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Validated High Performance Thin Layer Chromatography Method for Simultaneous Estimation for Gallic Acid and Quercetin in Polyherbal Blend and Their Quantitative Estimation

Simple, sensitive high performance thin layer chromatography method for the estimation of gallic acid and quercetin in in-house polyherbal blend has been developed and validated. Methanolic solution of herbal blend comprising of *Emblica officinalis*, *Camellia sinensis* and *Garcinia cambogia* was used for analysis. The separation was performed on TLC aluminum plates precoated with silica gel G60 F_{254} and toluene: ethyl acetate: formic acid (5:1.5:1 v/v/v) at 254 nm scanning wavelength. The system gave well resolved peaks for gallic acid and quercetin at R_f 0.14 and R_f 0.29 respectively. The method validated as per ICH Q2R1 guidelines which shows regression co-efficient 0.9939 for gallic acid and 0.9988 for quercetin in range of 2–6 μg/ml. Recovery of gallic acid and quercetin was found in range of 98–102 % which confirms the accuracy of method. Precision study (interday & intraday) showed that the relative standard deviation is less than 2 %, showing method is well precise. Proposed validated HPTLC method is simple, precise, specific, robust and accurate, and could find application in routine quality-control analysis. The method was used for quantitative estimation of gallic acid and quercetin in the polyherbal blend and was found as 1.648 % w/w and 3.165 % w/w respectively.

Keywords: gallic acid, quercetin, simultaneous estimation, high performance thin layer chromatography, validation, quantification, polyherbal, herbal, extracts.

Introduction

During past decades, public interest in herbal has increased exponentially. According to WHO, mass population (about 80 %) in developing countries depends essentially on herbal plants for primary health care needs owing to efficacy and lower side effect. Also now researchers are exploring plants as source for new lead structure against different diseases [1–2]. Many herbal formulations as single and polyherbal have been proved to be active and are widely available in market. The major cause of concern with herbal plants is biodiversity and quality of the plants which ultimately affects the efficacy of the formulation. High performance thin layer chromatography (HPTLC) has become a widely acceptable analytical tool for the quality control of herbal drugs. It serves as a low operation- cost and quick analysis tool in herbal analysis.

The polyherbal mixture prepared for the study comprised of three plants viz. *Emblica officinalis, Camellia sinensis* and *Garcinia cambogia*, each of which are known to have therapeutic value [3–5]. The polyherbal blend was prepared with the aim to enhance the overall potential of the herbal extracts as these are used as antioxidant, anti-inflammatory and anti-obesity agent. These herbal plants consist of various phytoconstituents as alkaloids, flavanoids, tannins and polyphenols which contribute to their pharmacological activity. To be specific quercetin, gallic acid, ellagic acid and ascorbic acid are present in polyherbal blend and known for its effect.

Some methods are reported which includes HPTLC method for simultaneous estimation of quercetin and gallic acid in *Leea indica* [6], *in Eclipta alba* and *Guiera senegalensis* simultaneous HPTLC method is developed for estimation of quercetin and gallic acid [7], HPTLC simultaneous estimation of gallic acid and quercetin is also reported in single plant extract of *Abutilon indicus* [8] *and* HPTLC method is also reported in literature for estimation of gallic acid, rutin and quercetin in aqueous extract of *Terminalia chebula* [9] But simultaneous estimation of gallic acid and quercetin is not reported in polyherbal blend viz. three herbal plant mixture. Hence the present study aims to determine gallic acid and quercetin simultaneously in polyherbal blend. Same developed and validated method can be used for quantification of the biomarker in herbal mixture consisting of gallic acid and quercetion.

Gallic acid is phenyl propanoid, chemically it is 3,4,5-Trihydroxybenzoic acid, and possesses antioxidant, anti-inflammatory and astringent activity [10–11]. Quercetin is 3,3,4,5,7-pentahydroxyflavone and possesses anti-inflammatory, antihypertensive, vasodilator effects, antiobesity, antihypercholesterolemic and antiatherosclerotic activities [12–13].

