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# Simultaneous Quantification of Glycyrrhetinic acid and Apigenin using HPTLC from *Glycyrrhiza glabra* Linn

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**Abstract**

*Glycyrrhiza glabra* Linn. commonly known as Mulethi was employed medicinally as an expectorant and carminative in ancient times. There are no reports of simultaneous quantification of Glycyrrhetinic acid and Apigenin from this plant. Hence a TLC densitometric method was developed and validated for quantification of these marker compounds. Glycyrrhetinic acid and Apigenin were quantified from 2N methanolic-HCl extract and methanolic extract respectively using the Solvent System of Ethyl Acetate: Ethanol: Water: Ammonia (6.3: 2: 0.4: 0.1 v/v). The method was validated using ICH guidelines in terms of precision, repeatability and accuracy. Linearity range for Glycyrrhetinic acid and Apigenin was found to be 160 to 960 ng spot$^{-1}$ and 32 to 96 ng spot$^{-1}$ respectively; and the content of Glycyrrhetinic acid and Apigenin was found to be 0.65 ± 0.059% and 0.0074 ± 0.0004% respectively. This simple, precise and accurate method gave good resolution from other constituents of extract.

***Keywords:***

HPTLC, *Glycyrrhiza glabra*, TLC densitometric Method, Glycyrrhetinic acid, Apigenin

## 1. Introduction

*Glycyrrhiza glabra* Linn. commonly known as licorice and sweet wood in English; Mulethi and Jethi-madh in Hindi; Yashti-madhu in Sanskrit (family Leguminosae) native to the Mediterranean and certain areas of Asia is a genus of perennial herbs and under shrubs distributed in the subtropical and warm temperate regions of the world. Glycyrrhiza is derived from the ancient Greek term “Glykos”, means sweet, and “Rhiza”, means root. Historically [1], the dried rhizome and root of this plant were employed medicinally by the Egyptian, Chinese, Greek, Indian, and Roman civilizations as an expectorant and carminative. In modern medicine, licorice extracts are often used as a flavoring agent to mask bitter taste in preparations, and as an expectorant in cough and cold preparations. Licorice extracts have been used for more than 60 years in Japan [2] to treat chronic hepatitis, and also have therapeutic benefit against other viruses, including human immunodeficiency virus (HIV), cytomegalovirus (CMV), and *Herpes simplex*. It is one of the oldest and most frequently employed folk medicine in China and Europe, are widely used to treat diseases of the respiratory tract, gastrointestinal and cardiovascular system, etc. [3]. Deglycyrrhizinated licorice (DGL) preparations are useful in treating various types of ulcers [4, 5], while topical licorice preparations have been used traditionally to sooth and heal skin eruptions, such as psoriasis and herpetic lesions.

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### 1.1. Principal Constituents

The principal constituent of liquorice to which it owes its characteristic sweet taste is glycyrrhizin, besides glycyrrhizinic acid [6] a glycoside which on hydrolysis yields glycyrrhetinic acid. Other constituents present in liquorice are triterpene saponins, flavonoids, iso-flavonoids, polysaccharides, pectins, simple sugars, amino acids and coloring matter. The yellow color is due to the anthoxanthin glycoside [7], isoliquiritin (chalcone) which undergoes partial conversion to liquiritin during drying and storage of roots. Isoliquiritin gives on hydrolysis isoliquiritigenin, while liquiritin gives liquiritigenin as a glucone. The presence in the inner bark of a hemolytically active saponin has been reported. The plant contains phytoestrogens in the form of isoflavones such as formononetin; glabrone, neoliquiritin and hispaglabridin A and B have significant antioxidant activity [8] and both glabridin and glabrene possess estrogen-like activity [9].

