Introduction
Licorice, the root of the leguminous Glycyrrhiza plant species, has been consumed for over 4,000 years, also known as “sweet root.” Glabridin [4-[(3R)-8,8-dimethyl-3,4-dihydro-2H-pyrano[6, 5-f]chromen-3-yl]benzene-1,3-diol] (Figure 1) is a major polyphenolic flavonoid specific for *Glycyrrhiza glabra* L. Recent studies have proven the anticancer activity of glabridin in inhibiting migration, invasion and angiogenesis of MDA-MB-231 human breast adenocarcinoma cells and human non-small cell lung cancer A549 cells by inhibiting focal adhesion kinase/Rho signaling pathway (1, 2). Moreover, glabridin has been reported to possess several pharmacological activities such as antioxidant (3), anti-*Helicobacter pylori* (4), antifungal (5), estrogenic, anti-proliferative (6, 7) and antipneumocytic activity (8). In addition, glabridin inhibits serotonin reuptake (9), melanogenesis and inflammation (10).

The Unani system of medicine is commonly practiced in India and many other countries. Different types of herbal formulations are used in the Unani system, such as solid (Qurs, Habbs, Safoof), semi-solid (Khamira, Itrifal, Majoon) and liquid (Sharbat) preparations (11, 12). Qurs-e-Gul is a tablet formulation mentioned in the National Formulary of Unani Medicines (NFUM), which has been commonly used in the Unani system of medicine as a deobstruent drug and prescribed for the treatment of jaundice and cardiac-related problems. It is composed of five ingredients; namely, *Glycyrrhiza glabra* (28.57% w/w), *Pistacia lentiscus* (4.7% w/w), *Bamboosa bambos* Druce (4.76% w/w), *Nardostachys jatamansi* (14.28% w/w) and *Rosa damascena* (47.6% w/w) and excepients (13).

Several analytical methods have been reported for the quantification of glabridin using different analytical techniques, such as high-performance liquid chromatography (HPLC) (14–16), capillary electrophoresis (17) and liquid chromatography–mass spectrometry (LC–MS) (18). The previously reported isocratic HPLC method was found to have a very narrow linearity range (10–100 μg/mL) and the recovery of the method was also found to be poor, i.e., 92% (14). Moreover, the reported isocratic method was found to be inappropriate to separate the immediate impurities from the main constituents in polyherbal formulations. Hence, it was thought worthwhile to develop and validate a gradient reversed-phase (RP)-HPLC method for the quantification of glabridin in crude drug and in polyherbal formulations, which can separate the glabridin with good resolution in multicomponent formulations with better validation parameters. The newly proposed and validated HPLC method can be used more easily and efficiently for the quantification of glabridin in polyherbal formulations than earlier reported methods.

Experimental
Reagents and chemicals
Glabridin (98%) was obtained as a gift sample from Sami Labs (Bangalore, India). HPLC-grade acetonitrile and methanol were purchased from Merck (India). Milli-Q water used throughout the experiment was prepared using a Millipore water purification system.

Instrumentation and general conditions
Chromatographic experiments were conducted on a YL9100 HPLC system (South Korea) that comprised quaternary YL9110 pumps, a variable wavelength programmable YL9120 ultraviolet (UV)-visible detector, YL9130 column oven and a system controller. The instrument was controlled by use of YL-Clarity software installed with the equipment. Samples were injected by using a rheodyne injector fitted with a 20-μL fixed loop. Standard and sample solutions were filtered through a 0.22-μm syringe filter before injection. The
separation was achieved by using a C18 reversed-phase column (Merck Lichrocart 250-4, Lichrosphere 100 RP-18e, 5 μm, Sorbent lot number L57020637). The mobile phase consisted of acetonitrile and water in gradient elution method from 50 to 80% in 20 min. The flow rate was kept at 1.0 mL/min. All the analyses were performed at room temperature and detection was carried out at a wavelength of 230 nm using a UV-visible detector.

