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HPTLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF ELLAGIC ACID, GLYCYRRHIZIC ACID AND KAEMPFEROL IN HERBAL FORMULATION

Ms. Dipali Dilip Suryawansh*, Dr. Mrs. Varsha Milind Jadhav, Dr. Vilasrao J. Kadam,
Bharati Vidyapeeth's College of Pharmacy, C.B.D. Belapur, District Thane, Navi Mumbai 400614.
Maharashtra, India.

ABSTRACT

A simple, precise and accurate high performance thin layer chromatographic (HPTLC) method was developed and validated for the simultaneous determination of Ellagic acid (EA), Glycyrrhizic acid (GLY) and Kaempferol (KAE) in the developed herbal formulation. The separation was carried out on Merck TLC aluminum sheets of silica gel G60 F_{254} of 200 µm thickness, using Toluene: Ethyl acetate: Formic acid (4.5:4.5:1 v/v/v) as mobile phase and densitometric analysis of compound was carried out in absorbance mode at 265 nm. The aforesaid mobile phase gave well defined peaks at Rf value of 0.41± 0.03 for Ellagic acid, 0.28 ± 0.03 for Glycyrrhizic acid and 0.76 ± 0.03 for Kaempferol. .The linear regression analysis data for the calibration plots for EA, GLY and KA showed a good linear relationship with regression coefficient (r^2) of 0.9936, 0.9923 and 0.9919 respectively; in the concentration range of 300-900 ng/spot for all the compounds. The limit of detection and quantitation were 62.5197 and 189.4539 ng/spot, respectively for EA, 126.6847 and 383.8930 ng/spot, respectively for GLY and 44.9678and 136.2661 ng/spot, respectively for Kaempferol. The method was validated for linearity, accuracy, precision, specificity, and robustness, the limit of detection (LOD) and limit of quantification (LOQ) as per ICH guidelines. In conclusion, the statistical analysis of the data showed that the method is reproducible and selective for the estimation of all compounds. The proposed developed HPTLC method can be applied for identification and quantitative determination of EA, GLY and KAE in their extracts and herbal formulation.

Keywords: Ellagic acid, Glycyrrhizic acid, Kaempferol, Herbal formulation, Estimation, HPTLC, Standardization, Method development and validation.

INTRODUCTION

A large number of people depend on herbal medicine for their primary healthcare treatment mainly in underdeveloped or developing countries. Indian traditional medicinal systems like Ayurveda, Siddha and Unani have a very rich history of their effectiveness but modern research also confesses the importance of such medicine. Indian traditional medicine and medicinal plants are also considered as active sources of a new drug. Several steps have been taken in India to encourage such types of medicine and to integrate them into clinical practice. Evidence based incorporation of Indian traditional medicine in clinical practice will help to provide quality wellness programs to all. [1]

Currently, the major pharmaceutical companies have demonstrated a renewed interest in investigating higher plants as sources for the development of standardized Phytotherapeutic agents with proven efficiency, safety and quality. [2]

DRUG PROFILE

***Punica granatum* (Pomegranate) [3, 4]:** Pomegranate peel of *(Punica granatum*) from the punicacea family. This plant grows as 3 m to 5 m tall tree or shrub that is indigenous to Iran, Afghanistan, China and Indian subcontinent. It is used externally for the treatment of skin disease because of its astringent and antibacterial properties. This folkloric herb is believed to treat a variety of ailments like bacterial infections (Antidiarrheal), Anti-cancer (skin cancer), Chronic Obstructive Pulmonary Disease (COPD) and in chronic periodontitis. Its antimicrobial properties have been found useful in treating various dental and dermatologic conditions. The high antioxidant content of the fruit is due to the presence of phenolic acids like Gallic acid, ellagic acid, punicalagin A and punicalagin B.

Figure 1 *Punica granatum*

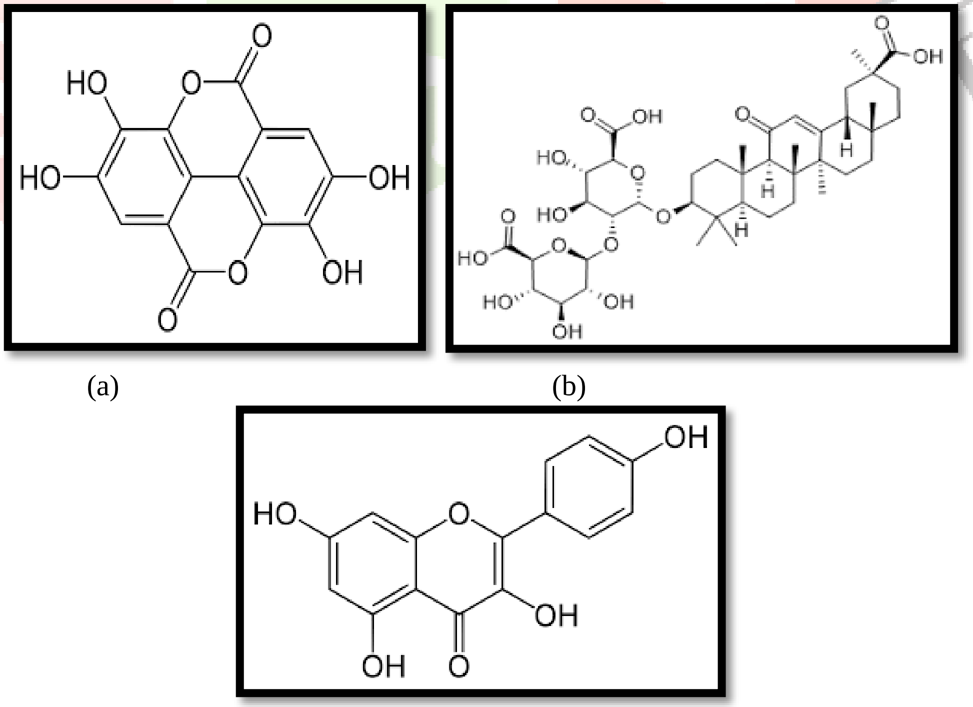
***Glycyrrhiza glabra L.* (Licorice) [4, 5]:** *Glycyrrhiza glabra*, is a sweet root, moist, soothing, flavoring herb commonly known as Licorice belonging to the family Fabaceae. The plant is widely used as a medicine from the ancient medical history of Ayurveda. Licorice roots are used as a flavoring agent in beverages and also in tobacco products. Medicinally it is used in the treatment of menstrual cramps, menopause symptoms, upper respiratory tract ailment, and hypoglycemia. Glycyrrhizin (Glycyrrhizinate; Glycyrrhizic acid), a triterpenoid saponin; constitutes 10-25% of licorice root extract, is considered as its primary active ingredient.

Figure 2 *Glycyrrhiza glabra*

***Nyctanthes arbor-tristis* (Night flowering Jasmin)** [6]**:** *Nyctanthes arbor-tristis* leaves from the Oleaceae family. Nyctanthes arbor-tristis is a shrub or a small tree growing 10 m to 15 m tall with, brown-grey bark. Commonly known as Parijata. It is mainly found in sub-tropical regions in the Himalayas like Nepal and southern parts of India and is mostly cultivated in tropical and sub-tropical regions all over the world. Other than Parijata it is also known as Har-shinghar. [10] Different parts of this plant are used for different medical purposes. Its leaves have broad-spectrum medicinal use such as anti-bacterial, anti-inflammatory, anti-pyretic and anti-helminthic effects.

