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HPTLC METHOD DEVELOPMENT AND VALIDATION FOR QUANTITATIVE ESTIMATION OF ELLAGIC ACID, GLYCYRRHIZIC ACID AND HESPERIDIN FROM *PUNICA GRANATUM* PEEL, *GLYCYRRHIZA GLABRA* ROOT AND *CITRUS SINENSIS* PEEL EXTRACT

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Keywords:

HPTLC, Method development and Validation, Ellagic acid, Glycyrrhizic acid and Hesperidin

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ABSTRACT: The present study focuses on developing and validating a high-performance thin-layer chromatography (HPTLC) densitometry method for identifying and quantifying ellagic acid, glycyrrhizic acid and hesperidin in the ethanolic extract of *Punica granatum, Glycyrrhizic glabra* and *Citrus sinensis*. The separation was achieved by mobile phase for ellagic acid was Methanol: Water: Formic acid (3.8:4.7:0.8) and spotting was done on RP TLC Al plate silica gel 60, for glycyrrhizic acid was ethyl acetate: Formic acid: Glacial acetic acid: Water (14.7:0.8:0.5:1) and mobile phase for hesperidin was n – butanol: Acetic acid: Water (2.8:0.6:0.9) on TLC aluminium plate pre-coated with silica gel 60 F254. Densitometric scanning was carried out at a wavelength of 254nm and 287nm in absorbance mode. The peak corresponding to reference standard and that of the extract had the same RF of 0.19 for ellagic acid, 0.14 for glycyrrhizic acid and 0.58 for hesperidin. Following the International Conference of Harmonization (ICH) requirements for precision, accuracy, and reproducibility, the method was verified. The regression equation showed a linear function with a correlation coefficient of 0.999, 0.997 and 0.999, respectively. The limit of detection was 39, 48.26 and 22.39ng/spot respectively and limit of quantification was 120, 146.2 and 67.87 ng/spot for Ellagic acid, Glycyrrhizic acid and Hesperidin, respectively. The content of ellagic acid from the *Punica granatun* extract was found to be 0.78% w/w, glycyrrhizic acid from the *Glycyrrhiza glabra* extract was found to be 0.35% w/w and hesperidin from the *Citrus sinensis* extract was found to be 0.134% w/w. The validated HPTLC method can be routinely used for the quality control and standardization of the marker Ellagic acid, Glycyrrhizic acid and Hesperidin and formulations containing *Punica granatun*, *Glycyrrhiza glabra* and *Citrus sinensis.*

INTRODUCTION: Herbal medicine is used by a huge number of individuals in poor and emerging countries for their primary healthcare treatment. Traditional Indian medicinal systems such as Ayurveda, Siddha and Unani have a long history of efficacy, but modern science confirms the value of such medicine. Traditional Indian medicine and medicinal herbs are also thought to be active ingredients in a new treatment.

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In India, several initiatives have been done to foster and incorporate these types of medicine into clinical practice. Incorporating evidence-based Indian traditional medicine into clinical practise will aid in delivering high-quality wellness programs to everybody. The big pharmaceutical corporations are currently rekindling their interest in higher plants as potential sources for the creation of standardized phytotherapeutics [1, 2].

Drug Profile:

***Punica granatum* (Pomegranate) - *Punica granatum* (Pomegranate):** The punicacea family's pomegranate peel (*Punica granatum*). Because of its astringent and antibacterial characteristics, it is used externally to treat skin conditions.

This folkloric plant is thought to help with bacterial infections (antidiarrheal) [3], anti-cancer (skin cancer) [4], antioxidant [3, 5] and chronic periodontitis [6], among other things. Its antibacterial qualities have been proven to be beneficial in treating various dental and dermatologic ailments. The presence of phenolic acids such as gallic acid, ellagic acid, punicalagin A and punicalagin B contributes to the fruit's high antioxidant value [2, 7, 8].

Glycyrrhiza glabra L. (Licorice) - Glycyrrhiza glabra is a sweet, moist, relaxing and flavouring sweet root. It belongs to the Fabaceae family. The herb has been widely used as a medication since Ayurveda's ancient medical history. Licorice roots are utilised in beverages and tobacco goods as a flavouring ingredient. Menstrual cramps [9], menopause symptoms [9], upper respiratory tract sickness [10], anticancer [10] and hypoglycemia [11] are all treated with it medicinally. The principal active element is glycyrrhizin (Glycyrrhizinate; Glycyrrhizic acid), a triterpenoid saponin that makes up 10-25 percent of licorice root extract [2, 7, 12].