Carbenoxolone, one of the oleandane derivatives prepared from *G. glabra* possess considerable mineralocorticoid activity [10] which may be due to similarity in structure of glycyrrhetic acid to the structure of hormones secreted by the adrenal cortex [11]. The roots and rhizomes of *G. glabra* has been studied with respect to spatial learning and passive avoidance [12] in rats and found to have memory enhancement activity [13]. It was also found to have anxiolytic activity [14] preliminary free radical scavenging [15]; cerebral ischemia [16] and antioxidant capacity towards LDL oxidation [17].

It has been reported that licorice inhibits growth and cytopathology of many unrelated DNA and RNA viruses, while not affecting cell activity or cellular replication [18]. *In vitro* research has also demonstrated glycyrrhizic acid inhibits cyclooxygenase activity and prostaglandin formation (specifically prostaglandin E2), as well as indirectly inhibiting platelet aggregation, all factors in the inflammatory process [19, 20].

Licorice and its constituents, specifically glycyrrhizin, have antiviral activity against *Herpes simplex* and are capable of irreversibly inactivating the virus [21]. Glycyrrhizin has also been shown to inhibit viral replication and infectivity of HIV [22] and to be effective in the treatment of eczema [22]. In the present paper, we have reported our work on simultaneous quantification of Glycyrrhetinic acid (Fig. 1) and Apigenin (Fig. 2) from roots and rhizomes of *G. glabra*.

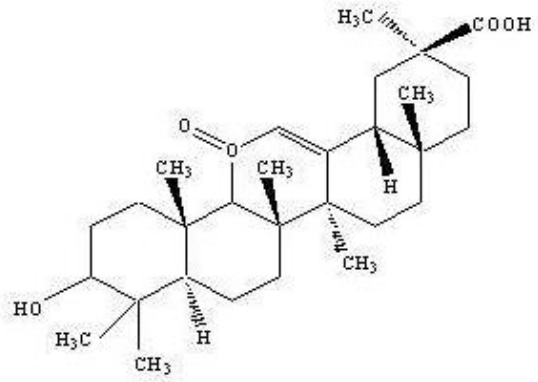


**Fig. 1**: Structure of Glycyrrhetinic acid

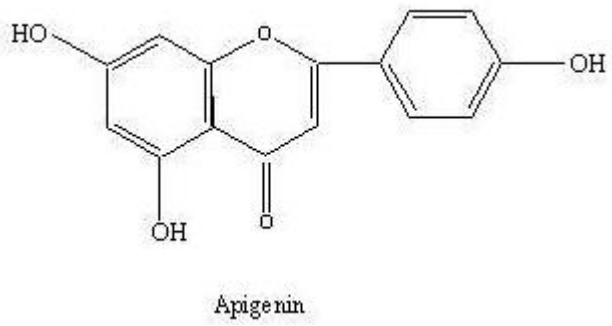


**Fig. 2**: Structure of Apigenin

## 2. Experimental

### 2.1. Collection of plant material

The roots and rhizomes of *G. glabra* were collected from Rohtak local market. The sample was dried and stored in air tight glass container and powdered to 40 mesh size when required.

### 2.2. Standard compounds

Glycyrrhetinic acid and Apigenin was procured from SPCI, Chennai, India.

### 2.3. Chemicals

All chemicals used were of analytical grade.

### 2.4. TLC conditions

TLC conditions were as shown in Table 1.

**Table 1.** TLC conditions

| | |
|---|---|
| TLC Plates | 20 ×10 cm, precoated with silica gel 60 F254 TLC plate (E. Merck) (0.2mm thickness) |
| Spotting device | CAMAG Linomat V Automatic Sample Spotter; Camag (Muttenz, Switzerland) |
| Syringe | 100 µL (Hamilton) |
| Developing chamber | CAMAG glass twin trough chamber (20 × 10 cm) |
| Densitometer | CAMAG TLC Scanner 3 linked to winCATS software; Camag |
| Experimental condition | Temperature 25 ± 2ºC, relative humidity 40 % |

### 2.5. TLC fingerprinting profile

#### 2.5.1. Sample Solutions

Preparation of Sample Solution was optimized to achieve good fingerprinting and also to extract the marker compounds efficiently. Of these, the preparation of selected Sample Solutions is given below:

#### 2.5.1.1. Sample Solution 1

Methanolic extract for quantification of apigenin.

