Quantitative Analysis of Total Phenolic, Flavonoid Contents and HPTLC Fingerprinting for Standardization of *Glycyrrhiza glabra* Linn. Roots

**Abstract**

The *Glycyrrhiza glabra* Linn is an important medicinal plant and its roots are commonly used in various indigenous system of medicine to cure many acute and chronic diseases. The current study was designed to quantify total phenolic, flavonoid contents in *G. glabra* Linn roots to evaluate the antioxidant potential and to carry out the pharmacognostical investigations along with HPTLC fingerprinting in order to develop the quality control parameters for the standardization of this important medicinal plant. A variety of pharmacognostical investigations e.g., extractive values, total ash, water soluble ash, and acid insoluble ash, moisture content, loss on drying, pH and phytochemical screening of *G. glabra* Linn roots were analyzed as per the standard methods. The content of total phenolics, flavonoids, pesticide residues, aflatoxin and heavy metals were also determined as per the reported methods. The HPTLC fingerprinting of methanolic extract of the *G. glabra* roots was also carried out using CAMAG-HPTLC system. The results of phytochemicals investigations revealed the presence of carbohydrates, phenolic compounds, flavonoids, alkaloids, proteins, saponins, lipids, sterols and tannins in various solvent extracts. Total phenolic and flavonoid contents in methanolic extract were found to be 7.47 mg/gm and 2.25 µg/gm, respectively. Heavy metals concentrations were found to be under the standard limits. Aflatoxins and pesticides residues were not detected in the extract. The standard parameters established through this study may prove important tools for authentication, identification, purification and standardization of *G. glabra* Linn roots in herbal pharmaceutical industries.

**Keywords:** *Glycyrrhiza glabra* roots; HPTLC fingerprint; Liquorice; Standardization

**Theoretical Background**

*Glycyrrhiza glabra* (*G. glabra*) Linn commonly known as Sweet wood in English is the most valuable medicinal herb that belongs to pea family “Leguminosae”. *Glycyrrhiza* is derived from two Greek words “glycos” which means sweet and “rhiza” means root. *G. glabra* consist of unpeeled dried roots and stolon [1]. It is an important medicinal herb which is known since ancient time and it is also one of the most widely used herbes in different traditional systems of medicines worldwide [2]. It is widely cultivated in China, France, Germany, India, Italy, Russia, United Kingdom and United State of America etc. [3]. *G. glabra* is used as a medicine as well as a flavoring agent. The *G. glabra* roots contain an important active constituent called as glycyrrhizin; a saponin (Figure 1) which is reported to be 60 times sweeter than cane sugar [4]. The roots are used in traditional medicine since many centuries in the treatment of a variety of diseases in different indigenous systems of medicine including folk medicines. It is...
The cleaned roots were used to prepare the extracts in different solvents. The roots were dried below 60°C in a hot air oven, powdered and passed through sieve 14 to obtain a uniform size powdered material. The dried powder (500 gm) was used for the continuous hot extraction with different solvents like petroleum ether, n-butanol, chloroform, acetone and methanol. The dried powdered material was placed in a soxhlet apparatus on a water bath for six hours. The extracts so obtained were filtered and dried with the help of rotary evaporator (Buchi, Rotavapor-R-210, Switzerland) and the final polar extract kept at low temperature for further investigations for the development of standard parameters.

**Physicochemical standardization**

The physicochemical standardization studies of the *G. glabra* roots extracts were carried out according to WHO guidelines on the quality control methods for medicinal plant materials and various other pharmacopeial procedures. A variety of physicochemical parameters like extractive values in different solvents, total ash value, water soluble ash value, acid insoluble ash value, moisture contents, loss on drying, pH values (1% and 10% solutions) were carried out for the development of the standard parameters of *G. glabra* root extracts. Apart from these, analysis on aflatoxins, pesticides residues and heavy metals were also carried out as per standard methods [24,25].

**Preliminary qualitative phytochemicals analysis**

The various solvent extracts were subjected to qualitative chemical analysis. The extracts were screened for the presence of carbohydrates, phenolic compounds, flavonoids, alkaloids, proteins, saponins, lipids, steroids and tannins [25].

**Estimation of total phenolic and flavonoid contents**

The total phenolic content in the methanolic extract was determined by the colorimetric assay of phenols by Folin Ciocalteu’s method. A calibration curve of standard “gallic acid” was plotted for calculation of total phenolic contents in the sample [26,27] and the total phenolic contents in the roots extract is reported as Gallic acid equivalent (GAE) (mg/g of dry mass). The total flavonoid contents of methanolic extract roots was determined by the Aluminum chloride colorimetric method using double beam UV spectrophotometer. A calibration curve of standard “Quercetin” was plotted for calculation of the total flavonoid contents in the sample [28]. The flavonoid contents in the sample of roots extract is reported as Quercetin equivalent (µg/gm of dry mass).

