifera* extract from the peak area and regression equation mentioned in Table 5.15 and 5.16. The developed method, due to its good performance, proved suitable for routine analysis of quercetin in the herbal dosage.

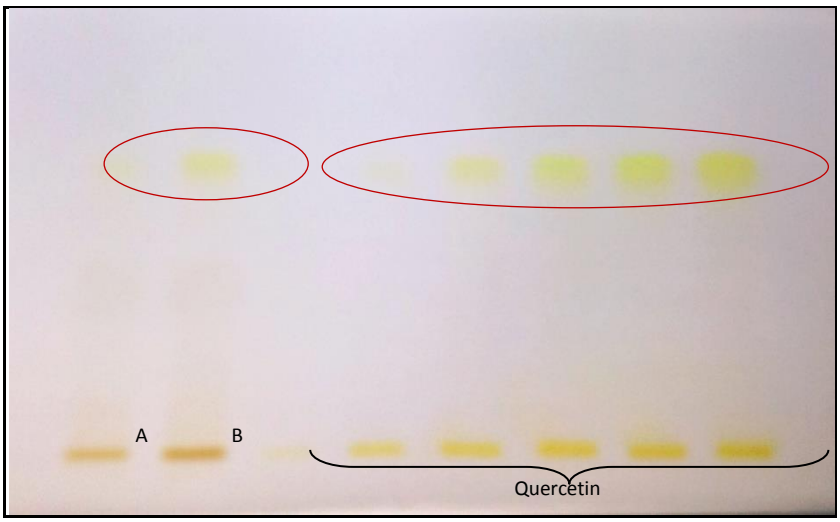

Fig 5.13 Image of the TLC plate before derivatization at 254nm

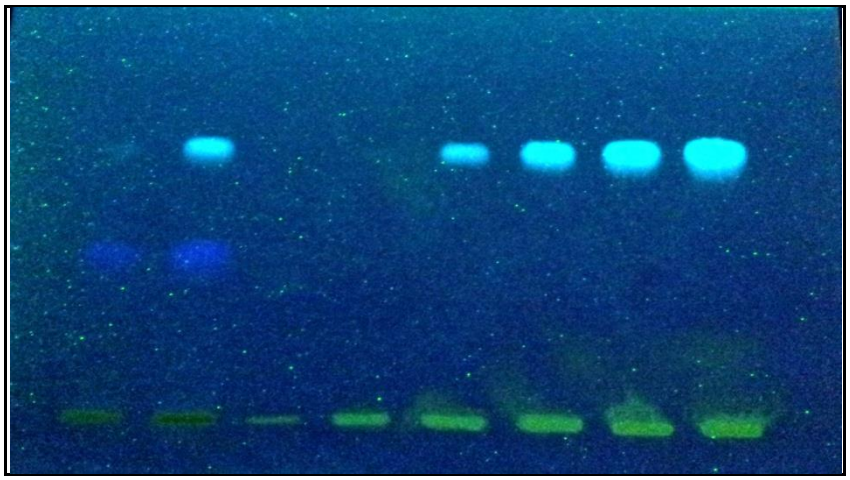

Fig. 5.14 Image of the TLC plate after derivatization with 10 % $AlCl_3$ at 546 nm