Figure 3 *Nyctanthes arbor-tristis*

Ellagic acid, Glycyrrhizic acid and Kaempferol are constituents present in the extract of *Punica granatum*, *Glycyrrhiza glabra* and *Nyctanthes arbor-tristis* leaves respectively, can be used as markers for their HPTLC simultaneous determination. As yet, no report on simultaneous determination and validation of Ellagic acid, Glycyrrhizic and Kaempferol in extract and in the formulation by HPTLC method is found in the literature. So, the present work aims to develop and validate the HPTLC method for simultaneous estimation of Ellagic acid, Glycyrrhizic acid and Kaempferol in extract and herbal formulation.



(a) (b)

(c)

Figure 4: Structures of (a) Ellagic acid, (b) Glycyrrhizic acid and (c) Kaempferol

Table I: Physicochemical properties of Ellagic acid, Glycyrrhizic acid and Kaempferol

Properties	**Ellagic acid**	**Glycyrrhizic acid**	**Kaempferol**
Chemical name	2,3,7,8-Tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione	6-[6-carboxy-2-[(11-carboxy-4,4,6*a*,6*b*,8*a*,11,14*b*-heptamethyl-14-oxo-2,3,4*a*,5,6,7,8,9,10,12,12*a*,14*a*-dodecahydro-1*H*-picen-3-yl)oxy]-4,5-dihydroxyoxan-3-yl]oxy-3,4,5-trihydroxyoxane-2-carboxylic acid	3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one
pH	5.5	4.5	7.73
pKa	5.54	2.96	6.33
Solubility	Soluble in alcohol and water	Freely soluble in alcohol and hot water	Slightly soluble in water and highly soluble in hot ethanol, ethers, and DMSO.
Melting point	>360°C	220°C	276–278 °C
Molecular formula	$C_{14}H_6O_8$	$C_{42}H_{62}O_{16}$	$C_{15}H_{10}O_6$
Molecular weight	302.19 g/mol	822.9 g/mol	286.24 g/mol
Half life	8.4h +/- 1.8 h	3.5 h	2.93-3.79min.
λmax	265nm	255nm	265nm, 365nm

MATERIALS AND METHODS

Reagents and materials:

Analytical grade ethanol, methanol, toluene, ethyl acetate and formic acid were purchased from SD Fine Chemicals, Mumbai, India. Standard Ellagic acid and Glycyrrhizic was procured from Yucca Enterprises, Mumbai.

Plant material:

The *Punica granatum* peel and *Glycyrrhiza glabra* root in powdered form was purchased from 'Yucca Enterprises' Wadala (e), Mumbai 400 037. *Nyctanthes arbor-tristis* leaves collected from Pharmacognosy garden of Bharati Vidyapeeth's college of Pharmacy Belapur, Navi Mumbai 400614. All drugs were authenticated by ***Alarsin House***. A/32, Street No. 3 M.I.D.C. Andheri (East). Mumbai 400093 India.
Phone: 02228216836/02228367804.
Email: alarsinmumbai@gmail.com

INSTRUMENTATION

Quantitative HPTLC method development and validation was performed using CAMAG HPTLC system: specifications are given in table II.

Table II: HPTLC system details

Sr. no	**HPTLC System components**	**Specification**
1	Make and model	CAMAG. Switzerland
2	Sample applicator	CAMAG Linomat V
3	Densitometric scanner	CAMAG TLC Scanner 3
4	Sampling mode	Manual with Linomat applicator 5
5	syringe	Hamilton (100µl)
6	Detection	Ultraviolet (UV) detector
7	software	WinCATS (Ver.1.4.1.8)

EXPERIMENTAL WORK

Experimental work is presented in two sections, namely analytical method development and analytical method validation.

Analytical method development [7]

- **Selection of detection wavelength:**
  UV spectra of 10 ppm solution of each Ellagic acid, Glycyrrhizic acid and Kaempferol were generated separately by scanning over the range of 300-900 nm using a Double Beam Spectrophotometer (Shimadzu UV1801). All three spectra were overlaid and analyzed for selection most suitable detection wavelength (Isobestic point) to be used for simultaneous estimation of Ellagic acid, Glycyrrhizic acid and Kaempferol.

- **Selection of stationary phase:**
  TLC plates pre-coated with silica gel G60 F_{254}were selected as a chromatographic layer.

- **Preparation of extracts:**
  The crude drugs were washed with distilled water, sun dried and then ground to powder. The individual drug weighing 50 g each of Punica granatum, Glycyrrhiza glabra and Nyctanthes arbor-tristis were extracted with ethanol by hot continuous percolation method in Soxhlet apparatus. The ethanolic extracts were filtered, evaporated and dried on an Electric water bath.

- **Preparation of extracted solution:**
  Accurately weighed 100 mg of each dried extract was dissolved in 100 ml of methanol to get final stock solution containing 1000 µg/ml each. The solutions were filtered through 0.45 µ membrane filter and further used for HPTLC analysis.

- **Preparation of standard solution:**
  A stock solution of Ellagic acid, Kaempferol and Glycyrrhizic acid were prepared by dissolving 10 mg of accurately weighed standard drugs in methanol individually and volume was made up to 10ml in a volumetric flask and labeled as Stock A, B and Stock C respectively. From this, 1.0ml of stock solution was transferred individually in 10ml volumetric flask and the volume was adjusted to 10ml to obtain a standard solution containing 100 µg /ml of Ellagic acid, kaempferol and Glycyrrhizic acid respectively and was sonicated for 5 minutes.

- **Preparation of sample solution:**
  A 100 mg gel formulation was weighed and transferred into another volumetric flask containing 10 ml of methanol and kept in the ultrasonicator for 20 min for extraction of all three drugs from the formulation. From the resultant solution, 1 ml of a solution was withdrawn and transferred into 10 ml of volumetric flask and then volume was made up to the mark with methanol to get a final concentration of 1 mg/ml.

- **Optimization of chromatographic conditions:**
  TLC aluminum plates (10 x 10 cm) percolated with, silica gel G60 F_{254} of 200 µm thickness (Merck, Mumbai, India) was used as stationary phase. Standard solutions of markers and samples were applied to the plates as bands of 6.0 mm wide, 10.0 mm from the bottom edge to the same chromatographic plate by use of a Camag (Muttenz, Switzerland) Linomat 5 sample applicator equipped with a 100 µl Hamilton syringe. The slit dimension was selected at 5 x 0.45 mm. Ascending development to a distance of 80 mm was performed at room temperature (28 ± 2°C), with Toluene: Ethyl acetate: Formic acid (T: Et: F) in the ratio of 4.5:4.5:1(v/v/v) as mobile phase, previously Camag glass twin-trough chamber saturated with mobile phase vapor (T: Et: F) for 20 min. After development, the plates were dried and then scanned at 265 nm with a Camag TLC Scanner 3 using the deuterium lamp with win CATS software.