Experimental

Chemicals and solvents

Reference standard quercetin was purchased from Cayman Chemical Company, USA and gallic acid from Natural Remedies, India. All chemicals and solvents used were of analytical grade.

Plant material

Herbal plant powder extracts of *Emblica officinalis, Camellia sinensis* and *Garcinia cambogia* were procured from Kisalaya Herbals Ltd., Indore, Madhya Pradesh.

Polyherbal blend composition

A polyherbal blend was prepared by mixing equal amount of fruit of Emblica officinalis, leaves of Camellia sinensis and fruit of Garcinia combogia extracts.

HPTLC analysis

a) Preparation of standard solution of gallic acid and quercetin

Standard stock solution of Gallic acid and Quercetin was prepared separately by dissolving 10 mg of Gallic acid and Quercetin up to 10 ml of methanol, to get stock solution containing 1000 µg/ml of Gallic acid and Quercetin. 5 µl of the above solution was applied on plate to obtain standard densitogram of Gallic acid and quercetin.

b) Preparation of sample solution of Herbal blend

Sample stock solution was prepared by dissolving 50 mg of mixture in 1ml methanol sonicated for 10 min any insoluble fraction was removed by filtration. 30 µl of the above solution was applied on plate to obtain standard densitogram of blend. Presence of Gallic acid and Quercetin in blend was confirmed by overlay spectra.

c) Chromatographic condition

Based on sample solubility, stability and suitability various mobile phase compositions were tried to get a good resolution and sharp peaks. The standard and sample solution was run in various mobile phases, showed that Toluene, Ethyl acetate and Formic acid in proportion of 5:1.5:1 (v/v/v) was best suitable for Herbal mixture. Chromatography was performed using commercially-prepared, pre-activated (110 °C) silica gel 60 F_{254} TLC plates (10×10 cm). A Linomat IV (Camag, Muttenz, Switzerland) semi-automatic TLC applicator was used to apply samples and standards onto the TLC plate under a flow of nitrogen gas. After the application of sample, the chromatogram was developed in twin trough glass chamber 10×10 cm saturated with previously equilibrated mobile phase for 15 min. The chromatographic conditions were optimized to obtain the best peak shape. The plates were fixed in the scanner stage (CAMAG TLC SCANNER) and scanning was done at UV 254 nm. The peak table, peak display, spectrum mode were recorded. The retention factor (Rf) was calculated by WINCAT'S software version 1.4.3.6336

d) Validation

ICH Q2 (R1) guidelines were followed for the validation of the analytical method developed. Calibration curve for gallic acid and quercetin was obtained from the system as graph of concentration versus absorbance. The precision of the method was determined by interday and intraday precision by analyzing sample solutions at different time intervals on the same day and on three different days, respectively. System precision was evaluated from six replicate application of standard as 6µl of gallic acid and quercetin at 3 tracks and method precision was carried out from six replicate applications (30 µl application at 3 tracks) and was expressed as % relative standard deviation. Recovery studies were performed using standard addition