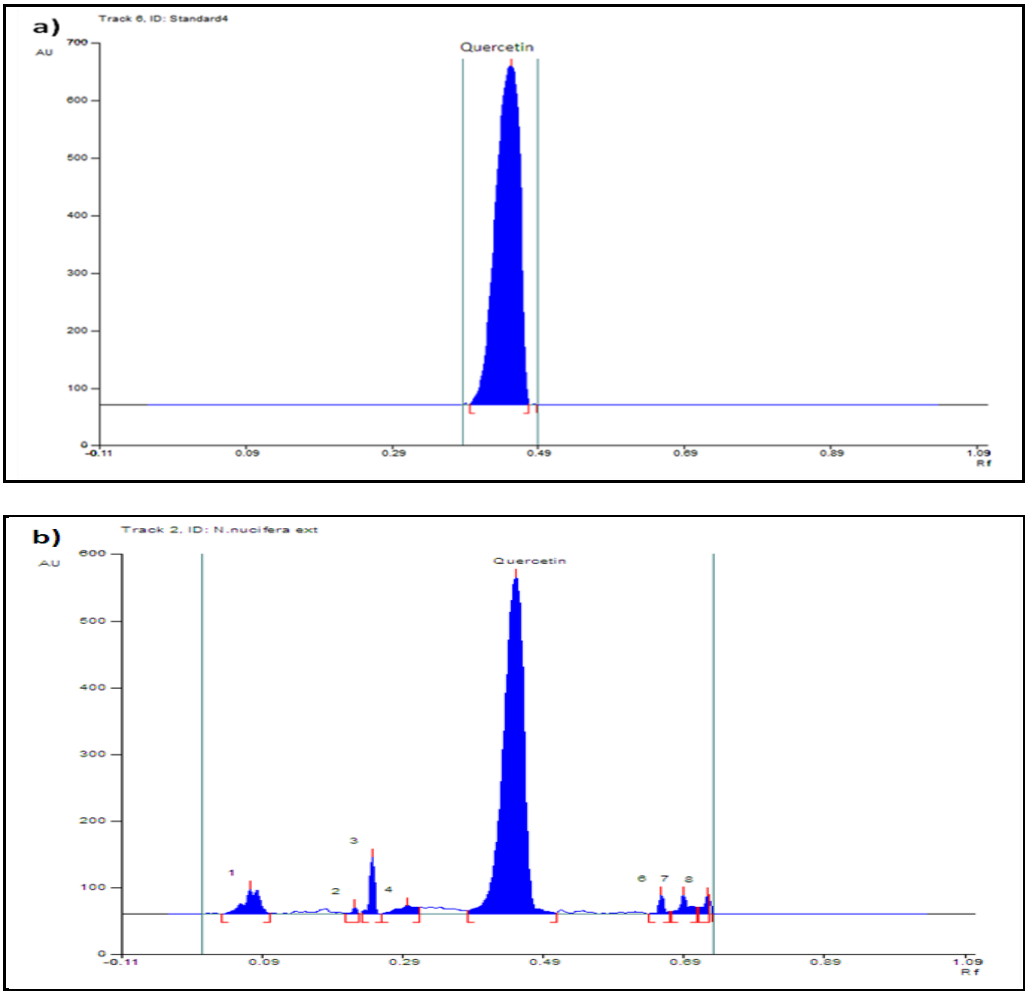

Fig. 5.15 Representative HPTLC Chromatograms of (a) Quercetin standard and (b) *N. nucifera* extract

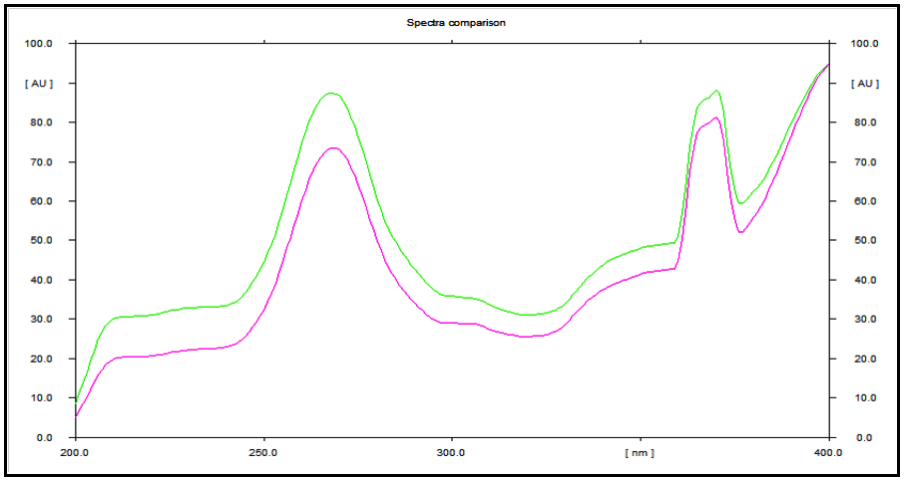

Fig. 5.16 Spectral comparison of *N. nucifera* extract with standard quercetin

5.2.4) HPTLC method development for simultaneous estimation glycyrrhizin, quercetin, rosmarinic acid and betulinic acid (Method IV)

5.2.4.1) Development of the optimum mobile phase

Different standards and formulation solution were spotted on the TLC plate and run in different solvent systems. Primarily, toluene, ethyl acetate and methanol in varying ratios were employed. When toluene: ethyl acetate: methanol in ratio of 8:2:0.5 v/v was used as solvent system merged spot of glycyrrhizin and quercetin was obtained. Although, changing ratio of solvent system to 7:1:0.5 v/v gave good resolution, Rf value obtained was less. Additionally, diffusion of spot lead to slight distortion of the typical peak nature. The spot characteristics were improved by addition of 0.5 ml of formic acid. The spot was analysed by densitometry at 546nm after derivatisation with anisaldehyde sulphuric acid reagent. Furthermore, methanol pretreatment and activation at 100 °C for 5 min improved resolution. The chamber was saturated for 20 min with the mobile phase at room temperature (Fig. 5.17) to prevent diffusion of the spots.