Analytical method validation [7]

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. Parameters to be considered during validation of the developed method as per ICH guidelines based on Q2 (R1) validation of analytical procedure: text and methodology are as follows

Table III: summary of analytical method validation parameters with its method to be followed according to ICH revised Q2 (R1) guidelines

Parameter	**Purpose**	**Recommendation as per ICH revised Q2(R1) guideline**	**Method/ procedure to be followed**
1. Accuracy	Assay (Content/ potency): Recovery studies	Accuracy was established across the specified range of analytical procedure by adding known added quantities of analyte to the synthetic mixture of drug product components and to the combined dosage form. As per ICH, Accuracy should be assessed using a minimum of 9 determinations over a minimum of three concentration levels covering the specified range i.e. 3 concentration levels in triplicate. (e.g. 3 Concentration/3 replicates each). Accuracy of the method is reported as percent recovery of known added amount of analyte in sample.	Accuracy was assessed using a minimum of 9 determinations over a minimum of 3 concentrations covering specific range. In the present work percent recovery was calculated by performing recovery studies in triplicates of three concentration levels viz. 80%, 100% and 120% by adding known amount of standard solution of standards.
Acceptable criteria: 98-102 % Recovery of known added amount of analyte.			

<table>
<tr><td>2. Precision</td><td>Assay (Content/ potency): Repeatability and Reproducibility</td><td>Repeatability</td><td>Intermediate Precision</td><td>Method/ procedure to be followed</td></tr>
<tr><td></td><td></td><td>Repeatability was assessed by using minimum of 9 determination covering the specified range for the procedure (e.g. 3 concentration/3 replicates each)</td><td>Intermediate precision was established to study the effects of random events i.e. days, on the precision of the analytical procedure. Intraday and Interday precision studies were performed by taking 9 determinations of 3 concentration/3 replicates each, at 3 times in a same day and on 3 different days, respectively.</td><td>Three replicate analysis were performed at three different concentration mixture levels low (LQC:30µg/ml), mid (MQC: 60 µg/ml) and high (HQC: 90 µg/ml) for Ellagic acid and low (LQC: 30µg/ml), mid (MQC: 60 µg/ml) and high (HQC:90µg/ml)for Glycyrrhizic acid and low (LQC: 30µg/ml), mid (MQC: 60 µg/ml) and high (HQC:90µg/ml)for Kaempferol respectively within the same day at three different times (Session 1, 2, 3).</td></tr>
<tr><td colspan="5">Acceptable criteria: RSD ≤ 2%
Precision is reported as relative standard deviation (coefficient of variation) for each type of precision investigation.</td></tr>
<tr><td>3. Specificity</td><td>Identification, Testing for impurities and Assay (Content/potency)</td><td colspan="2">As per ICH revised Q2 (R1), Specificity should be carried out ensure identification tests, the determination impurity and assay.</td><td>To determine specificity densitogram were obtained for standards, extract of all three drugs and placebo formulation.</td></tr>
<tr><td colspan="5">Acceptable criteria: No interference In analyte determination</td></tr>
</table>

4. **Detection Limit and Quantification Limit.**	Testing for impurities Sensitivity of analytical method and Assay: Determination of minimum detectable and quantifiable concentration of analyte solution.	Detection limit and quantification limit is determined based on the standard deviation of the response and the slope. **LOD**=3.3×σ/S **LOQ**=10×σ/S σ = Standard deviation of response estimated based on the calibration curve. S = Slope of the calibration curve.	The values of limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the response and the slope of calibration graph.
Acceptable criteria: NA			
5. **Linearity**	Testing for impurities and Assay (Content/potency): To check linear relationship of performed concentration.	As per ICH, for the establishment of linearity, a minimum of 5 concentrations are approved. Linearity is reported by the value of the correlation coefficient, y-intercept, and slope of the regression line along with a plot of the data.	Experimental determinations were carried out on seven serial dilutions of working solution prepared using methanol as diluting solvent. Linear relationship was checked by plotting average peak areas against sample concentrations. It was evaluated across the range of 30-90µg/ml for Ellagic acid, Glycyrrhizic acid and Kaempferol from their mixture.
Acceptable criteria: $R^2 \geq 0.99$			
6. **Robustness**	To establish reliability of analytical method.	Robustness was evaluated for proving the reliability of an analytical method with respect to deliberate variations in method parameters. To establish robustness of analytical method	Robustness was evaluated for proving the reliability of an analytical method with respect to deliberate variations in method parameters. To establish robustness of analytical method following parameters were studied. ➢ Change in mobile phase ratio. ➢ Change in chamber saturation time.
Acceptable criteria: Pooled RSD ≤ 3% in every change item			

RESULT AND DISCUSSION

Analytical method development:

- **Selection of wavelength:**

The UV spectra of were recorded by scanning the solution of individual drug10 ppm solution of each Ellagic acid (EA), Glycyrrhizic acid and Kaempferol (KAE) in range of 380-220 nm. The wavelength at which all the drugs show quantitable absorbance was selected for simultaneous determination of EA, GLY and KAE by plotting the overlay graph of individual UV spectra. The overlay UV spectra of three drugs showed isoabsorptive point at 265 nm; hence 265 nm was selected as wavelength for analysis.

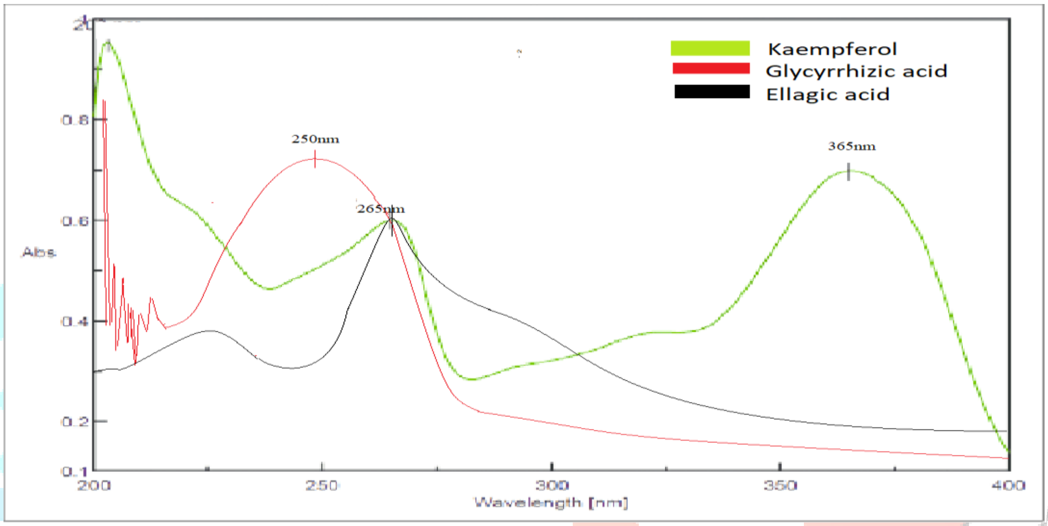


Figure 5: Overlay UV Spectra of EA, GLY and KAE

- **Optimization of chromatographic conditions**

Based on literature survey polarity and solubility of Ellagic acid, Glycyrrhizic acid and Kaempferol many preliminary trials were carried out for selection of mobile phase composition, some are represented in table.