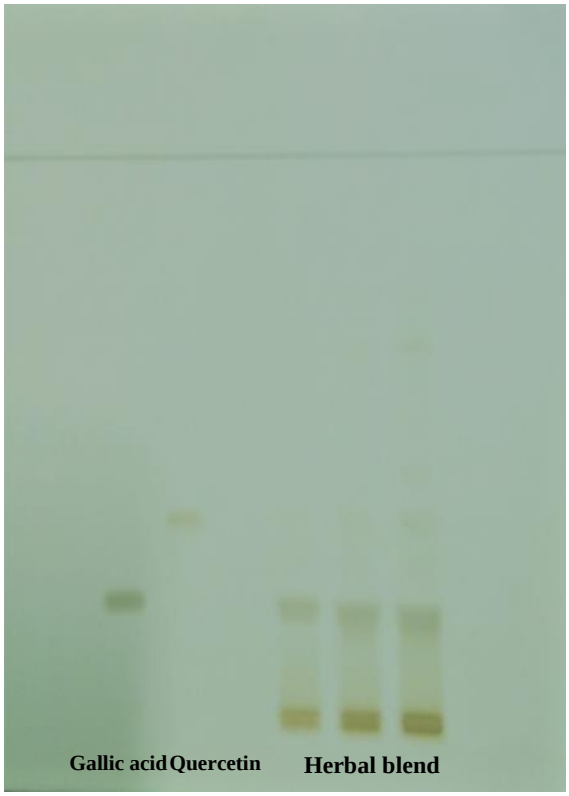
method and at three different levels viz. at 80, 100 and 120 % of the test concentration as per ICH guidelines. Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined using the formula based on the standard deviation of the response and the slope. To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters such as composition of the mobile phase and chamber saturation time in the range of ±0.2 ml and ±5 min, respectively was carried out. The effect of these changes on R_f values and peak area were studied.

e) Quantification of Standard Gallic acid and Quercetin in Herbal blend

Concentration of Gallic acid and Quercetin in Herbal blend was calculated using linearity equation of gallic acid and Quercetin.

Result and Discussion

Figure 1 shows developed HPTLC plate. Optimized chromatographic conditions are shown in Table 1.



Tracks — Blank, Gallic acid, quercetin and herbal blend at different concentrations

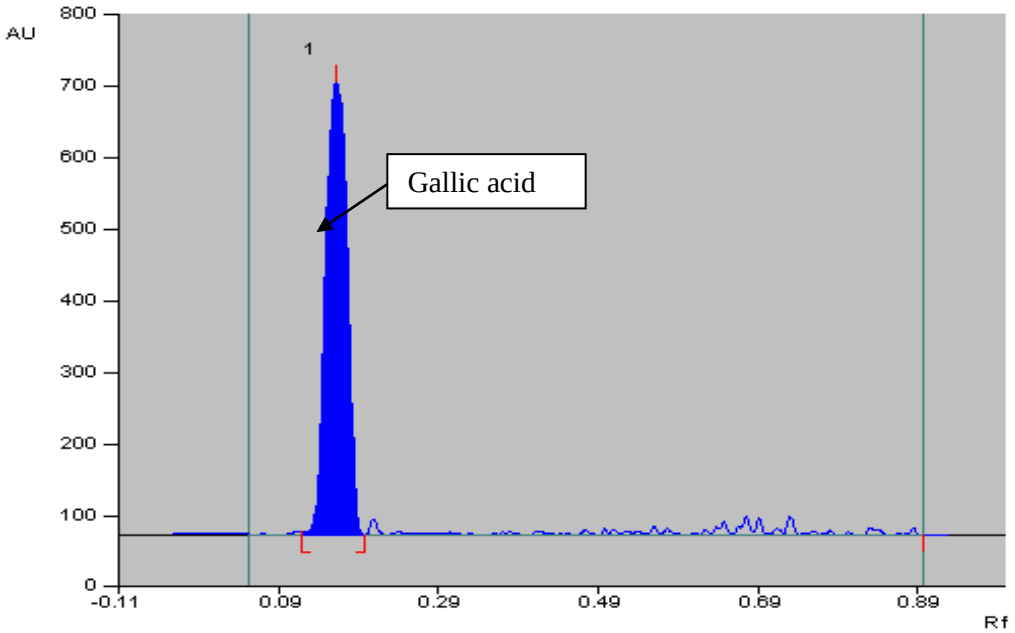
Figure 1. Developed HPTLC plate under UV light at 254 nm