5.2.4.2) Validation of the method

a) Linearity

The calibration curve was in adherence to Kubelka Munk theory and linear within 400–1900 ng/spot. Linear regression analysis was performed and correlation coefficient was derived which was about 0.9994 with SD value <2 for intercept. 100 µg/ml of standard mixture solution was used to evaluate linearity in triplicate. No significant difference was observed in the slopes of standard plots.

Table 5.20 Linearity data of quercetin analysis by HPTLC

Track	Vial	Rf				Amount/Fraction (ng)	Height				X (calc) ng				Area				X (calc) ng				Sample ID/Remark
		GLY	RA	QUR	BA		GLY	RA	QUR	BA	GLY	RA	QUR	BA	GLY	RA	QUR	BA	GLY	RA	QUR	BA	
1	1		0.68		0.87	400		17.6		30.9						556.9		873.8					
2	1	0.14	0.65	0.74	0.86	700	33.3	25.9	15.7	44.2					485.8	859.4	632.5	1347.7					
3	1	0.14	0.65	0.74	0.87	1000	41.8	36.1	22.4	54.9					5707	1125.4	801.7	1777.9					
4	1	0.14	0.65	0.74	0.87	1300	50.6	47.7	30.8	66.3					648.2	1379	981.9	2213.3					
5	1	0.14	0.65	0.74	0.87	1600	61.5	56.4	38.1	76.9					729.3	1658.2	1165.6	2642.6					
6	1	0.14	0.65	0.74	0.87	1900	69.8	66.9	44.5	88.1					799.7	1920.9	1329.5	3069.5					
7	2	0.13	0.65	0.74	0.87		63.5	61.2	31.4	21.6	1691.58	1733.42	1346.86	1124.67	653.5	1576.8	1108.1	880.1	1325.94	1512.38	1514.71	389.97	Formulation
8	2	0.13	0.65	0.74	0.87		65.1	66.3	43.8	32.9	1743.36	1886.57	1855.05	423.62	667.4	1862.6	1246.6	989.5	1378.97	1829.27	1751.06	465.06	Formulation

Table 5.21 Regression analysis for calibration plots (n=3)

Parameters	Results			
	GLY	**RA**	**QUR**	**BA**
Linearity Range	400– 1900 ng/spot	400– 1900 ng/spot	400– 1900 ng/spot	400– 1900 ng/spot
Coefficient of Determination (Height)	0.9981	0.9983	0.9981	0.9989
Coefficient of Determination (Area)	0.9991	0.9995	0.9997	0.9997
Regression equation (Height)	y = 0.0309x + 11.23	y = 0.0333x + 3.477	y = 0.0244x - 1.4633	y = 0.0377x + 16.9
Regression equation (Area)	y = 0.2621x + 305.97	y = 0.9019x + 212.78	y = 0.586x + 220.48	y = 1.457x + 311.91
Slope (Height)	0.0309	0.0333	0.0244	0.0377
Slope (Area)	0.2621	0.9019	0.586	1.457
Intercept (Height)	11.23	3.477	1.4633	16.9
Intercept (Area)	305.97	212.78	220.48	311.91

b) Precision

Six replicate determinations were performed and % RSD was calculated for sample application and peak area which indicated repeatability of the analytical method. Also, intraday and interday precision in peak area of all four compounds at 700 and 1900 ng/spot was determined and the % RSD values are shown in Table 5.22

Table 5.22 Intra-day and inter-day precision of HPTLC method (n=6)

Components	Conc. (µg/ml)	Intra-day			Inter- day		
		Mean area	**SD**	**%RSD**	**Mean area**	**SD**	**%RSD**
GLY	700	571.73	5.65	0.99	578.40	8.16	1.41
	1900	795.73	7.66	0.96	799.07	11.86	1.48
RA	700	1121.87	11.13	0.99	1123.53	13.30	1.18
	1900	1923.67	15.63	0.81	1927.00	21.12	1.10
QUR	700	811.13	9.88	1.22	814.47	15.28	1.88
	1900	1332.00	7.76	0.58	1342.00	13.20	0.98
BA	700	1773.93	15.53	0.88	1777.27	20.16	1.13
	1900	3074.57	22.14	0.72	3077.90	27.67	0.90

c) Robustness of the method

As depicted in Table 5.23, standard deviation and % RSD of peak areas were calculated for each parameter. The % RSD value < 2% indicated robustness of the method.