Table IV: trials for optimization of mobile phase composition for Ellagic acid, Glycyrrhizic acid and Kaempferol

Mobile phase tried	Composition (V/V/V)	Applied volume (µl)	Observation	Inference
Hexane: Ethyl acetate: Formic acid: Methanol	6: 3: 0.2: 0.6	2 µl, 5 µl, 10 µl and 20 µl	No resolution	Toluene: Ethyl acetate: Formic acid 4.5:4.5:1 (V/V/V) were optimized for method development.
Toluene: Ethyl acetate: Methanol	4: 4: 1	2 µl, 5 µl and 10 µl	No spot for Ellagic acid was observed	
Toluene: Ethyl acetate: Formic acid: Methanol	5: 4: 0.8: 0.2	2 µl, 5 µl, 10 µl and 20 µl	Tailing was observed	
Toluene: Ethyl acetate: Formic acid	4.5:4.5:1	2 µl, 5 µl, 10 µl, 15 µl and 20 µl	Good resolution	

For HPTLC method, Chromatographic conditions were optimized to achieve the best resolution and peak shape for Ellagic acid, Glycyrrhizic acid and Kaempferol. Different mobile phases in different proportion were tried and the mobile phase containing Toluene: Ethyl acetate: Formic acid 4.5:4.5:1:(V/V/V) was selected as optimal for obtaining well-resolved peaks of Ellagic acid Kaempferol and Glycyrrhizic acid.

Table V: optimized chromatographic conditions for validation.

Parameters	Optimized chromatographic conditions
Stationary phase	Precoated silica gel aluminum plate G 60 F_{254} (10 x 10 cm)
Mobile phase composition	Toluene: Ethyl acetate: Formic acid 4.5:4.5:1(V/V/V)
Sample application a) Application volume b) Band width c) Distance between the tracks	 10µl 6mm 10mm
Chamber saturation time	20 min
Temperature (^{0}C)	25-28
Relative humidity (%)	55-58
Technique of separation	Ascending
Total amount of mobile phase used	9.3ml
Solvent front	90mm
Migration time	20min
Densitometric evaluation- detection wavelength	265 nm