#### 2.5.1.2. Methanolic extract

Since the marker compound Apigenin was soluble in methanol, we prepared a methanolic extract. Accurately weighed 1 g of the powdered drug was extracted with methanol (25 mL × 4) under reflux on a water bath. The methanolic extract was filtered through Whatman I filter paper, filtrates were combined, concentrated under vacuum and the volume was made upto 25 mL in a volumetric flask. This extract was used for TLC fingerprinting and co-chromatography with marker compound.

#### 2.5.1.3. Sample Solution 2

2N methanolic-HCl extract for quantification of glycyrrhetinic acid

#### 2.5.1.4. Hydrolyzed extract

Glycyrrhetinic acid is present in bound form in the drug. Hence the drug was subjected to hydrolysis by the following procedure: Accurately weighed 2 g of the powdered drug was hydrolyzed with 2N aqueous hydrochloric acid (50 mL) under reflux on a water bath for 2 hours at 100°C. The extract was filtered through Whatman I filter paper and the marc was washed with minimum amount of double distilled water (~ 5 to 6 mL) and filtered. The combined filtrates were transferred to a separating funnel and further extracted with methanol (25 mL × 4), dried over sodium sulphate, pooled, concentrated and the volume was made upto 25 mL with methanol. This extract was used for TLC fingerprinting and co-chromatography with Glycyrrhetinic acid.

**2.5.1.5. Standard Solution of Glycyrrhetinic acid**

2 mg of Glycyrrhetinic acid was dissolved separately in methanol and the volume was made upto 25 mL with methanol in volumetric flask.

**2.5.1.6. Standard Solution of Apigenin**

2 mg of Apigenin was dissolved separately in 2N methanolic-HCl and the volume was made upto 25 mL with 2N methanolic-HCl in volumetric flask.

**2.5.2. Solvent Systems**

Solvent system: Ethyl acetate : Ethanol : Water : Ammonia (6.3 : 2 : 0.4 : 0.1 v/v) was used for co-chromatography and simultaneous quantification of Glycyrrhetinic acid and Apigenin.

**2.5.3. Procedure**

For co-chromatography with Glycyrrhetinic acid and Apigenin, 10 µL of Sample Solution of methanolic extract and hydrolyzed extract along with the standard compounds was applied on a TLC plate and the plate was developed in Ethyl acetate : Ethanol : Water : Ammonia (6.3 : 2 : 0.4 : 0.1 v/v) Solvent System to a distance of 8 cm. The $R_f$ values and color of the resolved bands were noted.

**2.5.4. Simultaneous quantification of Glycyrrhetinic acid and Apigenin using HPTLC**

**2.5.4.1. Preparation of Standard Solutions of Glycyrrhetinic acid**

A Stock Solution of Glycyrrhetinic acid (160 µg $mL^{-1}$) was prepared by dissolving 16 mg of accurately weighed Glycyrrhetinic acid in methanol and making up the volume of the solution to 100 mL with methanol in a volumetric flask. The aliquots (1.0 to1.6 mL) of the Stock Solution were transferred to 10 mL volumetric flasks and the volume of each was adjusted to 10 mL with methanol to obtain Standard Solutions containing 16 µg $mL^{-1}$, 32 µg $mL^{-1}$, 48 µg $mL^{-1}$, 64 µg $mL^{-1}$, 80 µg $mL^{-1}$and 96 µg $mL^{-1}$of Glycyrrhetinic acid respectively.