**HPTLC fingerprinting of methanolic extract of *G. glabra***

A qualitative HPTLC fingerprinting analysis (CAMAG HPTLC system with a Linomat 5 sample applicator, Switzerland) of the methanolic extract was carried out for the development of characteristic fingerprint profile to confirm the presence of various phyto-pharmaceuticals. For excellent separation and sharp peaks, many solvent systems in different combinations were tried during the experiment. The solvent system of Hexane: Ethyl acetate: Methanol (9:1:1) exhibited the satisfactory resolution for the separation of phytochemicals in the methanolic extract. The whole HPTLC fingerprinting analysis was carried out in a well maintained air-conditioned room of temperature of 22°C and 55% humidity. The methanolic extract (5 µL) was spotted on the pre-coated silica gel 60F254 HPTLC aluminium plates (E-Merck, Germany) as bands of 6 mm width with the help of the auto
sampler fitted with a 100 µL Hamilton syringe. The Hexane: Ethyl acetate: Methanol (9:1:1) solvent system was transferred to CAMAG twin trough plate development chamber lined with filter paper and pre-saturated with mobile phase (30 mL). The resulted plates were air dried and scanned. A spectrodensitometer (Scanner 3, CAMAG) equipped with ‘win CATS’ planar chromatography manager (version1.3.0) software was employed for the densitometry measurements, spectra recording and data processing. Absorption/remission was then measured at a scan speed of 20 mm/s. Chromatograms were recorded at 254 and 366 nm. The R<sub>f</sub> value of each compound separated on plate and data of peak area of each band were recorded [28,29].

### Determination of heavy metals, pesticide residues and Aflatoxin

The *G. glabra* extracts were analysed to detect the presence of heavy metals (Cd, Pb, As and Hg), pesticides residues and aflatoxins by HPLC according to official methods of the American Organization of Analytical Chemists (AOAC) [30].

### Results

#### Physicochemical evaluations

A variety of physicochemical parameters such as extractive values, total ash, acid insoluble ash, water soluble ash, moisture content, loss on drying, pH (1% and 10% solutions) were analyzed to determine the purity of the drug. The results of the physicochemical evaluations are presented in Table 1.

#### Table 1 Results of physicochemical analysis of *G. glabra* Linn Roots.

<table>
<thead>
<tr>
<th>S No</th>
<th>Physicochemical Parameters</th>
<th>Average values % Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>Extractive Values</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Petroleum ether</td>
<td>4.67 ± 0.23%</td>
</tr>
<tr>
<td>2.</td>
<td>Chloroform</td>
<td>10.56 ± 1.53%</td>
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<tr>
<td>3.</td>
<td>n-butanol,</td>
<td>6.54 ± 0.84%</td>
</tr>
<tr>
<td>4.</td>
<td>Methanol</td>
<td>13.89 ± 2.42%</td>
</tr>
<tr>
<td>B.</td>
<td>Ash Values</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Total Ash</td>
<td>4.67 ± 0.35%</td>
</tr>
<tr>
<td>2.</td>
<td>Acid Insoluble Ash</td>
<td>0.56 ± 0.34%</td>
</tr>
<tr>
<td>3.</td>
<td>Water Soluble Ash</td>
<td>6.54 ± 0.22%</td>
</tr>
<tr>
<td>C.</td>
<td>Loss on Drying (LOD)</td>
<td>5.87 ± 0.65%</td>
</tr>
<tr>
<td>D.</td>
<td>Moisture contents</td>
<td>0.56 ±0.054%</td>
</tr>
<tr>
<td>E.</td>
<td>pH of the extract (1% solution)</td>
<td>5.04 ± 0.65</td>
</tr>
<tr>
<td>F.</td>
<td>pH of the extract (10% solution)</td>
<td>6.26 ± 0.54</td>
</tr>
</tbody>
</table>

#### Preliminary qualitative phytochemicals analysis

The results of preliminary qualitative phytochemical screening revealed the presence of alkaloids, carbohydrates, phenolic compounds, flavonoids, proteins, saponins, lipids, tannins and steroids. All mentioned phytochemicals were present in the methanolic extract of the roots. The results of the preliminary phytochemicals screening are presented in Table 2.