Table 5.23 Robustness of the method

Amount (ng/spot)	Mobile phase composition (Area% RSD)	
	toluene: ethyl acetate: methanol: formic acid (7: 1: 0.5: 0.5 v/v/v)	toluene: ethyl acetate: methanol: formic acid (7.5:1.5:1:0.2 v/v/v)
700	1.48	1.62
1900	1.69	1.91

d) LOD and LOQ

The LOD and LOQ were found to be 11.96 and 36.24ng/ml, respectively for glycyrrhizin, 0.88 and 2.68ng/ml respectively, for quercetin and 0.31 and 0.93ng/ml for betulinic acid, 3.6 and 11.05ng/ml, respectively for rosmarinic acid.

e) Specificity

The specificity was complying according to USP. Resolution was found to be greater than 2. The excipients and other components did not interfere in the estimation of rosmarinic acid, glycyrrhizin, quercetin and betulinic acid. Thus good resolution and absence of interference showed the specificity of HPTLC method

f) Accuracy

The proposed method when used for extraction and simultaneous estimation of all four compounds from pharmaceutical dosage form after spiking with 80, 100 and 120% of additional drug afforded recovery of 98–100% as listed in Table 5.24.

Table 5.24 Recovery studies (n=3)

Sample	Excess drug added to the analyte (%)	Theoretical content (ng)	% Recovery	% RSD
RA	0	700	98.11	0.84
	80	1260	99.19	0.96
	100	1400	100.02	1.07
	120	1540	99.07	1.14
GLY	0	700	98.24	0.89
	80	1260	99.46	0.97
	100	1400	101.14	1.04
	120	1540	100.24	0.93
QUR	0	700	101.01	0.92
	80	1260	99.45	1.09
	100	1400	99.32	0.96
	120	1540	99.61	1.07
BA	0	700	99.67	1.07
	80	1260	101.07	1.13
	100	1400	101.12	1.08
	120	1540	99.26	0.97

g) Spot stability

Decomposition was not observed during spotting and development.

5.2.4.3) Analysis of the developed polyherbal formulation

A compact spot with *R*f value of 0.14, 0.68, 0.74 and 0.87 was obtained for glycyrrhizin, rosmarinic acid, quercetin and betulinic acid respectively in the HPTLC chromatogram of extracted drug samples (Fig. 5.17). Other components of the extract did not interfere in separation of all four markers. The %w/w of glycyrrhizin, rosmarinic acid quercetin and betulinic acid were calculated using peak area and regression equation (mentioned in Table 5.20 and Table 5.21) were 25.11 ± 0.03%w/w, 6.23 ± 0.14 %w/w, 15.73 ± 0.08 %w/w and 14.59 ± 0.05 %w/w respectively. The developed method, due to its good performance, proved suitable for routine analysis of all four compounds in the herbal dosage.

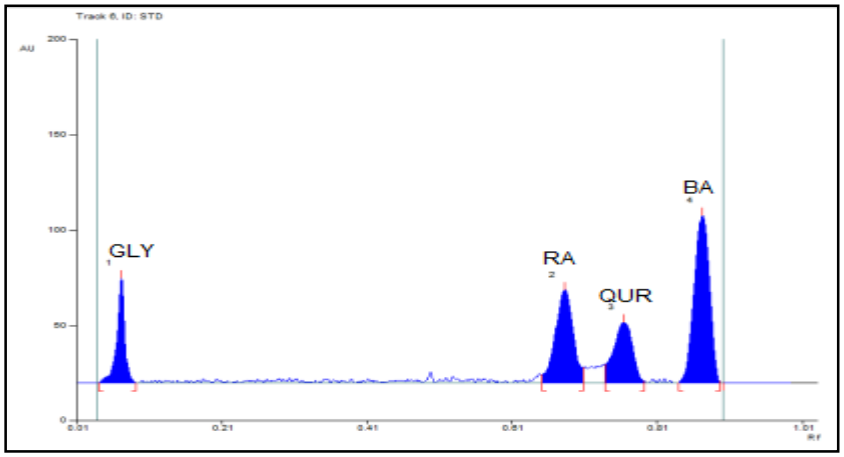

Fig. 5.17 Representative HPTLC Chromatograms of Glycyrrhizin, Rosmarinic acid, Quercetin and betulinic acid