**2.5.4.2. Preparation of Standard Solutions of Apigenin**

A Stock Solution of Apigenin (16 µg/mL) was prepared by dissolving 1.6 mg of accurately weighed Apigenin in methanol and making up the volume of the solution to 100 mL with methanol in a volumetric flask. The aliquots (2.0 to 6.0 mL) of the Stock Solution were transferred to 10 mL volumetric flasks and the volume of each was adjusted to 10 mL with methanol to obtain Standard Solutions containing 3.2 µg $mL^{-1}$, 4.8 µg $mL^{-1}$, 6.4 µg $mL^{-1}$, 8.0 µg $mL^{-1}$and 9.6 µg $mL^{-1}$of Apigenin respectively.

**2.5.4.3. Preparation of calibration curve of Glycyrrhetinic acid**

10 µL each of the Standard Solutions of Glycyrrhetinic acid (160 to 960 ng $spot^{-1}$) were applied (band width: 6 mm, distance between the tracks: 12 mm) in triplicates on a TLC plate using automatic sample spotter. The plates were developed in a twin trough chamber (20×10 cm) upto a distance of 8 cm using a Solvent System of Ethyl acetate : Ethanol : Water : Ammonia (6.3 : 2 : 0.4 : 0.1 v/v) at 25 ± 2°C and 40% relative humidity. It was then scanned densitometrically at 254 nm in absorbance mode. The area of the resolved peaks was recorded. Calibration curve of Glycyrrhetinic acid was obtained by plotting peak areas *vs* concentrations of Glycyrrhetinic acid applied.

**2.5.4.4. Preparation of calibration curve of apigenin**

10 µL each of the Standard Solutions of Apigenin (32 to 96 ng $spot^{-1}$) were applied (band width: 6 mm, distance between the tracks: 12 mm) in triplicates on a TLC plate using automatic sample spotter. The plates were developed in a twin trough chamber (20×10 cm) upto a distance of 8 cm using a Solvent System of Ethyl acetate : Ethanol : Water : Ammonia (6.3 : 2 : 0.4 : 0.1 v/v) at 25 ± 2°C and 40% relative humidity and scanned densitometrically at 254 nm in absorbance mode. The

area of the resolved peaks was recorded. Calibration curve of Apigenin was obtained by plotting peak areas *vs* concentrations of Apigenin applied.

#### 2.5.4.5. Quantification of glycyrrhetinic acid in the sample

15 µL of suitably diluted Sample Solution of methanolic extract was applied in triplicates on a TLC plate. The plate was developed and scanned as mentioned above. The peak areas were recorded and the amount of Glycyrrhetinic acid was calculated using the calibration curve.

#### 2.5.4.6. Quantification of apigenin in the sample

15 µL of suitably diluted Sample Solution of 2N Methanolic-HCl extract was applied in triplicates on a TLC plate. The plate was developed and scanned densitometrically at 254 nm in absorbance mode. The peak areas were recorded and the amount of Apigenin was calculated using the calibration curve.

### 2.6. Validation of the method

ICH guidelines were followed for the validation of the analytical method developed (CPMP/ICH/281/95 and CPMP/ICH/381/95) for precision, repeatability and accuracy. Instrumental precision was checked by repeated scanning (n = 7) of the same spot (320 ng $spot^{-1}$) and (32 ng $spot^{-1}$) of Glycyrrhetinic acid and Apigenin respectively and expressed as relative standard deviation (% R.S.D.). The repeatability of the method was affirmed by analyzing 320 ng $spot^{-1}$ and 32 ng $spot^{-1}$ of Glycyrrhetinic acid and Apigenin respectively individually on TLC plate (n = 5) and expressed as % R.S.D. Variability of the method was studied by analyzing aliquots of standard solution containing 320, 480, 640 ng $spot^{-1}$ and 48, 64, 80 ng $spot^{-1}$ of Glycyrrhetinic acid and Apigenin respectively on the same day (intra-day precision) and on different days (inter-day precision) and the results were expressed as % R.S.D.

