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Elaboration and Verification of the HPTLC Methodology for the Simultaneous Estimation of Glycyrrhizic Acid (GLY), Ellagic Acid, and Kaempferolin in Herbal Formulations

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Abstract: Glycyrrhizic acid (GLY), Ellagic acid (EA), and kaempferol (KAE) can be detected simultaneously using the suggested herbal formulation using a simple, precise, and proven high performance thin layer chromatography (HPTLC) method. Using Merck TLC aluminum sheets of silica gel G60 F254 with a 200 µm thickness, the separation was carried out using ethyl acetate: toluene: formic acid (4.5:4.5:1 v/v/v) as the mobile phase. The chemical was densitometrically measured in absorbance mode at 265 nm. For ellagic acid, glycyrrhizic acid, and kaempferol, the aforementioned mobile phase yielded discrete peaks with Rf values of 0.41±0.03, 0.28±0.03, and 0.76±0.03, respectively. The linear regression analysis results for the calibration plots for EA, GLY, and KA showed a reasonable linear association in the concentration range of 300-900 ng/spot for all the chemicals, with regression coefficients (r2) of 0.9936, 0.9923, and 0.9919, respectively. For kaempferol, the limits of quantitation and detection were 44.9678 and 136.2661 ng/spot; for GLY, they were 126.6847 and 383.8930 ng/spot; and for EA, they were 62.5197 and 189.4539 ng/spot, respectively. The established HPTLC approach that has been proposed can be used to identify and quantify measurement of EA, GLY, and KAE in their extracts and herbal formulations. In the end, the statistical analysis of the data showed that the approach is both selective and repeatable for estimating every molecule.

Keywords: Herbal formulation, HPTLC, Standardization, Estimation, Validation Kaempferol, Ellagic acid, Glycyrrhizic acid

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Introduction

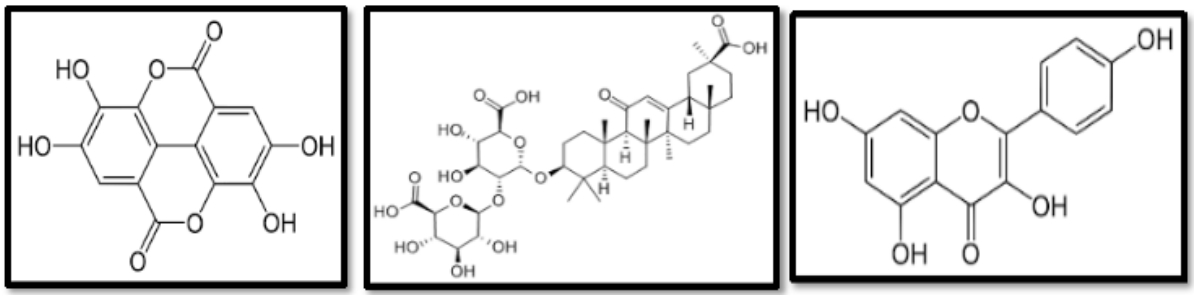
In many undeveloped or developing nations, herbal medicine serves as the primary source of healthcare for a sizable population. While there is a long history of success with Indian traditional medical systems like Ayurveda, Siddha, and Unani, contemporary research also confirms the significance of these therapies (Kagathara *et al.*, 2022). Medicinal herbs and Indian traditional medicine are regarded as potential active ingredients in novel pharmaceuticals. India has made a number of actions to support and incorporate these kinds of medicine into clinical practice (Kharat *et al.*, 2017). The use of evidence-based Indian traditional medicine in clinical settings will contribute to the provision of high-quality wellness initiatives for all (Satija *et al.*, 2020). The big pharmaceutical corporations are currently showing signs of increased interest in looking into higher plants as potential sources for the production of standardized phytotherapeutic compounds that have been shown to be effective, safe, and high-quality (Khairnar *et al.*, 2023).