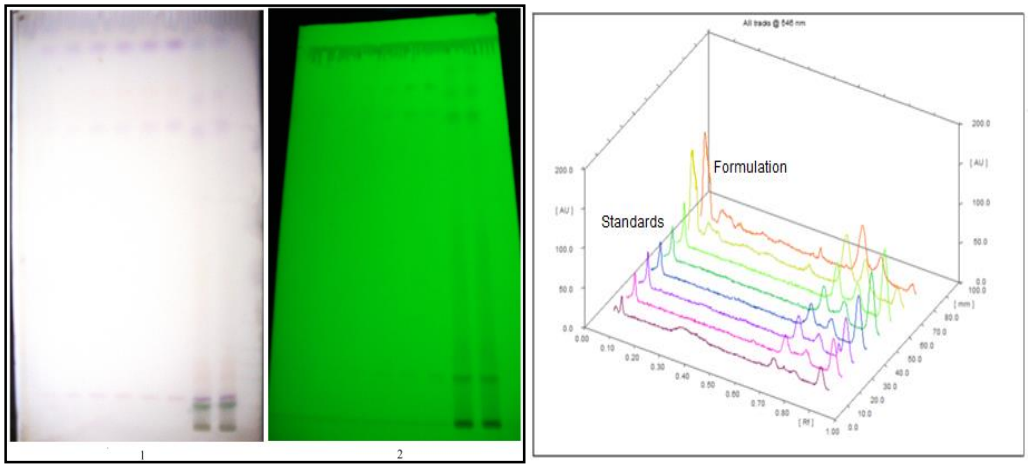

Fig. 5.18 (a)1- Photograph of TLC plate at 235nm, 2- Photograph of TLC plate at 254nm (b) Representative 3-dimensional overlaid HPTLC Chromatograms of Glycyrrhizin, Rosmarinic acid, Quercetin and betulinic acid and formulation

5.2.5) Spectrofluorometric method for estimation of quercetin in *Nelumbo* nucifera extract and in polyherbal formulation (Method V)

5.2.5.1) Excitation and emission spectra

Excitation and emission wavelength of quercetin was determined using its methanolic solution in concentration range of 10-70 ng/ml which were found to be at 242 nm and 515 nm respectively.

Table 5.25 Instrument Parameters

Parameters	Results
Excitation wavelength	242 nm
Emission wavelength	515 nm
Scan speed	Fast
Slit width	5 nm
Sensitivity	High

5.2.5.2) Validation of method

The developed method was validated according to ICH guidelines employing various parameters including linearity, precision, accuracy, sensitivity and robustness [19].

a) Linearity and range

Quercetin standard solutions were made in concentration range of 10-70ng/ml and linearity was evaluated (Y= 1.198x + 67.033; correlation coefficient R^2= 0.9997). The overlaid spectra are shown in figure 5.19.

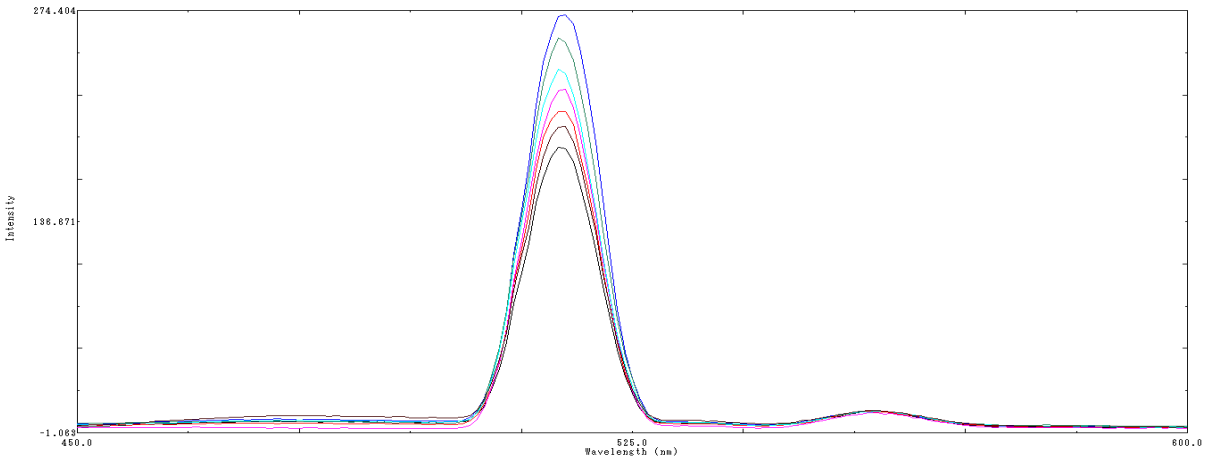

Fig. 5.19 Overlain spectra of Quercetin in methanol

Table 5.26 Data of calibration curve of Quercetin in methanol

Sr. No.	Concentration (ng/ml)	Fluoresence Intensity
1	10	185.139
2	20	198.831
3	30	211.521
4	40	229.217
5	50	242.023
6	60	256.37
7	70	271.703