Table 1

Optimized Chromatographic Condition

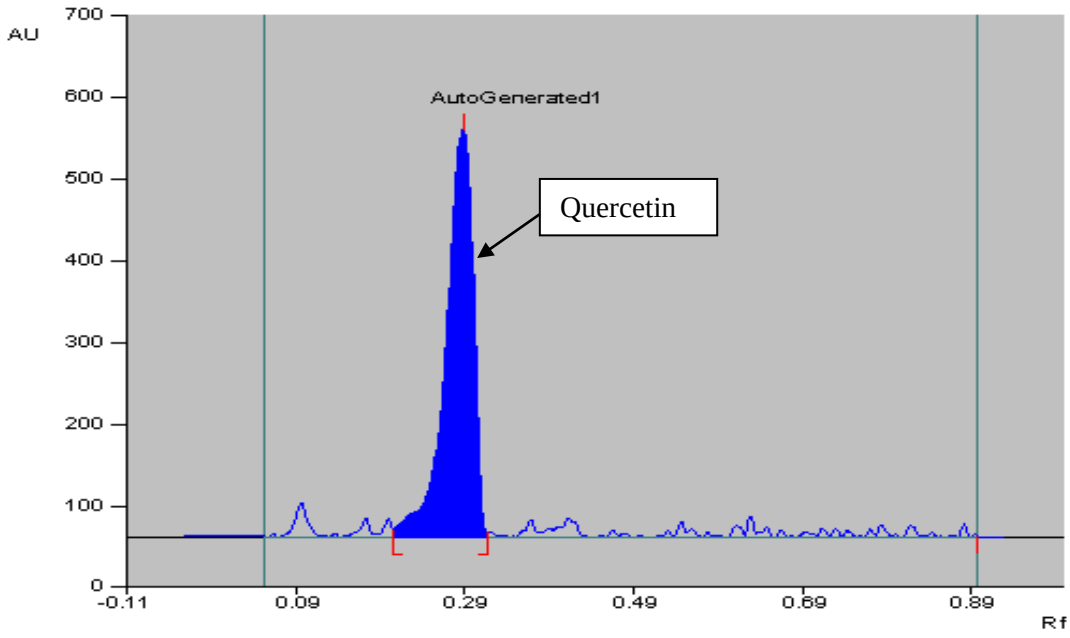
Sr. No.	Parameters	Details
1	Stationary phase	Silica gel 60 F_{254} plates
2	Mobile Phase	Toluene: Ethyl Acetate: Formic Acid
3	Sample Applicator	Camag linomat V applicator
4	Development chamber	Twin-through glass chamber, 10×10 cm with stainless steel lid
5	Saturation time	15 min
6	Scanning wavelength	254 nm
7	Syringe	Camag 100 ul syringe
8	TLC Scanner	Camag TLC scanner III
9	Software	WinCATs software version 1.4.3.6336

Optimized mobile phase gave sharp peak for gallic acid at R_f 0.14 (Figure 2) and Quercetin at R_f 0.29 (Figure 3). Herbal blend showed the presence of both actives at R_f 0.14 for gallic acid and R_f 0.29 for Quercetin (Figure 4). Presence of gallic acid and Quercetin in herbal blend was confirmed by overlay spectra as shown in Figure 5 and 6.