The peel of pomegranates, or *Punica granatum*, belong to the family Punicacea. This plant is native to Iran, Afghanistan, China, and the Indian subcontinent. It can grow to a height of 3 to 5 meters as a tree or shrub (Trivedi and Mishra, 2010). Due to its astringent and antibacterial qualities, it is applied externally to treat skin conditions (Lokhande *et al.*, 2023). Many diseases, including bacterial infections (antidiarrheal), skin cancer, chronic obstructive pulmonary disease (COPD), and chronic periodontitis, are said to be treated by this folklore herb. Numerous dermatological and dental disorders have been found to respond well to its antibacterial qualities (Tomar *et al.*, 2019). The fruit's high antioxidant concentration can be attributed to the presence of phenolic acids, specifically punicalagin A and B, gallic acid, and ellagic acid (Foudah *et al.*, 2021).

Glycyrrhiza glabra, a member of the Fabaceae family, is a sweet root that is moist, calming, and flavorful. It is generally known as liquorice. The plant has been utilized extensively in Ayurvedic medicine since ancient times. Liquorice roots are utilized in tobacco products and beverages as a flavoring ingredient (Vyas *et al.*, 2013). It is used medicinally to treat hypoglycemia, upper respiratory tract ailments, menopause symptoms, and cramping throughout the menstrual cycle. Its main active element is thought to be glycyrrhizin (also known as glycyrrhizinate or glycyrrhizic acid), a triterpenoid saponin that makes up 10–25% of the liquorice root extract (Shailajan *et al.*, 2019).

The Oleaceae family includes *Nyctanthes arbor-tristis* which is a small tree or shrub with brown-grey bark that can reach heights of 10 to 15 meters. Often referred to as Parijat (Thakker *et al.*, 2011). It is typically grown in tropical and subtropical climates worldwide, however, it is mostly found in subtropical Himalayan regions like Nepal and southern India. It is also referred to as Har-shinghar in addition to Parijata (Vyas *et al.*, 2011). This plant has several medicinal uses for its various sections. Its leaves possess a wide range of therapeutic properties, including antibacterial, anti-inflammatory, antipyretic, and anti-helminthic properties (Mistry *et al.*, 2015).

Ellagic acid, Glycyrrhizic acid, and Kaempferol are components that can be utilized as markers for their HPTLC simultaneous determination. These constituents are found in the extract of the leaves of *Glycyrrhiza glabra*, *Nyctanthes arbor-tristis*, and *Punica granatum*, respectively (Ali *et al.*, 2017). There are currently no published reports in the literature on the simultaneous determination and validation of kaempferol, elagic acid, and



(a) (b) (c)

Fig. 1: Structures of (a) Ellagic acid, (b) Glycyrrhizic acid and (c) Kaempferol.

Table 1: Details of HPTLC system

S. No.	HPTLC System components	Specification
1	Make and model	WATERS
2	Sample applicator	WATERS Linomat V
3	Densitometry scanner	WATERS Scanner 3
4	Sampling mode	Manual with Linomat applicator 5
5	Syringe	Hamilton (100µl)
6	Detection	Ultraviolet (UV) detector
7	software	WATERS (Ver.1.4.1.8)

glycyrrhizic acid in extract and formulation using the HPTLC method (Pathan *et al.*, 2023). In order to simultaneously estimate ellagic acid, glycyrrhizic acid, and kaempferol in extract and herbal formulation, the current study intends to design and evaluate the HPTLC method (Laila *et al.*, 2014). Figure 1 shows structures of Ellagic acid, Glycyrrhizic acid and Kaempferol.

Materials and Methods

Reagents and materials:

Loba Chemicals in Mumbai, India, supplied analytical grade ethanol, methanol, toluene, ethyl acetate, and formic acid. Standard ellagic acid and glycopyrrhizic acid were purchased from Loba Chemicals in Mumbai, India (Ahire *et al.*, 2023).

Instrumentation:

The CAMAG HPTLC system was used to build and validate the quantitative HPTLC method; the system's specifications are illustrated in Table 1.

Development Method:

Wavelength Selection:

We made different UV spectra of 10 ppm solutions of Ellagic acid, Glycyrrhizic acid, and Kaempferol by scanning over the range of 300–900 nm with a Shimadzu UV1801 double beam spectrophotometer. We put all three spectra on top of each other and looked at them to find the best detecting wavelength (isobestic point) for measuring Ellagic acid, Glycyrrhizic acid, and Kaempferol all at the same time (Surana and Mahajan, 2022; Yeola *et al.*, 2023).