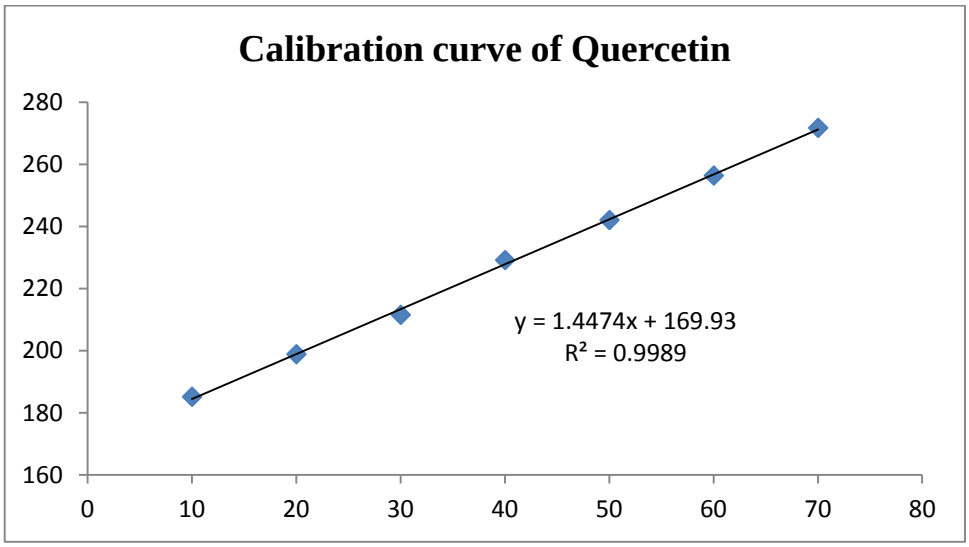

Fig. 5.20 Calibration curve of quercetin

b) Accuracy

For accuracy study, the samples were spiked with 80%, 100% and 120% of the standard markers and analyzed by the developed method in triplicate. The percentage recovery, calculated from the amount of drug found in solution, was between 98% and 101 %. The results demonstrated accuracy of the developed method as it remained unaffected in the presence of other extracts (Table 5.27).

c) Precision

Repeatability, assessed using triplicate determinations of three different concentrations, was employed to estimate both interday as well as intraday precision. The results showed that %RSD (Relative Standard Deviation) is less than 2.0 indicating good precision.

d) Detection and Quantitation limits

Calibration curve was performed in triplicate and SD (Standard Deviation) was calculated from the intercepts. Both limit of detection (LOD) and limit of quantitation (LOQ) were determined from SD of the response and the slope according to ICH guidelines (1966) using following formula:

LOD = 3.3*SD/slope of calibration curve

LOQ = 10* SD/slope of calibration curve

SD = standard deviation of intercepts

The LOD and LOQ for quercetin were found to be 0.28ng/ml and 0.83ng/ml respectively.

e) Stability

Both standard and sample methanolic solutions of quercetin were stored room temperature for 72 hrs and 5ºC in for 5 days. The results indicated that the solutions were stable in refrigerator as there were no changes in fluorescence.

f) Specificity

No other constituent from the extract affected the results of quercetin so the developed analytical method can be considered as specific for quercetin according to ICH guidelines for specificity. This can be applied to results of recovery of extracts and in formulation also.

5.2.5.3) **Analysis in *N. nucifera* extract and in developed polyherbal formulation**

The developed spectrofluorimetric method was employed for the determination of quercetin in hydro alcoholic extract of *Nelumbo nucifera* leaves and in formulation. The results obtained were satisfactory and in good agreement as shown in Table 5.28.

Table 5.27 Summary of validation parameters

Parameters	**Quercetin (λ_{em}=515nm)**
Calibration range (µg/ml)	10-70ng/ml
Detection limit (µg/ml)	0.28ng/ml
Quantitation limit (µg/ml)	0.83ng/ml
Regression equation	y = 1.4474x + 169.93
Correlation coefficient (R^2)	R^2 = 0.9989
Accuracy (% recovery ± standard deviation)	

80%	98.77 ± 0.21
100 %	99.63 ± 0.18
120%	100.19 ± 0.69
Precision (%RSD)*	
Intraday	0.81
Interday	1.59

Table 5.28 Percent yield of quercetin (%w/w) in *N. nucifera* leaves and in developed tablet formulation

Sample	Marker compound measured	% Yield of marker*
Lotus leaves extract	Quercetin	9.58
Tablet Formulation	Quercetin	7.54

*Found from regression equation stated in Table 5.27

5.2.6) Spectrofluorometric method for estimation of glycyrrhizin in *Glycyrrhiza glabra* extract and in developed polyherbal formulation (Method VI)

5.2.6.1) Excitation and emission spectra

Excitation and emission wavelength of glycyrrhizin was determined using its methanolic solution in concentration range of 100-600 pg/ml which were found to be at 272 nm and 545 nm respectively.