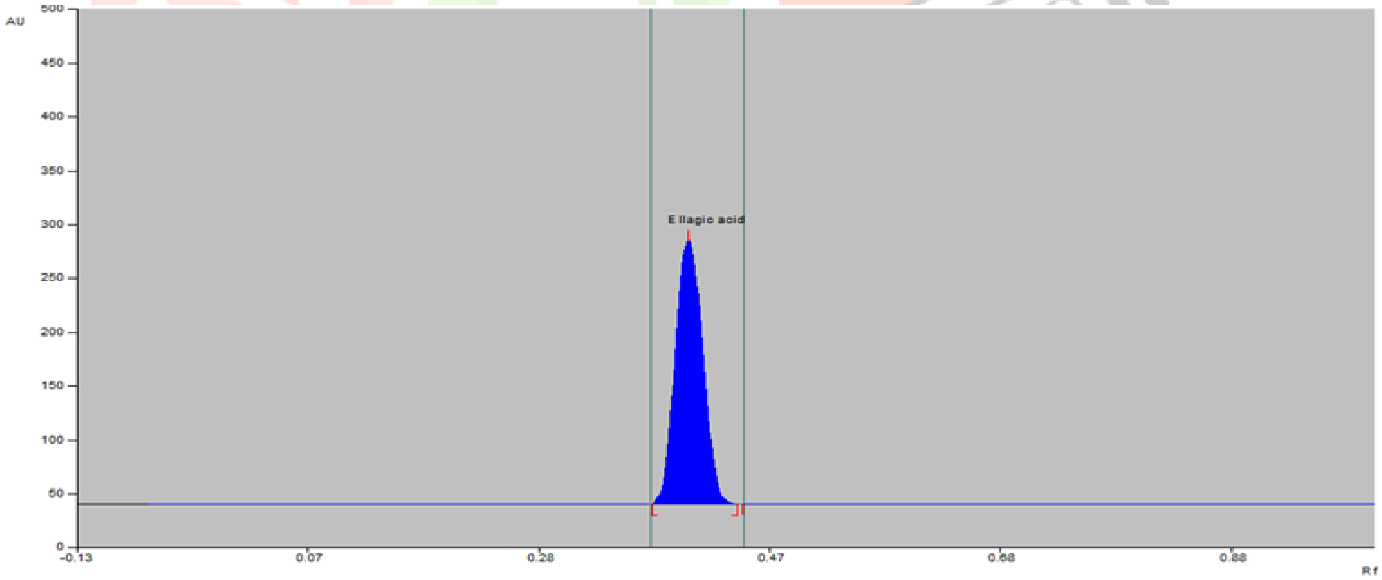


Figure 7: HPTLC Chromatogram of Ellagic acid Rf 0.41± 0.03

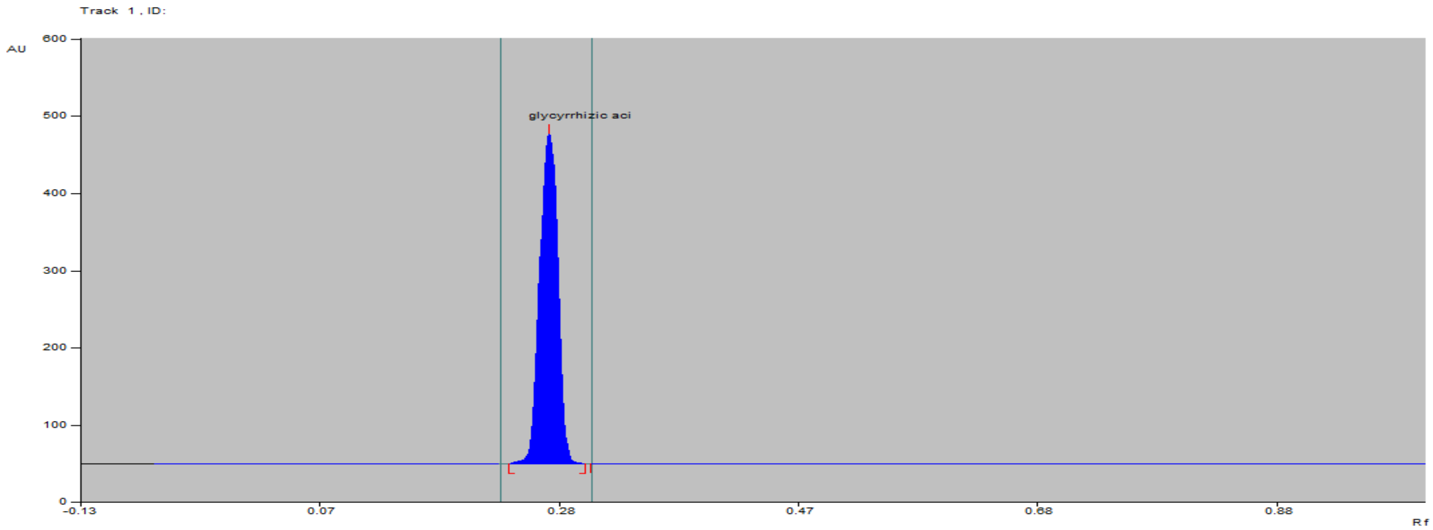


Figure 8: HPTLC Chromatogram of Glycyrrhizic acid Rf 0.28 ± 0.03

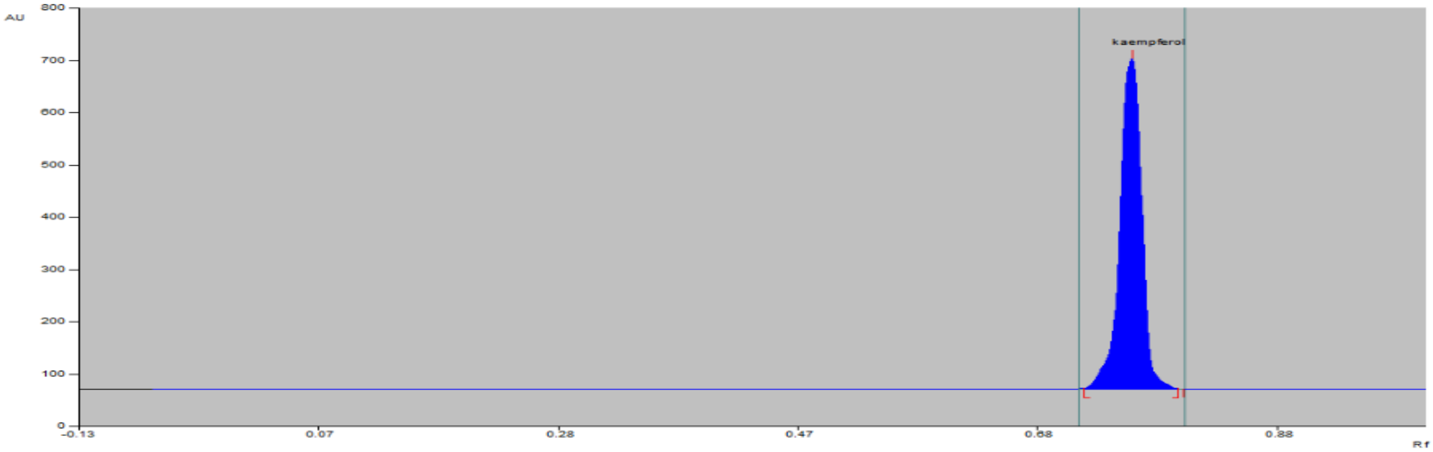


Figure 9: HPTLC Chromatogram of Kaempferol0.76 ± 0.03

- **Estimation of marker in extract (Quantification)**

The amount of Ellagic acid and Glycyrrhizic acid present in crude extract were calculated by using linear regression equation. The method was found to be selective and specific for quantification.

A. Quantification of Ellagic acid from pomegranate extract

The quantification of pomegranate extract was performed in triplicate. Average of all these was used for calculating amount of Ellagic acid present in it.

Table VI: Quantification of Ellagic acid from pomegranate extract

	Peak area 1	Peak area 2	Peak area 3	Average peak area (AU)
Pomegranate extract 1	9245.9	9526.8	9645.2	9472.6
Pomegranate extract 2	10143.7	9982.5	9875.4	10000.5
Pomegranate extract 3	10321.3	10122.4	10257.5	10233.7
Average				9902.2
Ellagic acid content in extract				900.85 ng/10 µl

The Ellagic acid content in pomegranate extract was found to be 0.900 % w/w

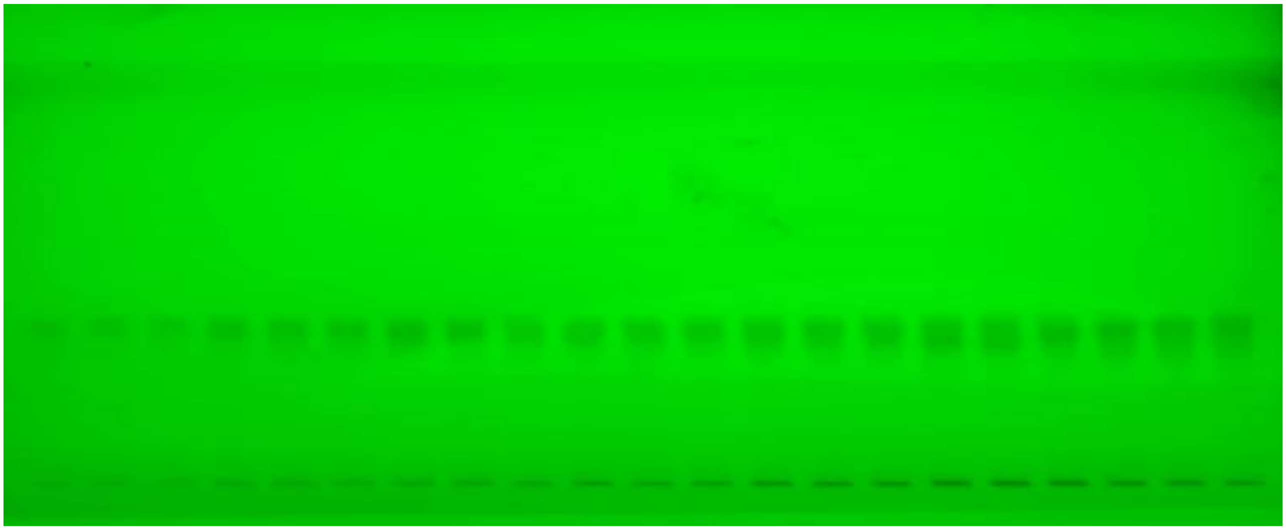


Figure 10: Photo-documentation of Ellagic acid with Pomegranate extract, at 265 nm

B. Quantification of Glycyrrhizic acid from Licorice extract

The quantification of licorice extract was performed in triplicate. Average of all these was used for calculating amount of Glycyrrhizic acid present in it.

Table VII: Quantification of Glycyrrhizic acid from licorice extract

	Peak area 1	Peak area 2	Peak area 3	Average peak area (AU)
extract 1	7807.5	7625.3	7819.2	7750.6
extract 2	7800.6	7986.4	8421.5	8069.5
extract 3	8544.5	8026.7	8039.6	8203.6
Average				8007.9
Glycyrrhizic acid content in extract				710.39 ng/10 µl