For the evaluation of limit of detection (LOD) and limit of quantification (LOQ) different concentrations of the Standard Solutions of Glycyrrhetinic acid and Apigenin were applied along with methanol and 2N methanolic- HCl respectively as blank and determined on the basis of signal to noise ratio.

The accuracy of the method was assessed by performing recovery study at three different levels (50 %, 100 % and 125 % addition of Glycyrrhetinic acid and Apigenin respectively). The percent recoveries and the average percent recoveries were calculated.

## 3. Results and discussions

There is no report of simultaneous quantification of Glycyrrhetinic acid and Apigenin respectively in *Glycyrrhiza glabra.* Hence a simple, precise method was developed for simultaneous quantification of these marker compounds.

### 3.1. TLC fingerprint and co-chromatography

Quality control and quality assurance of herbal drugs remains a challenge as they contain a myriad of compounds in complex matrices in which no single active constituent is responsible for the overall efficacy [23]. Hence a systematic consideration of all its constituents is as important as the quantification of the active constituents present in it. TLC fingerprint profile of herbal drugs represents a comprehensive qualitative approach for the purpose of species authentication, evaluation of quality and ensuring the consistency and stability of herbal drugs and their products. In the present study, we developed TLC fingerprint profile for *Glycyrrhiza glabra* and carried out co-chromatography with marker compounds Glycyrrhetinic acid and Apigenin.

Glycyrrhetinic acid and Apigenin were resolved at Rf 0.77 and Rf 0.42 (Table 2) along with compounds from Sample Solution of 2N methanolic-HCl and Methanolic extract when the plate was developed in Solvent System of Ethyl acetate : Ethanol : Water : Ammonia (6.3 : 2 : 0.4 : 0.1 v/v) at 25 ± 2°C and 40% relative humidity.

**Table 2:** TLC fingerprint profile of *Glycyrrhiza glabra* roots and rhizomes under UV 254 nm

| Sample No. | Sample Solution-1 | Sample Solution-2 |
|---|---|---|
| 1. | 0.21 | - |
| 2. | 0.35 | - |
| 3. | - | 0.42 (Glycyrrhetinic acid standard) |
| 4. | 0.71 | - |
| 4. | 0.77 (Apigenin standard) | - |

### 3.2. TLC densitometric quantification of Glycyrrhetinic acid and Apigenin using HPTLC

The simplicity of the sample preparation, and the possibility of analyzing several sample of herbal products simultaneously in a short time, make HPTLC the method of choice. In the present work Glycyrrhetinic acid and Apigenin were simultaneously quantified from *Glycyrrhiza glabra* by TLC densitometric method using HPTLC (Fig 3).