Table 5.29 Instrument Parameters

Parameters	Results
Excitation wavelength	272 nm
Emission wavelength	545 nm
Scan speed	super
Slit width	5 nm
Sensitivity	High

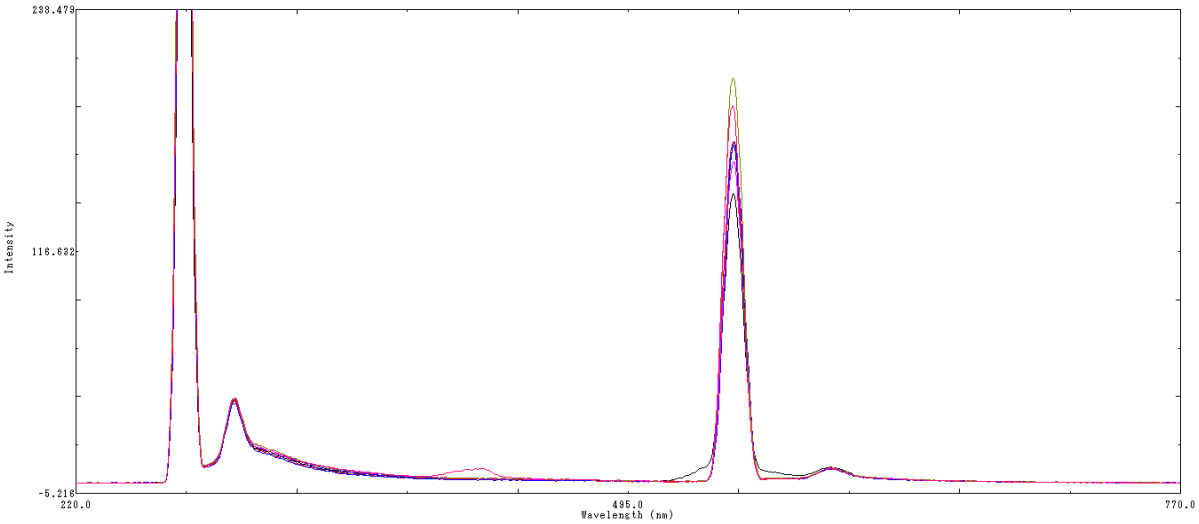

Fig. 5.21 Overlain spectra of Glycyrrhizin in methanol

Table 5.30 Data of calibration curve of Glycyrrhizin in methanol

Sr. No.	Concentration (pg/ml)	Fluoresence Intensity
1	100	135.787
2	200	146.626
3	300	157.701
4	400	165.315
5	500	176.366
6	600	184.971

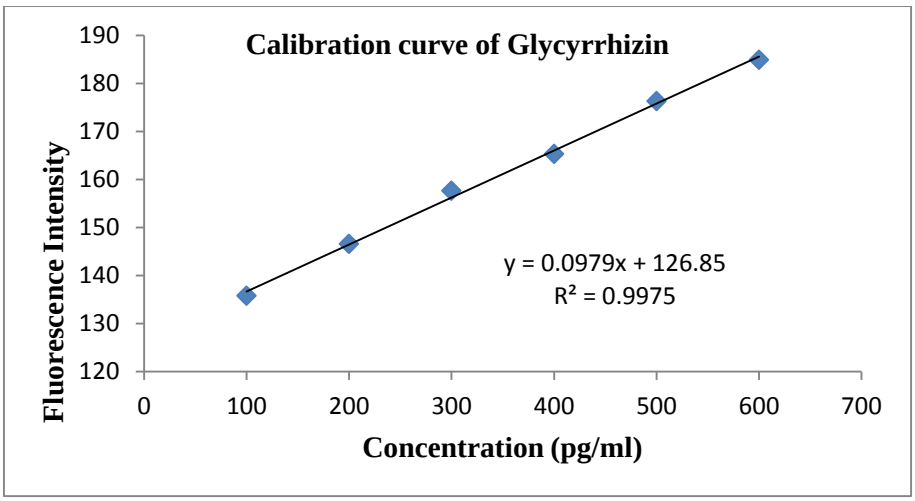

Fig. 5.22 Calibration curve of Glycyrrhizin

5.2.6.2) Validation of method

The developed method was validated according to ICH guidelines employing various parameters including linearity, precision, accuracy, sensitivity and robustness [19].