Citrus sinensis (Orange) -Orange peel from the rutaceae family (*Citrus sinensis*). Citrus peels are known for their antioxidant [13], anti-inflammatory [14], anticancer [15] and antibacterial [13] properties. Eriocitin, narirutin, hesperidin, and didymin are flavonoids found in citrus sinensis [16].

MATERIALS AND METHOD:

Materials:

Reagents and Materials: Analytical grade ethanol, methanol, toluene, ethyl acetate, glacial acetic acid, water, 1 – butanol and formic acid were purchased from SD Fine Chemicals, Mumbai, India. Standard Ellagic acid, Glycyrrhizic acid, and hesperidin were procured from Yucca Enterprises, Mumbai.

Plant Material: The *Punica granatum* peel and *Glycyrrhiza glabra* root and *citrus sinensis* peel in powdered form was purchased from 'Yucca Enterprises' Wadala (e), Mumbai 400 037.All drugs were authenticated by Alarsin House. A/32, Street No. 3 M.I.D.C. Andheri (East). Mumbai 400093 India.

Instrumentation and Condition: A Camag automatic TLC sampler 4(ATS4) applicator with a 100 l Hamilton syringe was used in the HPTLC system. At a distance of 15 mm, the samples and standard solutions of markers were spotted in the shape of bands (8 mm wide) at the bottom of the chromatographic plates.

For spots, we utilized aluminium TLC plates (2010 cm, 250mm thickness Merck) precoated with silica gel 60F254 and RP aluminium TLC plates silica gel 60 (for ellagic acid). With a scanning speed of 20 mm/s and a slit dimension of 6 mm 0.45 mm, the slit dimension was retained at 6 mm 0.45 mm.

Camag TLC ATS4 was used to scan at 254nm and 287nm, which were discovered to be the wavelengths of ellagic acid (254nm), glycyrrhizic acid (254nm) and hesperidin (287nm), respectively. Camag vision CATS software was used for application and scanning. Camag glass twin-trough a glass chamber (20×10 cm) saturated with mobile phase was used to develop the plates [2, 17, 18].

Preparation of Stock Solution: A standard stock solution of 1000 µg/ml was prepared by dissolving 5 mg of marker (ellagic acid, glycyrrhizic acid and hesperidin) in 10 ml methanol.

Preparation of Sample Solution: The methanolic extract of *Punica granatum* peel, *Glycyrrhiza glabra* root and *Citrus sinensis* peel Powder sample of 50mg was dissolved in 5ml methanol and centrifuged at 3000 rpm for 5 min. Volume was made up to 10 ml using methanol.

Chromatographic Condition: The mobile phase for ellagic acid was Methanol: Water: Formic acid (3.8:4.7:0.8) and spotting was done on RP TLC Al plate silica gel 60, mobile phase for glycyrrhizic acid was ethyl acetate: Formic acid: Glacial acetic acid: Water (14.7:0.8:0.5:1) and mobile phase for hesperidin was n – butanol: Acetic acid: Water (2.8:0.6:0.9) used for chromatogram development. Prior to the study, the chamber was saturated with the mobile phase for 20 min at room temperature.

Method Validation: The proposed analytical method was validated per the International Conference on Harmonization (ICH) guidelines Q2 (R1). The parameters checked were linearity, specificity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy (recovery) and robustness [18].

Linearity: The ability of an analytical process to generate test findings within a given range that are directly proportional to the concentration of the analyte in the sample is referred to as linearity. The drug concentration and peak area plotting graph for each standard revealed linearity.

The method linearity was determined by estimating the standard deviation (SD), coefficient of determination (r^2), slope and intercept of the calibration curves [18].

Precision: The precision of an analytical method is the closeness of a set of measurements acquired from serial sampling of the same homogenous sample under the defined conditions. For intraday and interday precision, the existing technique has been validated.

Ellagic acid (400ng/spot), Glycyrrhizic acid (500ng/spot) and Hesperidin (400ng/spot) intraday precision was determined in triplicate using the same procedure on the same day.

The method's interday precision was determined by repeating the procedure on different days with the same experimental conditions. The findings were presented as a Coefficient of Variation (CV%) [18].

Accuracy: Trueness, which represents the degree of agreement between the conventional true value, or an acknowledged reference value and the value found, is a measure of an analytical procedure's correctness.