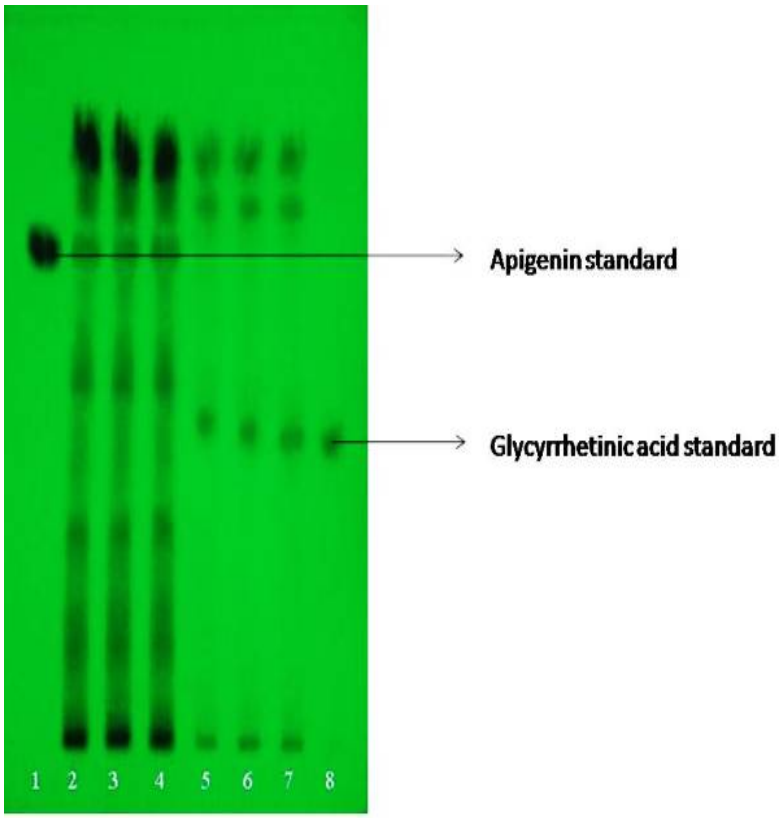


**Fig. 3**: TLC profile of Test solutions and Standard solutions of *Glycyrrhiza glabra* roots and rhizomes

The TLC densitometric method was validated in terms of precision, repeatability, and accuracy (Table 3, 4 and 5). The linearity range for Glycyrrhetinic acid and Apigenin was 160–960 ng $spot^{-1}$ and 32-96 ng $spot^{-1}$ with correlation coefficient (*r* values) of 0.999, 0.996 respectively. The TLC densitometric method was found to be precise with R.S.D for intraday in the range of 0.98–1.15 and

1.11-1.23 and for interday in the range of 1.03–1.25 and 1.13-1.28 for different concentrations of Glycyrrhetinic acid and Apigenin respectively (Table 3). This indicates that the proposed method was precise and reproducible. The limit of detection (LOD) values for Glycyrrhetinic acid and Apigenin were found to be 80 ng and 16 ng, and limit of quantification (LOQ) values were 160 ng and 32 ng respectively (Table 3). The average percent recoveries at 3 different levels of Glycyrrhetinic acid and Apigenin were found to be 100.28 % and 99.45 % respectively (Table 4).

**Table 3:** Method validation parameters for the quantification of Glycyrrhetinic acid and Apigenin by the proposed TLC densitometric method

| S. No. | Parameter | Glycyrrhetinic acid | Apigenin |
|---|---|---|---|
| 1 | Instrumental precision (% CV, n = 7) | 1.04 | 0.96 |
| 2 | Repeatability (% CV, n = 5) | 1.15 | 0.98 |
| 3. | Accuracy (average % recovery) | 100.28 | 99.45 |
| 4. | Limit of detection (ng) | 80 | 16 |
| 5. | Limit of quantification (ng) | 160 | 32 |
| 6. | Specificity | Specific | Specific |
| 7. | Linearity (Correlation coefficient) | 0.999 | 0.996 |
| 8. | Range (ng $spot^{-1}$) | 160-980 | 32-96 |

**Table 4.** Intra-day and Inter-day precision of Glycyrrhetinic acid and Apigenin

| Marker | Concentration (ng $spot^{-1}$) | Intra-day precision* | Inter-day precision* |
|---|---|---|---|
| Glycyrrhetinic acid | 320 | 1.15 | 1.23 |
| | 480 | 1.05 | 1.12 |
| | 640 | 0.98 | 1.11 |
| Apigenin | 48 | 1.25 | 1.28 |
| | 64 | 1.03 | 1.13 |
| | 80 | 1.12 | 1.21 |

* % R.S.D.; Mean (n=3)