The Glycyrrhizic acid content in licorice extract was found to be 0.710 % w/w

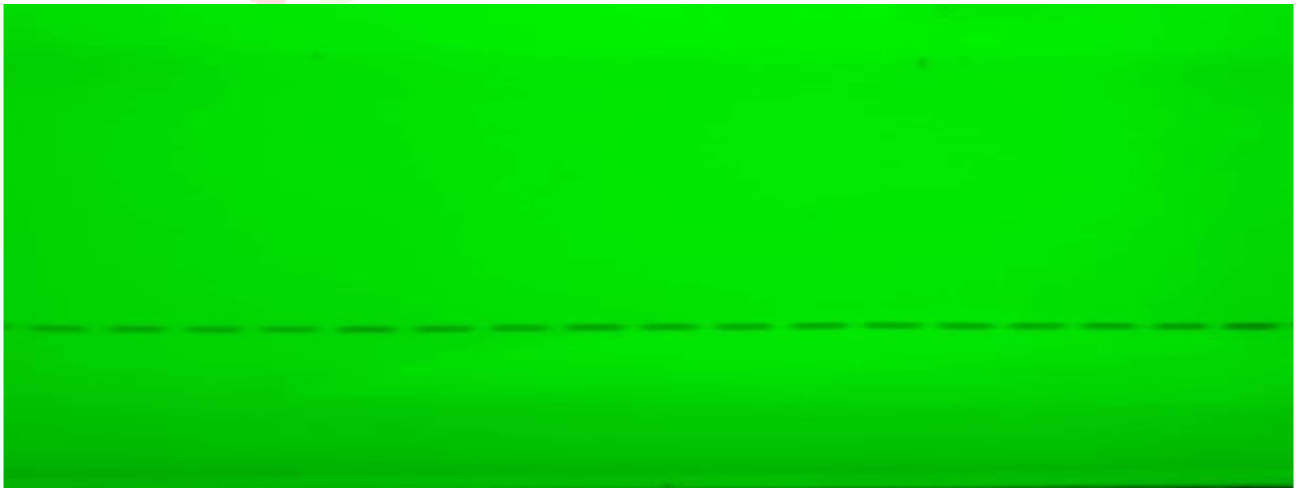


Figure 11: Photo-documentation of Glycyrrhizic with Licorice extract, at 265 nm

C. Quantification of Kaempferol from Night-flowering jasmine extract

The quantification of Night-flowering jasmine extract was performed in triplicate. Average of all these was used for calculating amount of Kaempferol present in it.

Table VIII: Quantification of Kaempferol from Night-flowering jasmine extract

	Peak area 1	Peak area 2	Peak area 3	Average peak area (AU)
extract 1	**6784.9**	**6825.4**	**6755.4**	**6788.56**
extract 2	**7021.5**	**7054.6**	**7154.6**	**7076.9**
extract 3	**6589.7**	**6675.5**	**6676.3**	**6647.16**
Average				**6837.54**
Kaempferol content in extract				**499.52 ng/10 µl**

The Kaempferol content in Night-flowering jasmine extract was found to be 0.499 %w/w

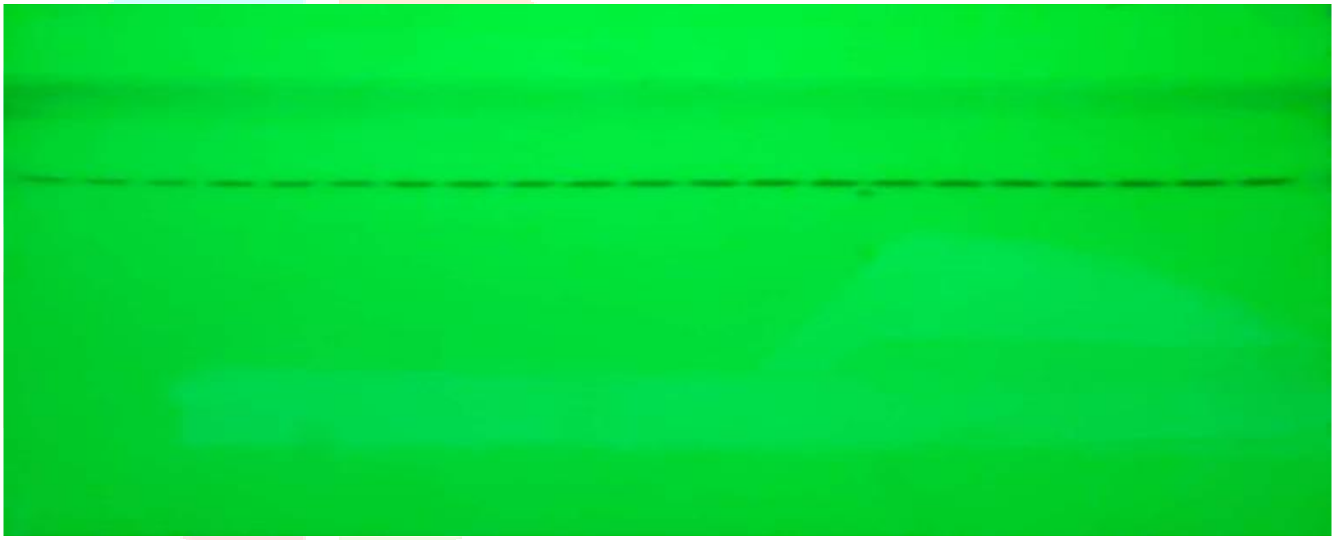


Figure 12: Photo-documentation of Kaempferol from Night-flowering jasmine extract, at 265nm

1. Accuracy

Accuracy of the method is reported as percent recovery of known added amount of analyte in the sample. The accuracy of the method was established by performing recovery studies in triplicates of three concentration levels viz. 80%, 100%, 120% by adding known amount of Ellagic acid , Glycyrrhizic acid and Kaempferol. Results obtained are given in table.

Table XI: Results for Accuracy

Drug	% Level	Initial amount (ng/spot)	Spiked amount (ng/spot)	Total expected amount (ng/spot)	Amount recovered (ng/spot)	% Recovery	Inference
Ellagic acid	80	600	480	1080	1087	100.65	Acceptable recovery hence accurate
	100	600	600	1200	1199	99.97	
	120	600	720	1320	1320	100	

Glycyrrhizic acid	80	600	480	1080	1065	98	Acceptable recovery hence accurate
	100	600	600	1200	1232	102	
	120	600	720	1320	1335	101	
Kaempferol	80	600	480	1080	1069	99	Acceptable recovery hence accurate
	100	600	600	1200	1210	100.87	
	120	600	720	1320	1297	98	

2. Precision

Precision was determined in triplicates by analyzing three quality Control (QC) samples of Ellagic acid, Glycyrrhizic acid and Kaempferol. At three distinct concentration levels (300 ng/spot, 600 ng/spot, and 900 ng/spot) each i.e. low quality control (LQC), mid quality control (MQC) and high quality control (HQC). The results obtained were further statistically analyzed for standard deviation and percent relative standard deviation (coefficient of variance). % RSD of the peak areas obtained was less than 2%, which indicates that the developed method was precise.