a) Linearity and range

Glycyrrhizin standard solutions were made in concentration range of 100-600pg/ml and linearity was evaluated (Y= 0.0993x + 126.24; correlation coefficient R^2= 0.9979). The overlaid spectra are shown in figure 5.21.

b) Accuracy

For accuracy study, the samples were spiked with 80%, 100% and 120% of the standard markers and analyzed by the developed method in triplicate. The percentage recovery, calculated from the amount of drug found in solution, was between 98% and 101 %. The results demonstrated accuracy of the developed method as it remained unaffected in the presence of other extracts (Table 5.31).

c) Precision

Repeatability, assessed using triplicate determinations of three different concentrations, was employed to estimate both interday as well as intraday precision. The results showed that %RSD (Relative Standard Deviation) is less than 2.0 indicating good precision.

d) Detection and Quantitation limits

Calibration curve was performed in triplicate and SD (Standard Deviation) was calculated from intercepts. Both limit of detection (LOD) and limit of quantitation (LOQ) were determined from SD of the response and the slope according to ICH guidelines (1966) using following formula:

LOD = 3.3*SD/slope of calibration curve

LOQ = 10* SD/slope of calibration curve

SD = standard deviation of intercepts

The LOD and LOQ for glycyrrhizin were found to be 4.65pg/ml and 14.11pg/ml

e) Stability

Both standard and sample methanolic solutions of glycyrrhizin were stored at room temperature for 72 h and 5ºC for 5 days. The results indicated that the solutions were stable in refrigerator as there was no change in fluorescence.

f) Specificity

No other constituent from the extract affected the results of glycyrrhizin so the developed analytical method can be considered as specific for glycyrrhizin according to ICH guidelines for specificity. This can be applied to the results of recovery of extracts and formulation also.

5.2.6.3) Analysis in extracts and in formulation

The developed spectrofluorimetric method was employed for determination of glycyrrhizin in aqueous extract of *Glycyrrhiza glabra* roots and in formulation. The results obtained were satisfactory and in good agreement as shown in Table 5.32.

Table 5.31 Summary of validation parameters

Parameters	Glycyrrhizin (λ_{em}=545nm)
Calibration range (µg/ml)	100-600pg/ml
Detection limit (µg/ml)	4.65pg/ml
Quantitation limit (µg/ml)	14.11pg/ml
Regression equation	y = 0.0993x + 126.24
Correlation coefficient (R^2)	R^2 = 0.9979
Accuracy (% recovery ± standard deviation)	
80%	99.14 ± 0.37
100 %	98.59 ± 0.36
120%	99.47 ± 0.21
Precision (%RSD)*	
Intraday	0.67
Interday	1.44

Table 5.32: Percent yield of glycyrrhizin (%w/w) in *G. Glabra* root extract and in developed tablet formulation

Sample	Marker compound measured	% Yield of marker*
Liquorice root extract	Glycyrrhizin	26.17
Formulation	Glycyrrhizin	24.19

*Found from regression equation stated in table 5.31

5.3 CONCLUSION

The chemical marker Glycyrrhizin, Rosmarinic acid, Quercetin and Betulinic acid were used for the standardization of *G. Glabra* roots, *P. vulgaris* aerial parts, *N. nucifera* leaves and *Z.*

jujuba fruits present in the formulation. Three methods using HPLC, HPTLC and spectrofluorimetry were developed for the determination of four markers. The methods were found to be applicable to developed polyherbal formulations.

The HPLC method (Method I) for simultaneous estimation of glycyrrhizin and betulinic acid was accurate, precise, sensitive and robust.

The developed HPLC and HPTLC methods (method II and IV) provide accurate and reproducible quantitative analysis for determination of glycyrrhizin, quercetin, betulinic acid and rosmarinic acid simultaneously. Statistically, the developed HPLC and HPTLC methods are less time consuming, easier and equally suitable for routine determination of glycyrrhizin, quercetin, betulinic acid and rosmarinic acid simultaneously in the developed polyherbal formulation.

HPTLC method (Method III) for rapid, simple, accurate quantitative estimation of quercetin present in the hydro-alcoholic extract of *N. nucifera* leaves was developed and validated which can be used as a QC standard. The developed method gave good peak shape and resolution of quercetin from plant constituents.

The spectrofluorimetric methods (Method V and VI) developed for quercetin and glycyrrhizin are effective with respect to sensitivity, linearity, reproducibility, accuracy and precision. The developed method was precise, accurate indicating application of this method for routine quality control of quercetin and glycyrrhizin in commercial herbal preparation. Owing to the detectability and sensitivity of the drug in nano and pico range, as an added benefit of fluorescence measurement, the method can also be used for bioanalytical purpose.

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