Using a method of standard spiking, recovery trials were conducted. The accuracy was calculated as the percent of analyte recovered when a sample of known concentrations was applied and subsequently spiked with 80, 100 and 120%w/w amounts of analyte in triplicate [18].

Robustness: The robustness of an analytical procedure is a measure of its ability to remain intact in the case of tiny but deliberate changes in method parameters and it indicates its reliability in routine use [18].

Specificity: Specificity is the ability to assess unequivocally the analyte within the presence of components that can be expected to be present. The specificity of the method was checked by overlaying the spectra of standards with the spectra of the sample.

The method was found to be specific since the spectra of standards matched with the spectra of the spots in the sample at the same Rf values as that of standards [18].

Limit of Detection and Quantitation Limit: The limit of detection of an analytical procedure is the lowest amount of analyte present in a sample which can be detected but not necessarily quantitated as an exact value. The detection limit (DL) may be expressed as:

$$DL = 3.3\ \sigma/S$$

(σ is a standard deviation and S is the slope of the calibration curve). The limit of quantitation of an analytical procedure is defined as the lowest amount of analyte within a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit (QL) may be expressed as:

$$QL = 10\ \sigma/S$$

(σ is a standard deviation and S is the slope of the calibration curve) [18].

Chromatographic separation was performed with standard solution of Ellagic acid, Glycyrrhizic acid and Hesperidin. The spots of these standards were applied on TLC plates.

For the development of the mobile phase, several trials were made using many solvents in different proportions by the linear ascending development method.

The optimized mobile phase consisting of Methanol: Water: Formic acid (3.8: 4.7: 0.8 v/v/v) showed satisfactory resolution at Rf 0.19 for Ellagic acid, Ethyl acetate: Formic acid: Glacial acetic acid: Water (14.7:0.8:0.5:1 v/v/v/v) showed satisfactory resolution at Rf 0.14 for Glycyrrhizic acid and n – butanol: Acetic acid: Water (2.8:0.6:0.9 v/v/v) showed satisfactory resolution at Rf 0.58 respectively. Densitometric measurements were obtained with Camag TLC scanner IV at 254nm and 287nm that was operated by vision CATS software.

RESULTS AND DISCUSSION:

HPTLC Method Development:

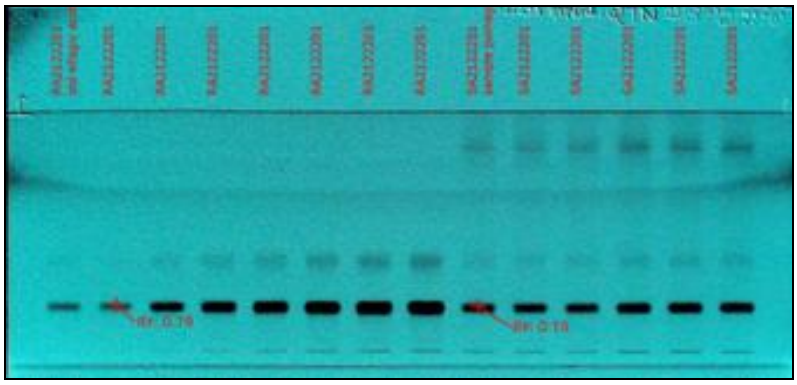

FIG. 1: DEVELOPED HPTLC PLATE OF ELLAGIC ACID & *PUNICA GRANATUM* PEEL EXTRACT AT 254nm

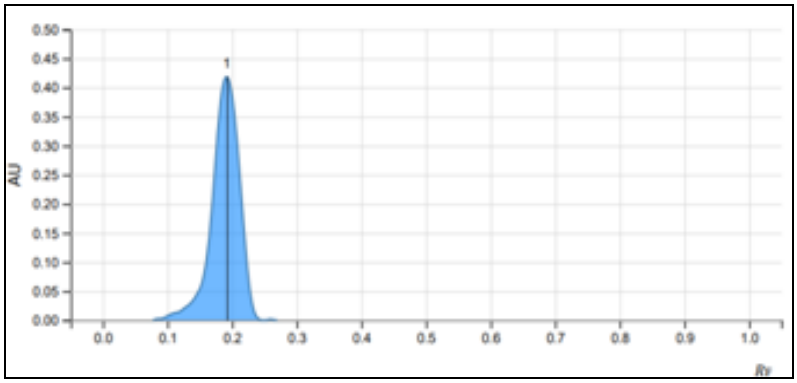

FIG. 2: HPTLC DENSITOGRAM OF ELLAGIC ACID OBTAINED USING OPTIMIZED CHROMATO-GRAPHIC CONDITION

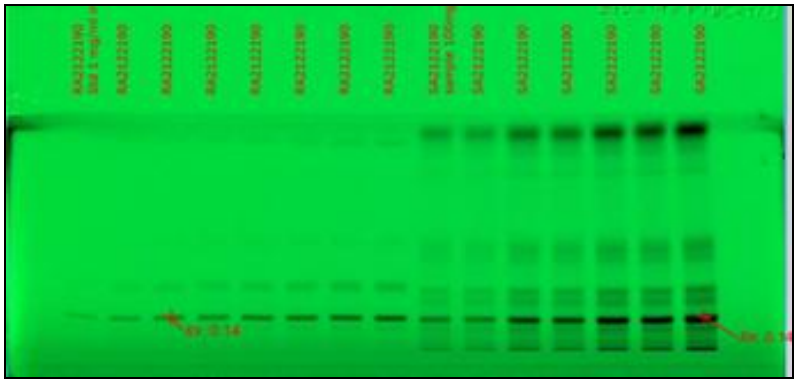

FIG. 3: DEVELOPED HPTLC PLATE OF GLYCYRRHIZIC ACID & *GLYCYRRHIZA GLABRA* PEEL EXTRACT AT 254nm

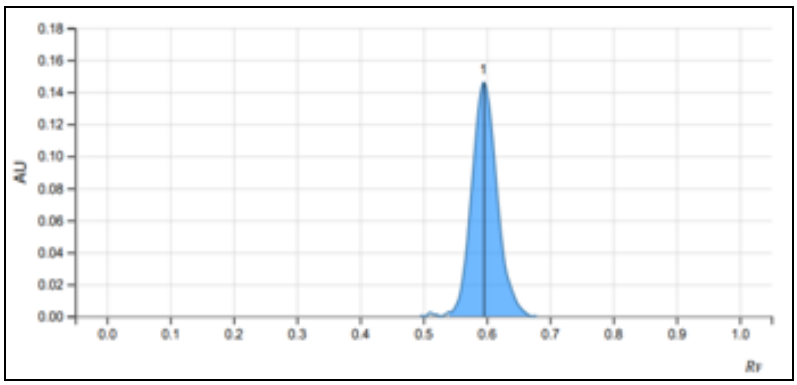

FIG. 4: HPTLC DENSITOGRAM OF GLYCY-RRHIZIC ACID OBTAINED USING OPTIMIZED CHROMATO-GRAPHIC CONDITION

Linearity: A five-point calibration curve was obtained by plotting peak area against concentrations. Linearity was evaluated by applying different concentrations of 400-800 ng/spot for Ellagic acid, 200 – 700 ng/spot for glycyrrhizic acid and 200 – 600 ng/spot for hesperidin respectively.

A good linearity relationship was found with r^2 values of 0.9962 (y = 11.107x + 666.07) for ellagic acid; for glycyrrhizic acid, r^2 value was found 0.9973 (y = 10.316x + 2346.3) and for hesperidin r^2 value was 0.9993 (y = 16.005x + 1048.5), respectively as shown in **Table 1** and **Fig. 7, 8** and **9.**