#### Total phenolic and flavonoid contents

The total phenolic contents represented as mean ± S.D in triplicate, was found to be 7.47 ± 0.05 mg/gm of Gallic acid equivalent (GAE) in the extract. The total flavonoids contents represented as mean ± S.D in triplicate, was found to be 2.25 ± 0.03 µg/gm quercetin equivalents (QE) in the extract.

#### HPTLC fingerprint profile

HPTLC fingerprinting of methanolic extract of the *G. glabra* roots was carried out by using Hexane: Ethyl acetate: Methanol (9:1:1) solvent system. A total number of 11 peaks at different R<sub>f</sub> values and peak area at 366 nm were observed in the HPTLC chromatograms (Figures 2 and 3; Table 3) while 10 peaks were observed in HPTLC chromatogram at 254 nm (Figures 4 and 5; Table 3). The total number of phytoconstituents (no. of peaks) in the extract and their retention factors (R<sub>f</sub>) are given in the Table 3 and chromatographic profile had been shown by Figures 2-5.

#### Analysis of heavy metals, pesticide residues and aflatoxins

The analysis of the heavy metals Cd, Pb, As, Hg in the extracts was carried out by atomic absorption spectrophotometer. All necessary safety precautions were taken to avoid potential contaminations. The Cadmium (Cd) concentration was found to be 0.28 ± 0.03 mg/kg which was under the permissible limit of 0.30 mg/kg according to WHO guidelines. Lead (Pb) was found to be 0.48 ± 0.12 mg/kg which was much lower than permissible limit of 10 mg/kg. Arsenic (As) and Mercury (Hg) were found to be 0.47 ± 0.05 mg/kg and 0.33 ± 0.08 mg/kg respectively in the sample of *G. glabra* roots extract. Both of these metals were found to be within permissible limits of 0.5 mg/kg and 1.0 mg/kg, respectively (Table 4). A total of 40 pesticides residues were analysed in the extracts by using GC-MS. All 40 pesticides were absent in all the samples of extract. Various aflatoxins B1, B2, G1 and G2 were analyzed and none of the aflatoxins were present in any samples of the extract.

#### Discussion

Standardization plays an important role in ensuring quality control of the herbal drugs. The quality control analysis of the medicinal plants is necessary to ensure therapeutic efficacy [31]. *G. glabra* is an important traditional medicinal plant used to treat wide range of ailments and diseases. Therefore, we standardized this important plant for the development of quality control parameters. A variety of physicochemical parameters were analyzed in order to standardize the *G. glabra* roots [28,32]. The phytochemical parameters like extractive values in different solvents, total ash value, water soluble ash value,
acid insoluble ash value, moisture contents, loss on drying, pH values (1% and 10% solutions) help in ensuring the purity of the crude plant drug vis-a-vis prevents from adulteration and substitution. The extractive values are used to evaluate the presence of active constituents in the crude drug. The highest percentage yield of *G. glabra* roots extract was found to be 13.89% in the methanol extract (Table 1). The *G. glabra* roots have higher concentration of fatty constituents as revealed in the analysis. The different ash values were determined to detect the presence of any foreign matters on the surface of the drug. The total ash comprises of physiological ash and non physiological ash. The physiological ash obtained from the plant tissues of the plant while non-physiological ash is obtained from the residue of the extraneous matter adhered to the surface of the plant. The acid insoluble ash generally includes silica and hence, high acid insoluble ash is an indicator of the earthly materials contamination. The water-soluble ash indicates the quantity of inorganic elements in the sample. Therefore, Ash values plays important role for the assessment of the quality and purity of the crude drugs. The low amount of total ash, acid insoluble ash and water soluble ash indicates that absence of the impurities in the crude drug. In our results, the amount of acid insoluble ash values was found to be the lowest in the extract of the *G. glabra* roots (Table 1), which indicates that extract was free from earthy materials. Moisture contents and loss on drying (LOD) are other important physicochemical parameters which are used to find out the amount of moisture and volatile contents in the tested drug. The high moisture content can cause hydrolysis of the active chemical ingredients of the drug leading to low therapeutic efficacy and quality. The dryness process and rate of moisture removal play a significant role in maintaining therapeutic efficacy of the drug. The moisture contents in the extract of *G. glabra* roots was found to be 0.56% (Table 1), hence indicating that the drug was properly dried and well stored.
Chromatogram of the methanolic extract of *G. glabra* at 366 nm.