**Preparation of sample solutions**

**Preparation of sample for crude**

One gram of the powdered crude drug was extracted with 25 mL of 30% aqueous ethanol by soaking overnight and then refluxing on a water bath for 45 min. It was filtered and evaporated to dryness under reduced pressure. The obtained residue was then reconstituted in HPLC-grade methanol and the volume was adjusted to 25 mL, which was filtered through a 0.22-μm syringe filter before injecting into the HPLC column.

**Preparation of sample for polyherbal formulation**

Twenty tablets were randomly selected from the formulation and average weight was determined. The tablets were triturated to get a uniform fine powder. Two grams, accurately weighed, of the powdered sample was extracted with 25 mL of 30% aqueous ethanol, as discussed previously. The obtained residue was then reconstituted in HPLC-grade methanol and volume was adjusted to 25 mL, which was filtered through a 0.22-μm syringe filter before injecting into the HPLC column.

**Validation methodology**

Method validation was carried out to confirm the suitability of the proposed analytical method for its intended use. The proposed method was validated as per International Conference on Harmonization (ICH) (19) guidelines for different parameters such as linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) and robustness, similar to the previously reported laboratory methods (20–23).

**Calibration curve for glabridin**

A stock solution of glabridin with a known concentration of 1,000 μg/mL was prepared in methanol and different aliquots were made to get six different desired concentrations from 1 to 500 μg/mL, which were injected (20 μL each) by rheodyne injector and chromatographed as per the previously mentioned protocol. The stock solution was kept in dark for storage to avoid possible degradation that may result from exposure to light.

**Accuracy as recovery**

The accuracy of the method was determined by recovery studies using the standard addition method. Preanalyzed samples were spiked with standard glabridin at three different concentration levels, i.e., 50, 100 and 150%, and the mixtures were reanalyzed by the proposed method. Data obtained was analyzed for percent recovery.

**Precision**

The precision of the method was carried out by performing repeatability and intermediate precision. In repeatability, six different injections of the same standard sample (three concentrations) were injected and calculated in the assay. The percent relative standard deviation (%RSD) of the area and retention time (Rt) were calculated. In intermediate precision, intra-day, inter-day, and inter-system precisions were performed. Intra-day and inter-day precisions were performed by preparing and applying three different concentrations of standard in triplicate six times a day and on six different days, respectively. Inter-system precision was performed by repeating the same procedure in a different HPLC system. Assay for each analysis was calculated and %RSD was determined.

**LOD and LOQ**

The LOD and LOQ were determined based on the basis of signal-to-noise ratio. The concentration of the sample with a signal-to-noise ratio of three was fixed as the LOD. The concentration of the sample with a signal-to-noise ratio of ten was fixed as the LOQ.

**Robustness of the method**

Robustness of the method was performed by introducing very small changes in the analytical methodology at a single concentration level (100 μg/mL). Robustness of the proposed method was determined in two different ways, i.e., by making deliberate changes in the flow rate and by changing the detection wavelength of the analysis. In the present study, the robustness was evaluated by using the Box-Behnken response surface design (24). The design simultaneously evaluated the effects of the three important parameters on peak area: flow rate of the mobile phase, detection wavelength and temperature of the column oven. Design Expert version 7.0.0.1 (Stat-Ease, Minneapolis, MN) was used to evaluate the results. Three-dimensional graphs represented peak area dependence on flow rate of the mobile phase and detection wavelength temperature of the column and flow rate of the mobile phase and temperature of the column and detection wavelength. Effects of the selected factors were evaluated over a range of

![Chemical structure of glabridin.](image-url)
conditions by determining the maximum area response of the glabridin peaks.

Results and Discussion

Method development

A variety of mobile phases was investigated for the development of the HPLC method suitable for analysis of glabridin in crude drug and polyherbal formulations. The investigated mobile phases included methanol–water, 50:50 (% v/v), acetonitrile–phosphate buffer (pH 3.5 adjusted with orthophosphoric acid), 80:20 (% v/v), acetonitrile–phosphate buffer (pH 3.5 with orthophosphoric acid), 60:40 (% v/v), acetonitrile–water, 50:50 (% v/v) and acetonitrile–water, 60:40 (% v/v). It was observed that acetonitrile and water 60:40 (% v/v) produces a sharp peak of glabridin at retention time of 9.1 ± 0.02 min. However, this mobile phase was not efficient to separate the compound in polyherbal formulation because of poor separation from the immediate impurities. Therefore, it was decided to make a gradient elution system with acetonitrile concentration from 50 to 80% within 20 min, which resulted in a sharp peak at the Rt of 14.9 ± 0.02 min with good separation from the impurities (Figure 2). Furthermore, chromatographic conditions were optimized and system suitability parameters like theoretical plates (7,739), tailing factor (0.982) and asymmetry (0.958) of glabridin were calculated using YL Clarity software, which proved the suitability of the proposed method.