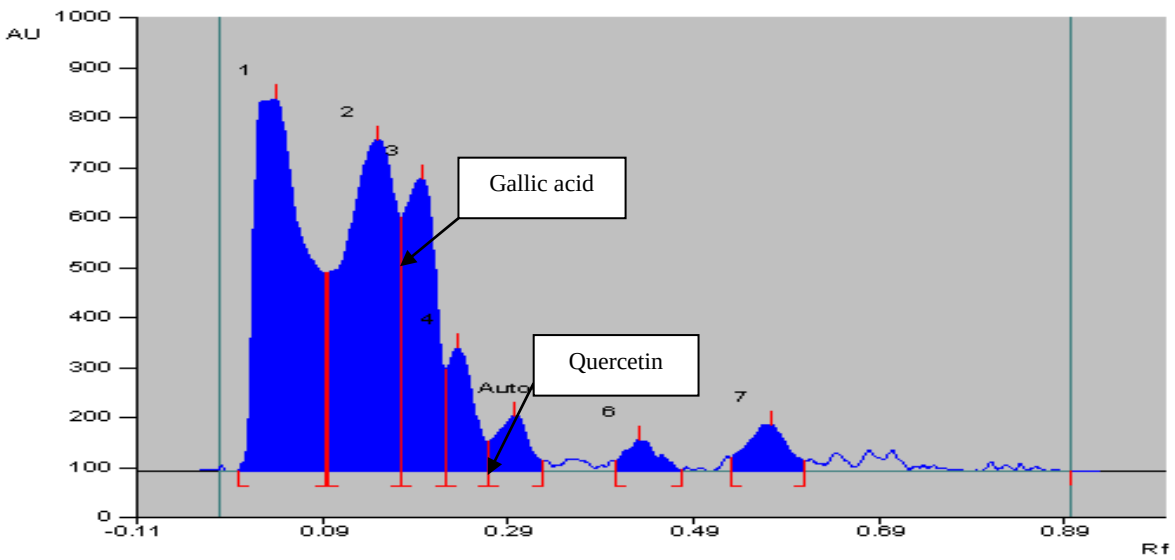
Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.09 Rf	0.1 AU	0.14 Rf	559.0 AU	100.00 %	0.17 Rf	9.5 AU	13281.0 AU	100.00 %

Figure 2. Densitogram of Standard Gallic Acid (5 µg/ml)



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.24 Rf	1.1 AU	0.29 Rf	517.3 AU	100.00 %	0.32 Rf	5.7 AU	11402.2 AU	100.00 %

Figure 3. Densitogram of Standard Quercetin (5 µg/ml)



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.00 Rf	2.0 AU	0.04 Rf	744.9 AU	29.67 %	0.09 Rf	35.5 AU	33630.9 AU	35.37 %
2	0.09 Rf	395.9 AU	0.15 Rf	663.4 AU	26.43 %	0.17 Rf	36.0 AU	31114.4 AU	32.72 %
3	0.17 Rf	506.5 AU	0.20 Rf	585.9 AU	23.34 %	0.22 Rf	39.8 AU	16480.7 AU	17.33 %
4	0.22 Rf	203.9 AU	0.24 Rf	247.5 AU	9.86 %	0.27 Rf	59.5 AU	5543.6 AU	5.83 %
5	0.27 Rf	60.3 AU	0.30 Rf	111.8 AU	4.45 %	0.33 Rf	21.5 AU	3099.4 AU	3.26 %
6	0.40 Rf	20.9 AU	0.43 Rf	62.9 AU	2.51 %	0.48 Rf	2.3 AU	1849.0 AU	1.94 %
7	0.53 Rf	26.9 AU	0.57 Rf	93.7 AU	3.73 %	0.61 Rf	20.4 AU	3370.6 AU	3.54 %

Figure 4. Densitogram of Herbal blend (1500 µg)