HPTLC photograph of methanolic extract of *G. glabra* at 254 nm.
the extract of 1% and 10% solutions were also measured using digital pH meter and was found to be 5.04 ± 0.65 and 6.26 ± 0.54, respectively (Table 1). The pH of the extracts indicated the acidic nature of the compounds. The results of preliminary qualitative phytochemical screening of methanolic extract of G. glabra roots showed the presence of alkaloids, carbohydrates, flavonoids, phenolic compounds, proteins, saponins, lipids, tannins and steroids (Table 2). Hence, the current preliminary qualitative phytochemicals analysis may find application in further quantitative investigations of these important therapeutically active phytochemicals. The phenols or phenolics and flavonoids, (polyphenolic compounds) are important secondary metabolites of plants and these compounds are natural antioxidants which have wide spectrum pharmacological potentials e.g., anti-allergic, antibacterial, anticancer, anti-inflammatory, neuroprotective activities. These compounds also protect the plants from pathogenic microbial attack. Therefore, the quantitative estimation of total phenolic and flavonoid contents in the G. glabra roots was an important step to develop the quality control parameters. Our study showed the presence of total phenolic contents (7.47 ± 0.05 mg/gm) and flavonoid contents (2.25 ± 0.03 µg/gm) in the extract. The HPLC technique is an important analytical tool for identification, detection, separation, and some other assessments of plants and their products [32]. Many phytochemicals were detected by analysing the HPLC fingerprinting profile of methanolic extract of G. glabra HPTLC chromatogram at 366 nm showed 11 peaks at different Rf values and peak area in Hexane: Ethyl acetate: Methanol (9:1:1) solvent system (Figures 2 and 3; Table 3), while there were 10 peaks in the HPTLC chromatogram at 254 nm (Figures 4 and 5; Table 3). The number of peaks indicated the constituent’s numbers in the extract and their retention factors (Rf), which are presented in Table 3 and chromatographic fingerprinting profiles have been shown in the Figures 2-5. The fingerprint images of G. glabra roots developed from these HPTLC study might be referred to as the standard reference fingerprints. These fingerprint images can be used for identification, authentication, purification, and to separate G. glabra roots from its adulterants for ensuring therapeutic efficacy. Heavy metals are highly toxic inorganic chemicals even at very low concentrations. Heavy metals stored in different parts of plants causes different toxicities in plants. Heavy metals toxicity pose a serious threat to the health of humans and animals. The most common toxic heavy metals are Cadmium, arsenic, mercury and lead. These heavy metals are present as contaminants in many herbal drugs. Exposure to lead (Pb) at higher concentration usually leads to anemia, hepatotoxicity, kidney damage, lower sperm count, miscarriage, and some neurological disorders [34]. Cadmium causes significant toxicity to many organs and systems especially on the kidney. Cadmium exposure causes anemia, cardiovascular diseases, cancers, hemorrhagic trauma, respiratory distress, rhinitis, bronchitis, and chronic obstructive pulmonary disease (COPD) [35]. Exposure to Arsenic (As) results in atherosclerosis,
cancer, gastrointestinal disturbances, hypertension, liver and renal diseases, reproductive diseases, neurological disorders, skin disorders [36]. Mercury (Hg) is poisonous in all its forms. Mercury causes much toxicity like inflammation in mouth and gums, gingivitis, salivary glands, depression, dementia, muscle tremors, loss of motor coordination, severe nausea, vomiting, abdominal pain, bloody diarrhea, and kidney failure. The analysis of Arsenic, Cadmium, Lead, and Mercury concentrations in the extracts of G. glabra roots were carried out to avoid all above mentioned toxicities and standardize this valuable plant. Pesticides also cause toxicity in humans; therefore, all herbal drugs should be free from pesticides [37]. We analysed a total of 40 pesticides in the extract but none of the pesticides was positive in the tested samples. Mycotoxins are secondary metabolites of fungi. Mycotoxins may contaminate the plant and many herbal products at the time of processing and production. Aspergillus flavus, A. parasiticus, and Fusarium verticillioides are the examples of mycotoxins producing fungi. Aspergillus species are reported to produce various type of aflatoxins viz aflatoxin B1, B2, G1 and G2. All these aflatoxins are reported to cause liver cancer in humans [38]. In our study, none of these aflatoxins was present in G. glabra roots extract.

Conclusion
Herbal drugs are used worldwide for variety of therapeutic purposes. Therefore, it is very important to ensure quality of herbal drugs and herbal products. Keeping this aim in mind, the standardization of G. glabra roots extract was carried out for the development of quality control parameters of these medicinal plants which possess wide range of therapeutic potentials. We have used reliable modern techniques of pharmacognostical studies for standardization of herbal drugs. The established parameters in this study may prove important tools for authentication, identification, purification and standardization of the roots of this plant. Therefore, the generated information might be useful for preparation of monograph of this plant and might provide useful piece of information pertaining to therapeutic efficacy and quality control of this plant.
References


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