Stationary Phase:

As a chromatographic layer, TLC plates that had silica gel G60 F254 already on them were chosen (Parekh and Jadhav, 2018).

Extracts Preparation:

The raw drugs were washed with clean water, dried in the sun, and then ground into a powder. We took 50 g of each drug from *Punica granatum*, *Glycyrrhiza glabra*, and *Nyctanthes arbor-tristis* and mixed them with ethanol using the hot continuous percolation method in a Soxhlet device. The products made from ethanol were

filtered, evaporated, and dried in an electric water bath (Ahamad *et al.*, 2014).

Extracted solution Preparation:

100 mg of each dried extract was carefully weighed out and mixed with 100 ml of methanol to make a stock solution with 1000 µg/ml of each. After going through a 0.45 µ membrane filter, the liquids were used for HPTLC analysis (Mehta *et al.*, 2021).

Standard solution Preparation:

Ellagic acid, kaempferol, and glycyrrhizic acid were prepared as a stock solution by dissolving 10 mg of precisely weighed standard medications in methanol. In a volumetric flask, the volume was made up to 10 ml, and the solutions were designated Stock A, Stock B, and Stock C, respectively. This allowed for the one-time addition of 1.0 ml of the stock solution per 10 ml volumetric flask. After that, the volume was reduced to 10 ml to create a standard solution that was sonicated for 5 min and contained 100 µg/ml of ellagic acid, kaempferol, and glycosyrrhizic acid, respectively (Nair *et al.*, 2017).

Sample solution Preparation:

We weighed out a 100 mg gel formulation and put it into a different volumetric flask that had 10 ml of methanol in it. The flask was then put into the ultrasonicator for 20 min to get all three drugs out of the formulation. One milliliter of the end solution was taken out and put into a volumetric flask that holds 10 ml. Methanol was then added until the volume reached the mark to get a final concentration of 1 mg/ml (Patel *et al.*, 2010).

Optimization of chromatographic conditions:

TLC metal plates (10 x 10 cm) were mixed with 200 µm thick silica gel G60 F254, which was used as a stationary phase. Bands of standard marker and sample solutions of 6.0 mm in width and 10.0 mm from the bottom edge to the same chromatographic plate were applied to the plates. A Camag Linomat 5 sample dispenser fitted with a 100 µl Hamilton syringe was used for this. It was decided to make the entire size 5 mm wide and 0.45 mm deep. We used a mobile phase consisting of 4.5:4.5:1 (v/v/v) of toluene, ethyl acetate, and formic acid (T: Et: F) to perform ascending development up to an 80-mm distance at room temperature (28 ± 2ºC). The mobile phase vapor (T: Et: F) was introduced into the Camag glass twin-trough chamber and allowed to stand for 20 min. The plates were next dried and scanned at 265 nm using a Camag TLC Scanner 3 with the win WATERS software and a deuterium light (Kaur *et al.*, 2019; Aher *et al.*, 2023).

Validation Method:

One goal of validating an analytical method is to show that it works well for what it was made for. These are the things that need to be thought about when validating the new method according to ICH standards based on Q2 (R1) validation of analytical procedure: text and methodology (Sonawane *et al.*, 2023).

Results and Discussion

Method development:

Wavelength Selection:

To get the UV spectra, a 10 ppm solution of each drug was scanned in the 380–220 nm range. The drugs were Ellagic acid (EA), Glycyrrhizic acid, and Kaempferol (KAE). We chose the wavelength at which all three drugs show quantifiable absorbance so that we could measure EA, GLY, and KAE all at the same time. This was done by drawing an overlay graph of each drug's UV spectrum. The overlap UV spectrum of three drugs showed an isoabsorptive point at 265 nm, so that is the wavelength that was chosen for the study.

Optimization of chromatographic conditions:

Numerous preliminary tests were conducted to determine the composition of the mobile phase, based on an analysis of the literature on the polarity and solubility of kaempferol, glycosyrrhizic acid, and ellagic acid. Table 2 displays a few of these.