Validation of analytical method

Linearity

To assess the linearity of the method, a stock solution of 1,000 µg/mL was prepared, and from this, different aliquots were prepared from 1–500 µg/mL. The stock solution was kept in the dark for storage. The linearity of the calibration for glabridin was assessed in the range of 1–500 µg/mL with excellent regression coefficient value 0.9992; the regression equation is $y = 26.683x - 142.17$. The slope ± SD and intercept ± SD were found to be 25.13633 ± 0.52 and 141.11 ± 1.2, respectively.

Accuracy

The accuracy of the method was determined by recovery studies. The preanalyzed samples were spiked with standard at three different concentration levels, i.e., 50, 100 and 150%. The mixtures were reanalyzed by the proposed method and found to be within the limit of 97.39–103.25%, which is better than the method reported by Shanker et al. (14). The values of recovery percent and %RSD are listed in Table I.
Precision of the proposed method was obtained by repeatability and intermediate precision in accordance with the ICH recommendations. In repeatability studies, six different injections of the same standard sample (three concentrations) were injected and calculated the assay. The %RSD of area and Rt were calculated. Inter-day and intra-day precisions were performed by preparing and applying three different concentrations of samples during the same day and on three different days, respectively. Inter-system precision was performed by repeating the same procedure by using a different system (Waters Co., Milford, MA) but on same column. The results from the determination of repeatability and intermediate precisions, expressed as %RSD, were listed in Table II and III.

### LOD and LOQ

The limits of quantification and detection were calculated by using the linearity curve method by using the formula LOD = 3.3σ/S and LOQ = 10σ/S, where σ is the standard deviation of the response and S is the slope of the calibration plot. For the developed method, LOD was found to be 0.35 μg/mL and LOQ was calculated at 1 μg/mL. Once the LOD and LOQ were determined, six replicates of blank and standard solutions at the levels of LOD and LOQ were applied and the %RSD was calculated.

### Robustness

The robustness was evaluated by using the Box-Behnken response surface design. The Design Expert software proposed the following polynomial equation for peak area:

\[
\text{Peak area} = 6572.00 + 0.76A + 35.22B - 11.42C - 24.68AB + 3.16AC - 16.77BC - 7.38A^2 - 73.39B^2 - 69.63C^2,
\]

where A is the detection wavelength (nm), B is the flow rate (mL/min) and C is the temperature (°C). According to the equation, flow rate appeared to have more effect on the peak area than detection wavelength and temperature. As the flow rate increases, peak area also increases. The detection wavelength was also found to increase the peak area, as indicated by the positive coefficient value. However, the lower magnitude of the coefficient indicated that the effect of detection wavelength is less than the flow rate on peak area. The temperature

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>Peak area Mean peak area ± SD</th>
<th>% RSD</th>
<th>Retention time Mean Rt ± SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1,135.6 ± 13.31</td>
<td>1.17</td>
<td>14.97 ± 0.06</td>
<td>0.40</td>
</tr>
<tr>
<td>100</td>
<td>2,231.3 ± 30.65</td>
<td>1.37</td>
<td>15.06 ± 0.10</td>
<td>0.66</td>
</tr>
<tr>
<td>200</td>
<td>5,273 ± 34.40</td>
<td>0.65</td>
<td>15.00 ± 0.08</td>
<td>0.53</td>
</tr>
</tbody>
</table>

### Table II

Repeatability of the Method (n = 3)