Table XII: Intraday Precision Studies

		Observation								
		Ellagic acid			**Glycyrrhizic acid**			**Kaempferol**		
Levels		**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
Amount (µg/ml)		**30**	**60**	**90**	**60**	**120**	**180**	30	60	90
Peak Area	**1**	5507	9552	11464	2284	4826	6234	5689	7762	9295
	2	5565	9595	11611	2287	4814	6223	5761	8063	9376
	3	5547	9567	11780	2280	4810	6216	5651	7749	9327
Average Peak Area		5539	9571	11618	2283	4816	6224	5700	7924	9332
S.D.		24.2395	217.8200	129.1106	2.8674	6.7986	7.4087	45.6167	145.054	33.3099
% RSD		0.4376	0.1861	1.1112	0.1256	0.1411	0.1190	0.8002	1.8305	0.3569
Inference		Precision is reported as standard deviation and relative standard deviation (coefficient of variation) for each type of precision investigation								

Table XIII: Interday Precision Studies

		Observation								
		Ellagic acid			**Glycyrrhizic acid**			**Kaempferol**		
Levels		**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
Amount (µg/ml)		**30**	**60**	**90**	**60**	**120**	**180**	**30**	**60**	**90**
Peak Area	**1**	5507	9552	11464	2292	4877	6020	5553	7471	8938
	2	5565	9595	11611	2327	4898	6083	5527	7463	8968
	3	5547	9567	11780	2276	4807	6099	5662	7411	9060
Average Peak Area		5539	9571	11618	2298	4860	5934	5580	7448	8988
S.D.		24.2395	17.8200	129.1106	21.296	38.9044	34.1011	58.4826	26.5992	51.9058
% RSD		0.4376	0.1861	1.1112	0.9267	0.8005	0.5746	1.0480	0.3571	0.5775
Inference		Precision is reported as standard deviation and relative standard deviation (coefficient of variation) for each type of precision investigation.								

3. Specificity

It was observed that excipients present in the formulation did not interfere with the peak of Ellagic acid and Glycyrrhizic acid. Therefore, the method is specific. The spectrum of standard Ellagic acid and Glycyrrhizic acid correspond with extract.

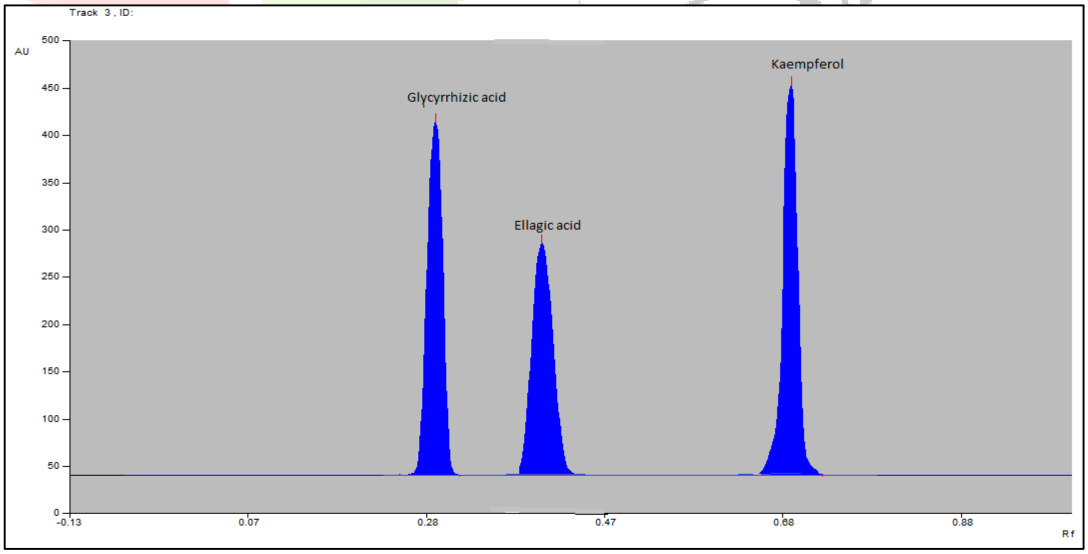


Figure 13: Chromatogram of combined GA, EA and KAE Standard Compound at 265 nm

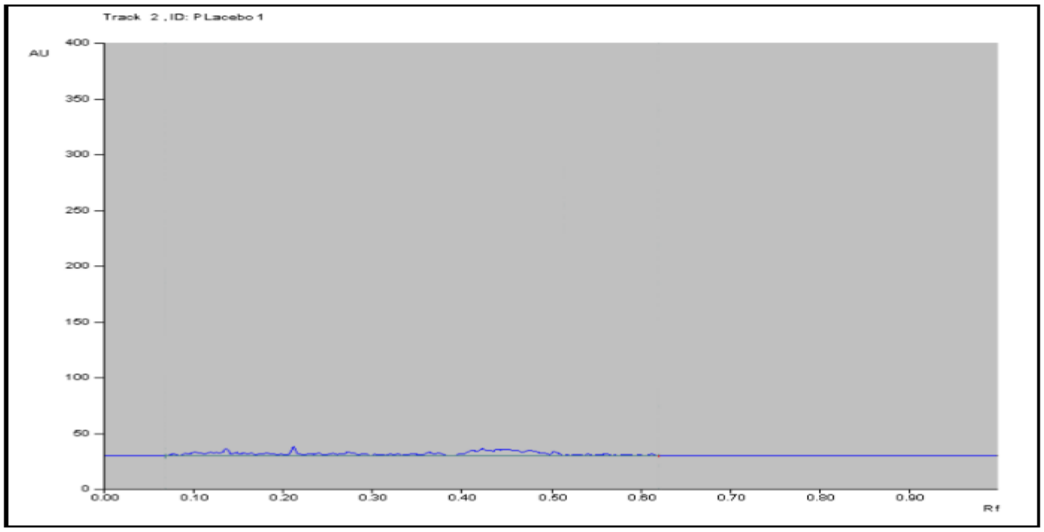
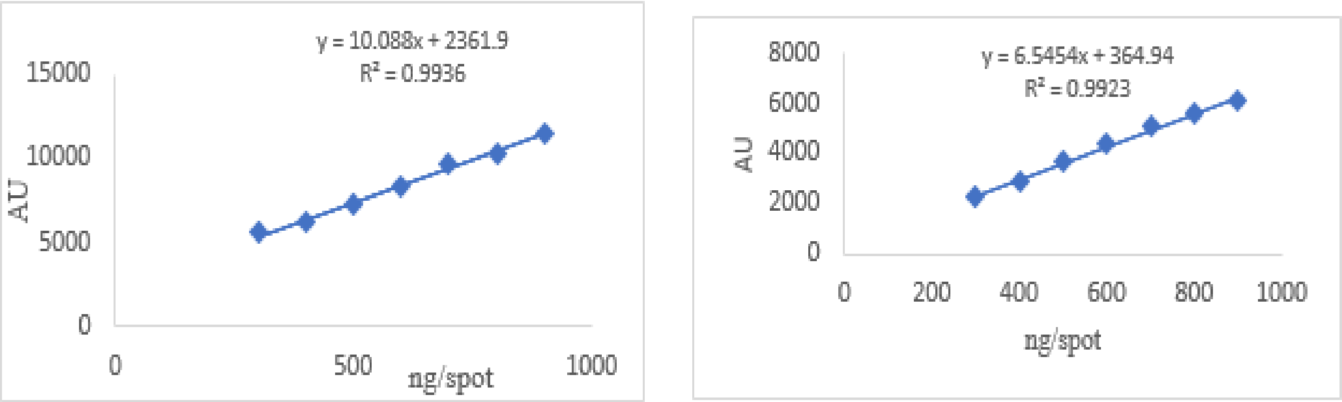