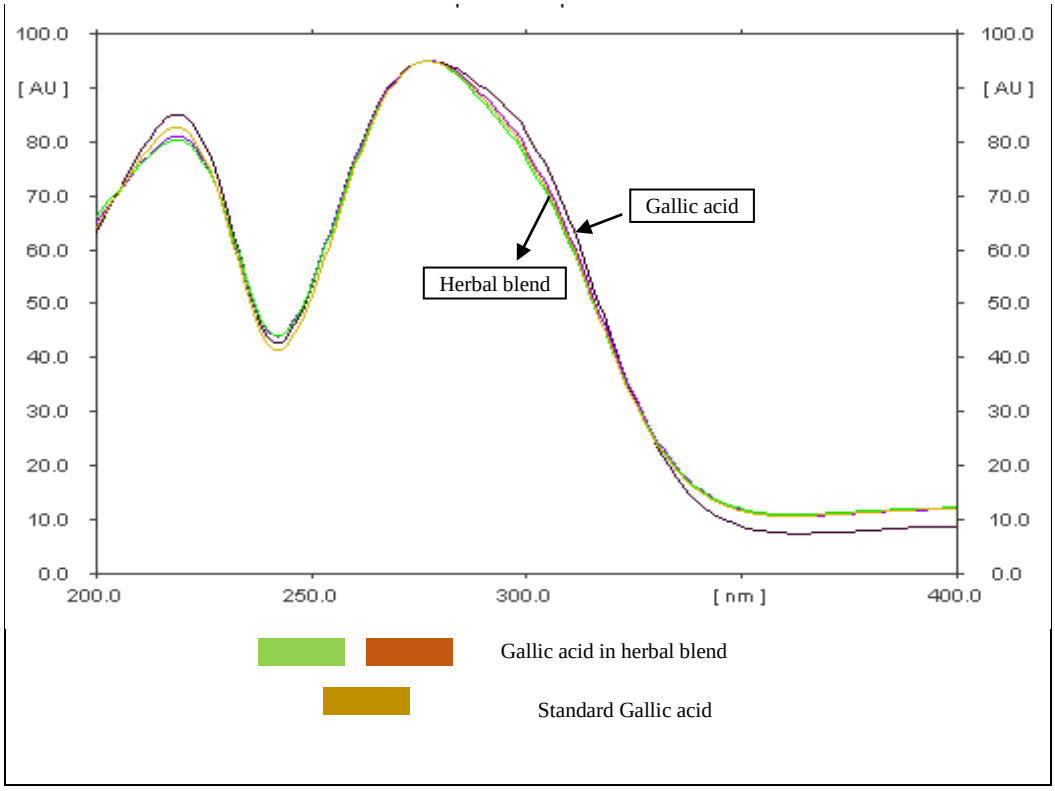


Figure 5. Overlay Spectra of Gallic acid standard and Gallic acid in Herbal blend