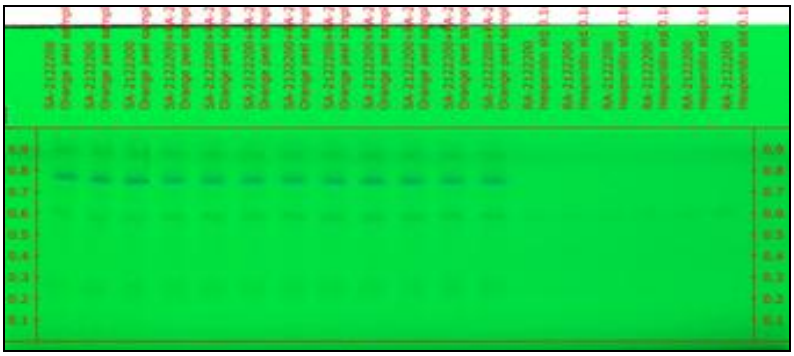

FIG. 5: DEVELOPED HPTLC PLATE OF HESPERIDIN & *CITRUS SINENSIS* PEEL EXTRACT AT 287nm

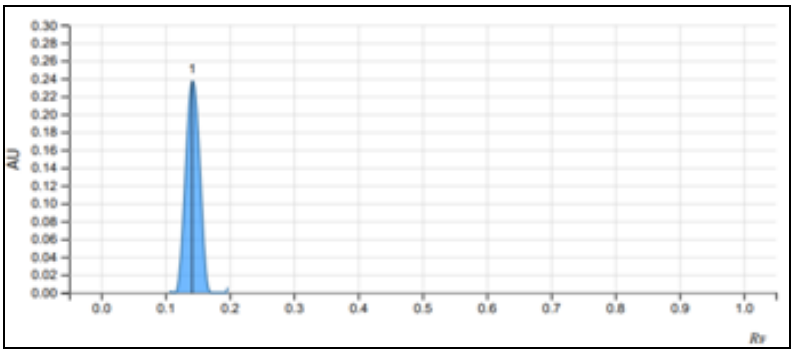

FIG. 6: HPTLC DENSITOGRAM OF HESPERIDIN OBTAINED USING OPTIMIZED CHROMATOGRAPHIC CONDITION

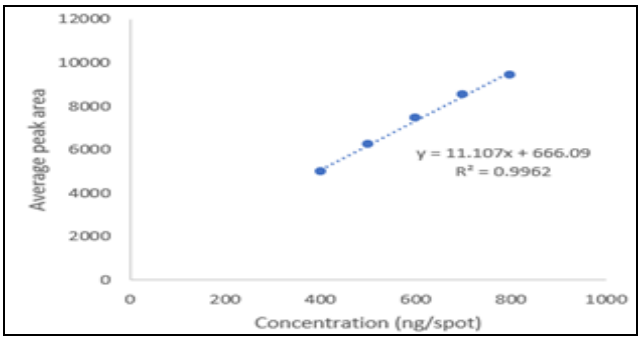

FIG. 7: GRAPH SHOWING LINEARITY OF ELLAGIC ACID

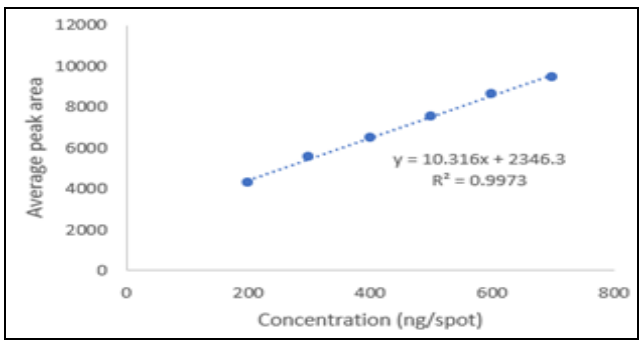

FIG. 8: GRAPH SHOWING LINEARITY OF GLYCYRRHIZIC ACID

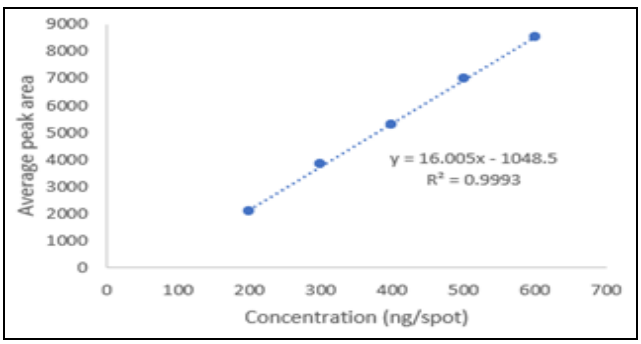

FIG. 9: GRAPH SHOWING LINEARITY OF HESPERIDIN

TABLE 1: LINEARITY RESULTS

Components	Concentration range (ng/spot)	Regression equation	Correlation coefficient (r^2)
Ellagic acid	400 - 800	y = 11.107x + 666.07	0.9962
Glycyrrhizic acid	200 - 700	y = 10.316x + 2346.3	0.9973
Hesperidin	200 - 600	y = 16.005x + 1048.5	0.9993

Precision: The presented method's intra-day and inter-day precision were calculated for each drug. The outcomes of intra-day and inter-day repeatability are shown in **Table 2**, which shows the % RSD.