Chromatographic settings were tuned for the HPTLC method in order to attain the optimal peak

Table 2: Optimized chromatographic conditions for validation

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

Table 3: Quantification of Ellagic acid from pomegranate extract

	Peak Area 1	Peak Area 2	Peak Area 3	Average Peak Area (AU)
Pomegranate extract 1	9246	9527	9645	9473
Pomegranate extract 2	10144	9986	9876	10001
Pomegranate extract 3	10322	10123	10256	10238
Average				9903
Ellagic acid content in extract				900.9 ng/10 ml

shape and resolution for kaempferol, lycyrrhizic acid, and ellagic acid. The mobile phase containing Toluene: Ethyl acetate: Formic acid 4.5:4.5:1:(V/V/V) was found to be the most effective for getting well-resolved peaks of Ellagic acid, Kaempferol, and Glycyrrhizic acid after other mobile phases in various proportions were attempted. Table 2 shows optimized chromatographic conditions for validation.

Quantification of marker in Extract:

A linear regression equation was used to figure out how much Ellagic acid and Glycyrrhizic acid were in the fresh extract. It was discovered that the method could pick out and quantify only certain things.

Quantification of Ellagic acid from Pomegranate Extract:

Three times, the amount of pomegranate juice was measured. The amount of ellagic acid in it was found by taking the average of all of these. Table 3 shows Quantification of Ellagic acid from pomegranate extract. The amount of ellagic acid in

Table 4: Quantification of Glycyrrhizic acid from liquorice extract

	Peak Area 1	Peak Area 2	Peak Area 3	Average Peak Area (AU)
Extract 1	7806	7626	7819	7751
Extract 2	7801	7987	8422	8069
Extract 3	8545	8027	8039	8204
Average				8008
Glycyrrhizic acid content in extract				710.40 ng/10 ml

Table 5: Quantification of Kaempferol from Night-flowering jasmine extract

	Peak area 1	Peak area 2	Peak area 3	Average peak area (AU)
Extract 1	6785	6826	6755	6789
Extract 2	7021	7055	7155	7077
Extract 3	6589	6676	6676	6648
Average				6838
Kaempferol content in extract				499.60 ng/10 ml

pomegranate juice was found to be 0.90% mass/weight.

Quantification of Glycyrrhizic acid from Liquorice extract:

Three times, the amount of liquorice extract was measured. The amount of Glycyrrhizic acid in it was found by taking the average of all of these. Table 4 shows Quantification of Glycyrrhizic acid from liquorice extract. Glycyrrhizic acid made up 0.71% of the weight of the liquorice extract.

Quantification of Kaempferol from Night-flowering jasmine extract:

Three times the amount of Night-flowering jasmine oil was measured. The amount of Kaempferol in it was found by taking the average of all of these. Table 5 shows Quantification of Kaempferol from Night-flowering jasmine extract. The Kaempferol content in Night-flowering jasmine extract was found to be 0.499 %w/w.

Accuracy:

The method's accuracy is shown as a percentage of the known amount of biomarker that was added to the sample. To make sure the method worked correctly, healing studies were done three times at three different concentration levels: 80%, 100%, and 120%. Ellagic acid, Glycyrrhizic acid, and Kaempferol were added in known amounts each time. The results are shown in Table 6.

Precision:

To determine the accuracy three times, Ellagic acid, Glycyrrhizic acid, and Kaempferol were employed as three quality control (QC) samples. There were three concentration levels that were utilized: 300, 600, and 900 ng/spot (Tables 7, 8). Low quality control (LQC), mid quality control (MQC), and high quality control (HQC) are the terms used to describe these stages. The standard deviation and per cent relative standard deviation (coefficient of variance) of the outcomes were then determined using statistical tests. The accuracy of the approach is demonstrated by the peak areas' per cent RSD, which was less than 2%.

Specificity:

It was seen that the add-ins in the mixture did not affect the peak of ellagic acid and Glycyrrhizic acid. This means that the method is unique. Standard Ellagic acid and Glycyrrhizic acid have the same range as the extract.