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>Peak area Mean peak area ± SD</th>
<th>% RSD</th>
<th>Retention time Mean Rt ± SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1,135.6 ± 13.31</td>
<td>1.17</td>
<td>14.97 ± 0.06</td>
<td>0.40</td>
</tr>
<tr>
<td>100</td>
<td>2,231.3 ± 30.65</td>
<td>1.37</td>
<td>15.06 ± 0.10</td>
<td>0.66</td>
</tr>
<tr>
<td>200</td>
<td>5,273 ± 34.40</td>
<td>0.65</td>
<td>15.00 ± 0.08</td>
<td>0.53</td>
</tr>
</tbody>
</table>
affected the peak area in the opposite direction to that observed for the detection wavelength and flow rate. The negative coefficient value of the temperature indicated that the peak area decreased with an increase in temperature. Although the software predicted that the different factors could affect the peak area, the values of peak area were in an acceptable range to demonstrate sufficient robustness of the analytical method.

The response surface plots represent the dependence of peak area on: (i) flow rate of the mobile phase and detection wavelength (Figure 3A); (ii) temperature of the column and flow rate of the mobile phase (Figure 3B); and (iii) temperature of the column and detection wavelength (Figure 3C). The interactive effects of the selected factors were evaluated over a range of conditions by determining the maximum area response of the glabridin peak using these response surface plots. The figures show that flow rate contributed more significantly toward the response of peak area in all the plots than detection wavelength and temperature. Figure 3A shows that at higher detection wavelength and flow rate, peak area is decreased compared to that observed at lower detection wavelength and flow rate. Figure 3B shows that the response surface is slightly influenced by the combined effects of temperature and detection wavelength. The result of their interaction resulted was that the peak areas were highest at the extremes of the temperature selected for the study and almost irrespective of the detection wavelength. The interaction of flow rate and temperature (Figure 3C) resulted in an increased peak area at the extremes of temperature and a decreased peak area at the extremes of flow rate.

**Analysis of crude sample and herbal formulation**

The newly developed and validated RP-HPLC method was applied for the analysis of glabridin in *Glycyrrhiza glabra* root powder and in a polyherbal Unani formulation (Qurs-e-Gul). The peak areas of triplicate samples were analyzed by regression equation obtained from the calibration plot to determine the content of glabridin in samples. No interference was found at the retention time of glabridin in samples (Figure 2). The content of glabridin in *Glycyrrhiza glabra* was found to be 0.098% w/w, which was supposed to vary between 0.07–0.8% w/w according to the literature (15). Similarly, glabridin content in polyherbal formulation (tablets) was found to be 0.016% w/w; the content was expected to be approximately 0.0196% w/w, because the formulation contains approximately 20.1% of *Glycyrrhiza glabra* root powder, including excipients used for the optimization of tablet formulation. Moreover, due to large biodiversity in plants, the confirmation of the presence of markers rather than their quantity is usually important in relation to identity and safety of traditional drugs.

**Conclusion**

A simple, economic, accurate, precise, reproducible and robust RP-HPLC–UV method for determination of glabridin was developed and validated in crude drug and polyherbal formulation over a wide concentration range. No interference was observed at the elution time of glabridin in both standard and samples. The validation data are indicative of good precision and accuracy, and proved the reliability of the method. The low LOD (0.35 μg/mL) and LOQ (1 μg/mL) values indicate the good sensitivity of the method, and prove that the developed method is much more sensitive than the existing HPLC method proposed by Shanker et al. (14) for the analysis of glabridin, with LOD and LOQ of 0.019 and 0.065 mg/mL. The proposed method was found to be excellent for the routine analysis of glabridin in crude drug and polyherbal formulation. This simple and validated analytical method will help in the upcoming research on this important anticancer moiety for quantification in different herbal formulations. The method can be used for quality control of *Glycyrriza* root and several polyherbal formulations that contain it, as an alternative to glycyrrhizin and glycyrrhetinic acid, which is a less specific marker than to glabridin (25).

**Acknowledgment**

Authors are thankful to CCRUM-AYUSH, Government of India for providing financial assistance.

**References**


7. Choi, E.M.; The licorice root derived isoflavon glabridin increases the function of osteoblastic MC3T3-E1 cells; Biochemical Pharmacology, (2005); 70: 363–368.


13. Kamal et al. 784