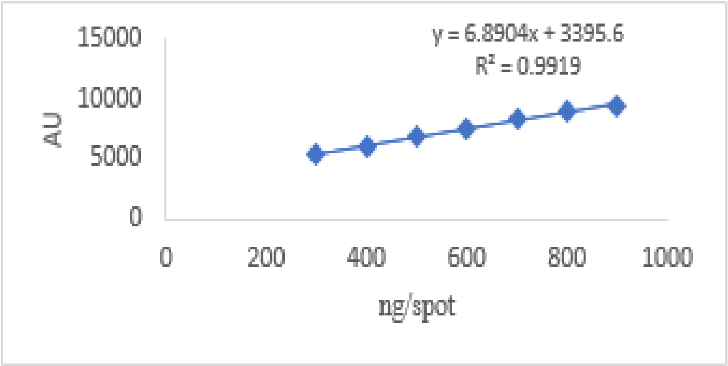
Figure 14: HPTLC chromatogram of placebo Gel at 265 nm

4. Linearity

Linear responses for all three markers Ellagic acid, Glycyrrhizic acid and Kaempferol were obtained at 300-900 ng/spot. Linearity was validated by interpreting regression line and test by calculating regression coefficient (R^2).



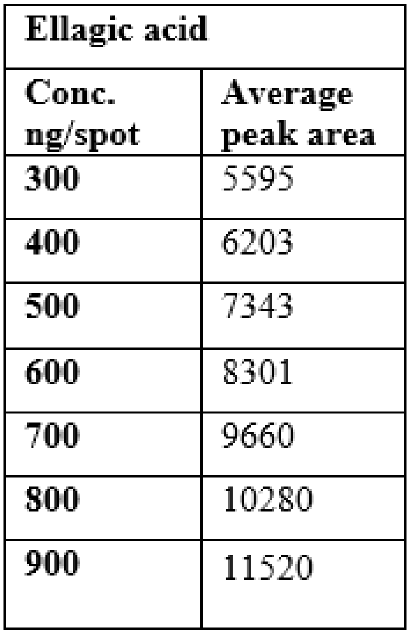
Graph showing linearity of Ellagic acid Graph showing linearity of Glycyrrhizic acid



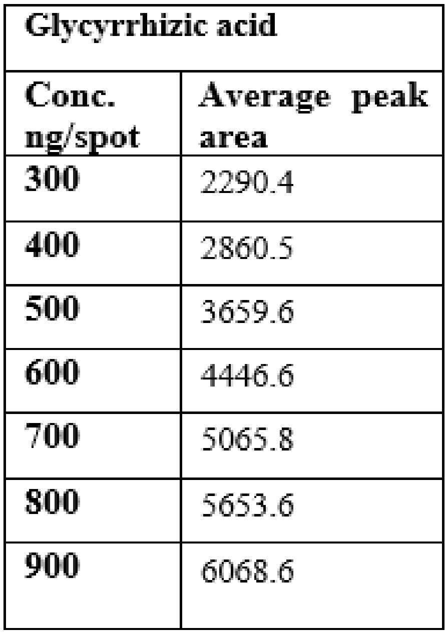
Graph showing linearity of Kaempferol

Figure 15: Calibration curve for linearity

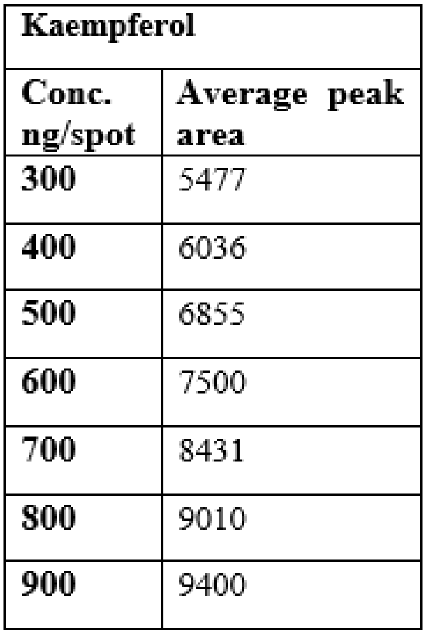
Table IX: Response data for the calibration curves (n=3)



Ellagic acid	
Conc. ng/spot	Average peak area
300	5595
400	6203
500	7343
600	8301
700	9660
800	10280
900	11520



Glycyrrhizic acid	
Conc. ng/spot	Average peak area
300	2290.4
400	2860.5
500	3659.6
600	4446.6
700	5065.8
800	5653.6
900	6068.6



Kaempferol	
Conc. ng/spot	Average peak area
300	5477
400	6036
500	6855
600	7500
700	8431
800	9010
900	9400

Table X: Linear regression data for the calibration curves

Parameters	**Ellagic acid**	**Glycyrrhizic acid**	**Kaempferol**
Linearity range (ng/spot)	300-900	300-900	300-900
Equation	y = 10.088x + 2361.9	y = 6.5454x + 364.94	y = 6.8904x + 3395.6
Correlation coefficient (r^2) ± SD	0.9936	0.9923	0.9919
Slope ± SD	10.088	6.5454	6.8904
Intercept± SD	2361.9	364.94	3395.6

5. Limit of Detection and Limit of Quantification

Values for detection limit and quantification limit were determined based on the standard deviation of the response and the slope of regression line. The calculated values of limit of detection (LOD) and limit of quantitation (LOQ) for Ellagic acid, Glycyrrhizic acid and Kaempferol are shown in table.

Table XIV: Results for (LOD&LOQ)

	Ellagic acid	**Glycyrrhizic acid**	**Kaempferol**
LOD	62.51979ng/spot	126.6847ng/spot	44.96781ng/spot
LOQ	189.4539ng/spot	383.8930ng/spot	136.2661ng/spot

6. Robustness

Robustness of method was studied by making slight but deliberate changes in chromatographic conditions such as changes in mobile phase composition and chamber saturation time. Effects of these changes on the retention factor (Rf) and peak area were evaluated by calculating the relative standard deviations (%RSD). From the results obtained it was concluded that the developed method was found to be robust.

Table XV: Results for Robustness

Parameters	EA		GA		KE	
	%RSD		%RSD		%RSD	
	300 ng/spot	400 ng/spot	300 ng/spot	400 ng/spot	300 ng/spot	400 ng/spot
Toluene : Ethyl acetate : Formic acid : Methanol (4:3.5:0.8:0.2)	0.02	0.87	0.37	0.40	0.42	0.48
Toluene : Ethyl acetate : Formic acid : Methanol (3:3:0.8:0.2)	0.03	0.78	0.93	0.94	0.52	0.58
Toluene : Ethyl acetate : Formic acid : Methanol (3.2:4:1:0.1)	0.15	0.70	0.56	0.31	0.60	0.56
Saturation time (min)						
18	0.09	0.29	0.23	0.57	0.48	0.50
20	0.11	0.63	0.63	0.98	0.93	0.87
22	0.23	0.20	0.11	0.42	0.45	0.65

CONCLUSION

The proposed HPTLC method was found to be economical, simple, accurate, precise, specific and robust and can be used successfully in the routine analysis of Ellagic acid, Glycyrrhizic and Kaempferol in raw materials, extracts and developed pharmaceutical preparations, without any interference.

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