TABLE 2: RESULT OF INTRADAY AND INTERDAY PRECISION OF ELLAGIC ACID, GLYCYRRHIZIC ACID AND HESPERIDIN

Marker	Concentration (ng/spot)	Intraday precision	Interday precision
		%RSD	%RSD
Ellagic acid	600	0.75%	0.81%
Glycyrrhizic acid	500	0.62%	0.69%
Hesperidin	400	1.25%	1.17%
Inference		Method was found to be precise	

The concentration levels used for the precision parameter was 600ng/spot for Ellagic acid and 400ng/spot for Hesperidin while 500ng/spot was used for Glycyrrhizic acid, RSD is relative standard deviation.

Accuracy: Results from accuracy studies, provided in **Table 3**, were in an acceptable range (98-102%), indicating that the recovery of the proposed method was good for all three markers.

TABLE 3: RECOVERY STUDY

	Ellagic acid			Glycyrrhizic acid			Hesperidin		
%Level	80	100	120	80	100	120	80	100	120
Initial amount (ng/spot)	600	600	600	500	500	500	400	400	400
Spiked amount (ng/spot)	480	600	720	400	500	600	320	400	480
Total amount (ng/spot)	1080	1200	1320	900	1000	1100	720	800	880
Amount recovered (ng/spot)	1087	1199	1320	874	994	1152	740	802	870
% Recovery	100.6%	99.9%	100%	97.1%	99.4%	104.7%	102.7%	100.2%	98.86%
Inference	Acceptable recovery			Acceptable recovery			Acceptable recovery		

Robustness: The effect of intentional changes in the saturation time of mobile phase was studied. The method was found to be robust for all the markers.

Method Parameters	Level of Variation	Modified Parameters	%RSD		
			Ellagic acid	Glycyrrhizic acid	Hesperidin
			600 ng/spot	500 ng/spot	400 ng/spot
Saturation time (20min)	+10	30 mins	0.44%	0.72%	1.0%
	-10	10 mins	0.45%	0.79%	1.29%

Specificity: The specificity of the method was determined by analysing reference and sample. The spot for ellagic acid, glycyrrhizic acid and hesperidin in the sample were confirmed by comparing Rf and spectra of spot with that of standard.

Limit of Detection and Quantification: The limit of detection (LOD) value for ellagic acid was 39ng, for glycyrrhizic acid was 48.26ng and for hesperidin was 22.39ng and the limit of quantification (LOQ) value for ellagic acid was 120ng, for glycyrrhizic acid was 146.2ng and for hesperidin was 67.87ng respectively, which shows the sensitivity of the method **Table 4.**

TABLE 4: RESULT OF LOD AND LOQ

Parameters	Ellagic acid	Glycyrrhizic acid	Hesperidin
LOD (ng/spot)	390	48.26	22.39
LOQ (ng/spot)	1200	146.2	167.85

The purity of the proposed method was determined by superimposing the spectrum of standard and sample peaks and confirmed for its purity. The samples were spotted on a TLC plate and developed. The peak of ellagic acid comes at Rf of

0.19 in the extract. The quantified amount of ellagic acid from the *punicagranatun* extract was found to be 0.78% w/w. The peak of glycyrrhizic acid comes at Rf of 0.14 in the extract. The quantified amount of glycyrrhizic acid from the *Glycyrrhiza glabra* extract was found to be 0.35% w/w. The peak of hesperidin comes at Rf of 0.58 in the extract. The quantified amount of hesperidin from the *citrus sinensis* extract was found to be 0.134% w/w. The HPTLC method was developed and validated in the present study to estimate ellagic acid, glycyrrhizic acid and hesperidin in a *Punica granatun, Glycyrrhiza glabra* and *Citrus sinensis* extract, respectively. The method was found simple, rapid, accurate, specific, and robust for the analysis of ellagic acid, glycyrrhizic acid and hesperidin in *Punica granatun* peel, *Glycyrrhiza glabra* root and *Citrus sinensis* peel extract, respectively. Any laboratory can adopt the developed method for the quality control of crude drugs that contains ellagic acid, glycyrrhizic acid and hesperidin as active marker.

CONCLUSION: The proposed HPTLC approach was proven to be cost-effective, easy, accurate, exact, specific, and robust, and it can be used to successfully analyze Ellagic acid, Glycyrrhizic acid and Kaempferol in raw materials, extracts, and finished pharmaceutical formulations.

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CONFLICTS OF INTEREST: Nil

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