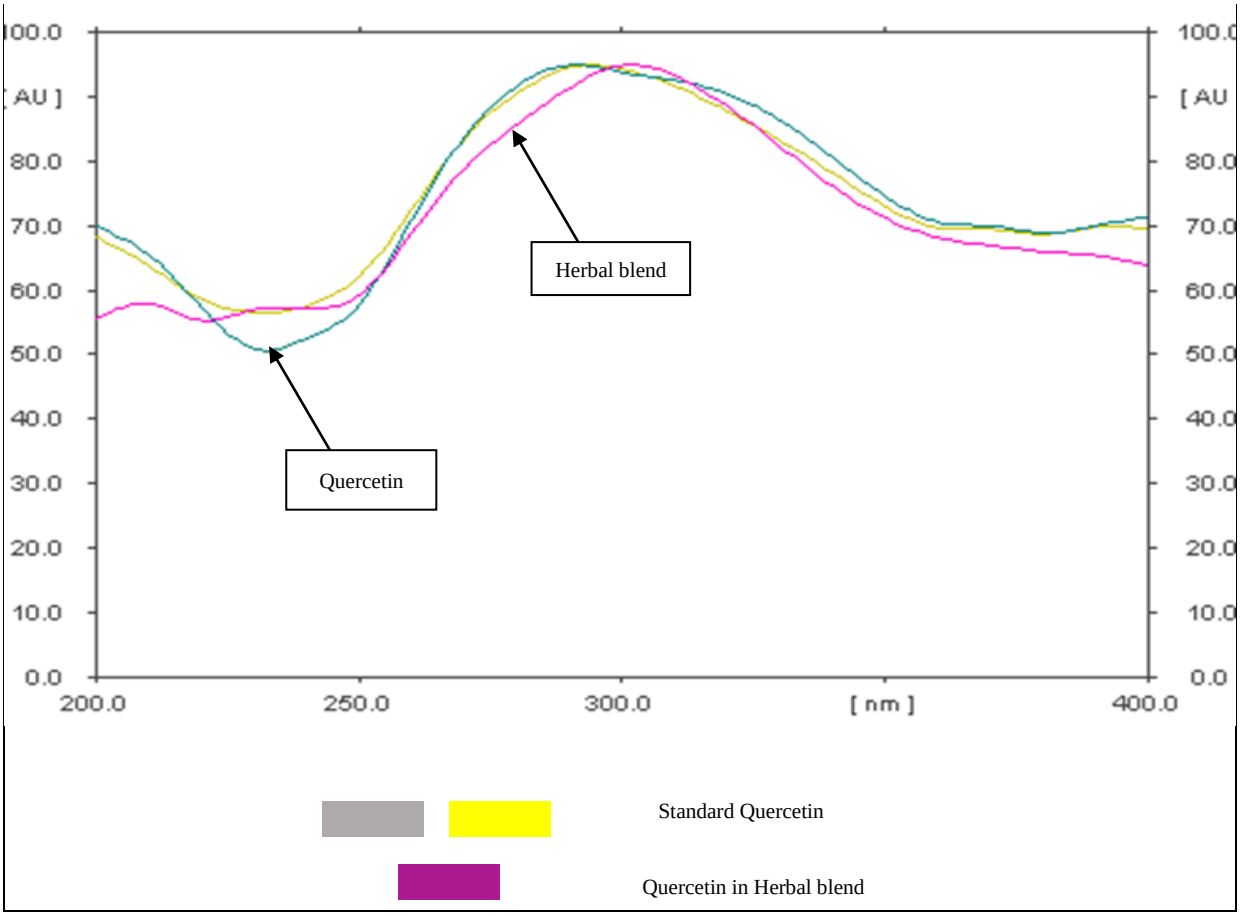


Figure 6. Overlay Spectra of Quercetin standard and Quercetin in Herbal blend

The linearity of calibration curve in pure solution, over the concentration range of 2-6 µl (1 mg/ml) through proposed HPTLC method was carried out and regression co-efficient was obtained 0.9939 (Figure 7) & 0.9988 (Figure 8) for Gallic acid and Quercetin respectively.