**Table 5.** Recovery studies of Glycyrrhetinic acid and Apigenin at 50 %, 100 % and 125 % addition by the proposed TLC densitometric method

| Marker | Amount of marker present (µg) | Amount of marker added (µg) | Amount of marker found (µg) | Recovery* (%) | Average Recovery (%) |
|---|---|---|---|---|---|
| Glycyrrhetinic acid | 650 | 325 | 966.7 ± 3.20 | 99.12 ± 0.23 | 100.28 |
| | 650 | 650 | 1324.5 ± 3.23 | 101.88 ± 0.38 | |
| | 650 | 810 | 1452.23 ± 5.43 | 99.4 ± 0.32 | |
| Apigenin | 160 | 80 | 242.37 ± 1.58 | 100.63 ± 2.16 | 99.45 |
| | 160 | 160 | 321.91 ± 0.25 | 100.9 ± 1.10 | |
| | 160 | 200 | 355.49 ± 0.47 | 98.39 ± 0.94 | |

*Mean ± SD (n=3)

The content of glycyrrhetinic acid and apigenin simultaneously quantified using TLC densitometric method was found to be 0.65 ± 0.059 and 0.0074 ± 0.0004 % w/w respectively (Table 5, Fig 4).

**Table 6.** Glycyrrhetinic acid and apigenin content estimated in *Glycyrrhiza glabra* roots and rhizomes by proposed TLC densitometric method

| Sample No. | Sample Solution | Glycyrrhetinic acid (% w/w)* | Apigenin (% w/w)* |
|---|---|---|---|
| 1 | 1 | - | 0.0074 ± 0.0004 |
| 2 | 2 | 0.65 ± 0.059 | - |

*Mean ± S.D. (n = 3)

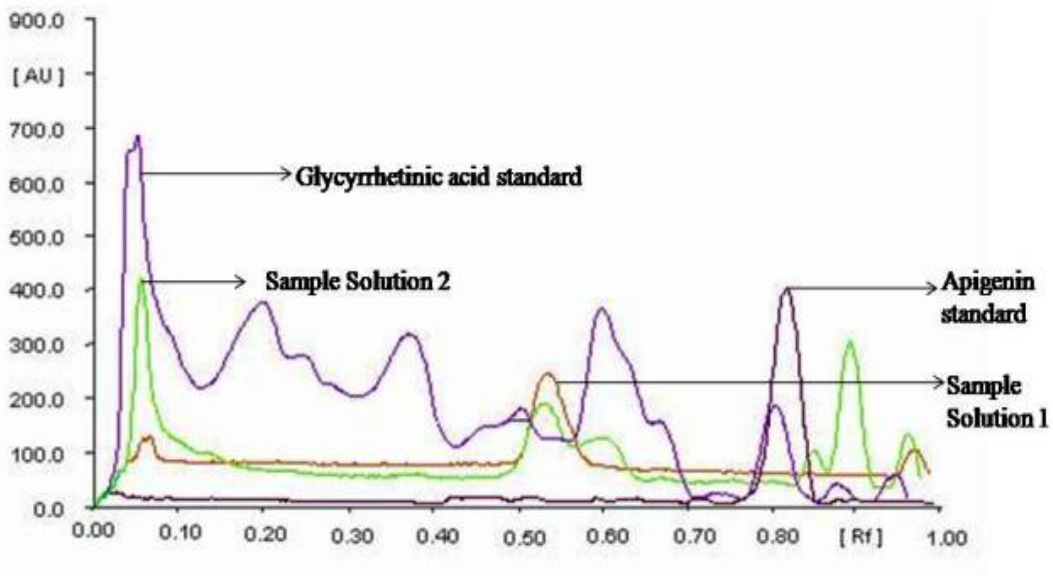


**Fig. 4**: TLC densitometric scan at 254 nm of test solutions and standard solutions of *Glycyrrhiza glabra* roots and rhizomes

## 4.Conclusion

A TLC densitometric method for the quantification of Glycyrrhetinic acid and Apigenin from roots and rhizomes of *Glycyrrhiza glabra* using HPTLC was developed and validated. The method was found to be simple, precise, specific sensitive and accurate and can also be used for the quantification of Glycyrrhetinic acid and Apigenin in the herbal raw materials. It can also be used in routine quality control of herbal materials as well as formulations containing any or both of these compounds.

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