Table 6: Results for Accuracy

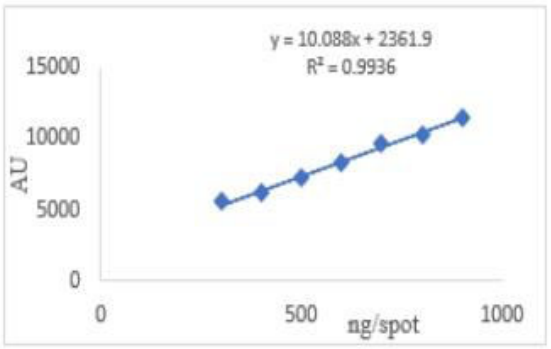
Drug	% Level	Initial Amount (ng/spot)	Spiked Amount (ng/spot)	Total Expected Amount (ng/spot)	Amount Recovered (ng/spot)	% Recovery	Inference
Ellagic acid	80	600	480	1080	1087	100.65	Acceptable recovery hence accurate
	100	600	600	1200	1199	99.97	
	120	600	720	1320	1320	100	
Glycyrrhizic acid	80	600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

Table 7: Intraday Precision Studies

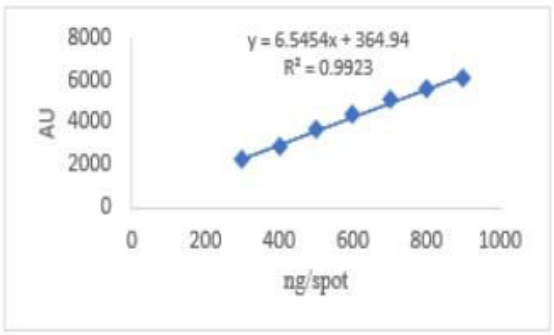
		Observation								
		Ellagic acid			Glycyrrhizic acid			Kaempferol		
Levels		**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
Amount (µg/ml)		**30**	**60**	**90**	**60**	**120**	**180**	30	60	90
Peak Area	**1**	5507	9552	11464	2284	4826	6234	5689	7762	9295
	2	5565	9595	11611	2287	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		For every kind of precise study, the results are given as standard deviation and relative standard deviation (coefficient of variation)								

Table 8: Interday Precision Studies

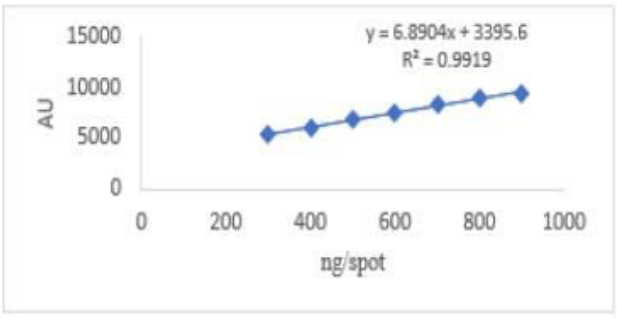
		Observation								
		Ellagic acid			Glycyrrhizic acid			Kaempferol		
Levels		**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
Amount (µg/ml)		**30**	**60**	**90**	**60**	**120**	**180**	**30**	**60**	**90**
Peak Area	**1**	5507	9552	11464	2292	4877	6020	5553	7471	8938
	2	5565	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		For each kind of precision study, precision is given as standard deviation and relative standard deviation, which is also known as coefficient of variation.								



Graph showing linearity of Ellagic acid



Graph showing linearity of Glycyrrhizic acid



Graph showing linearity of Kaempferol

Fig. 2: Calibration curve for linearity.

Table 9: Data from linear regression used to make the reference 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

Linearity:

At 300 to 900 ng/spot, linear reactions were seen for all three markers: ellagic acid, glycyrrhizic acid, and kaempferol. The linearity (Table 9; Fig. 2) was checked by looking at the regression line and testing it by finding the regression coefficient (R2).

Conclusion

It was discovered that the HPTLC approach being proposed is economical, simple to implement, accurate, precise, specific, and powerful. It is possible to use it to successfully analyze Ellagic acid, Glycyrrhizic acid, and Kaempferol in already-made pharmaceutical formulations, extracts, and raw materials without encountering any difficulties in doing so.

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