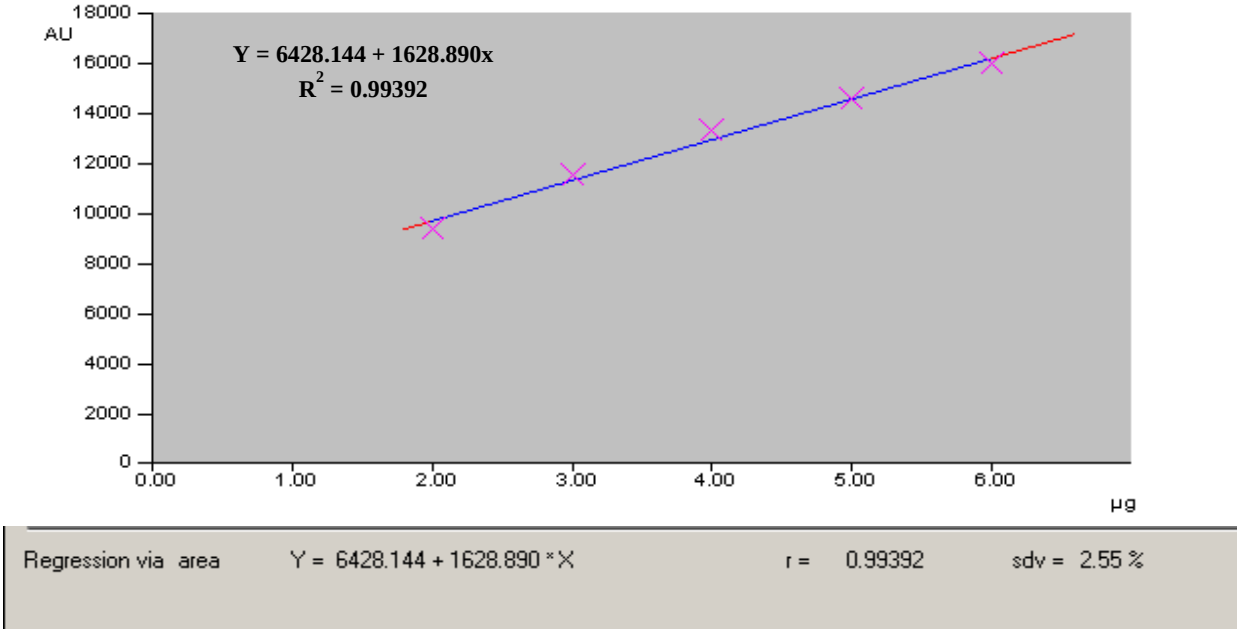


Figure 7. Calibration Curve of Gallic acid (R^2 = 0.9939)

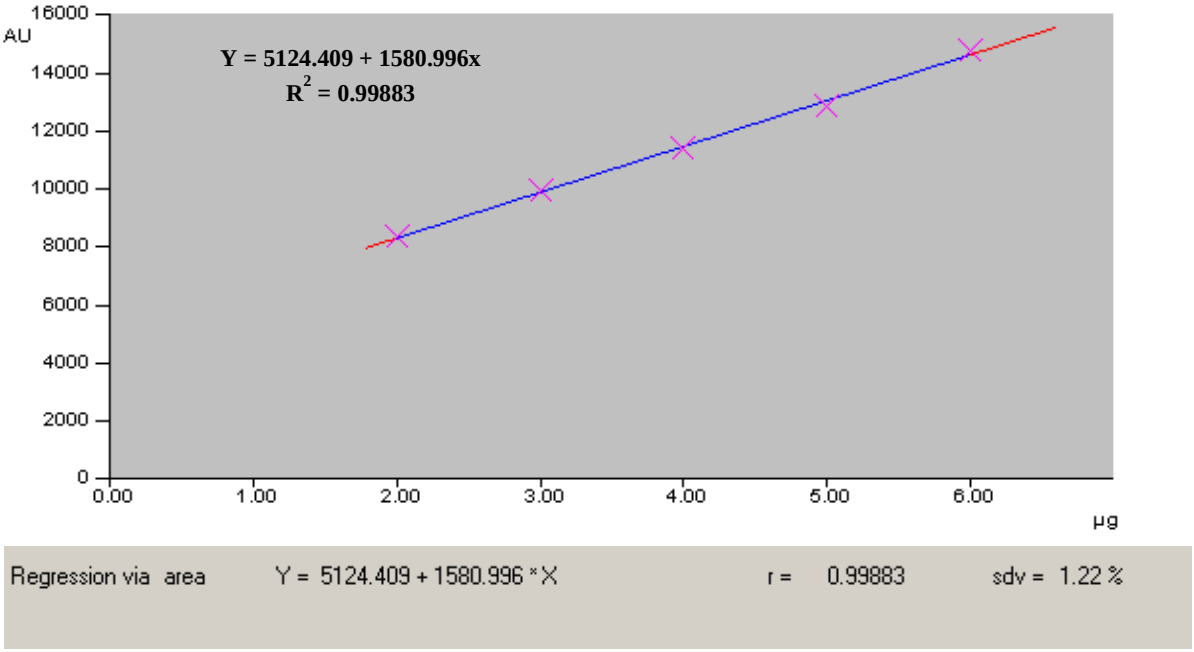


Figure 8. Calibration Curve of Quercetin (0.9988)

3D densitogram of linearity of standard gallic acid and quercetin is shown in Figure 9.

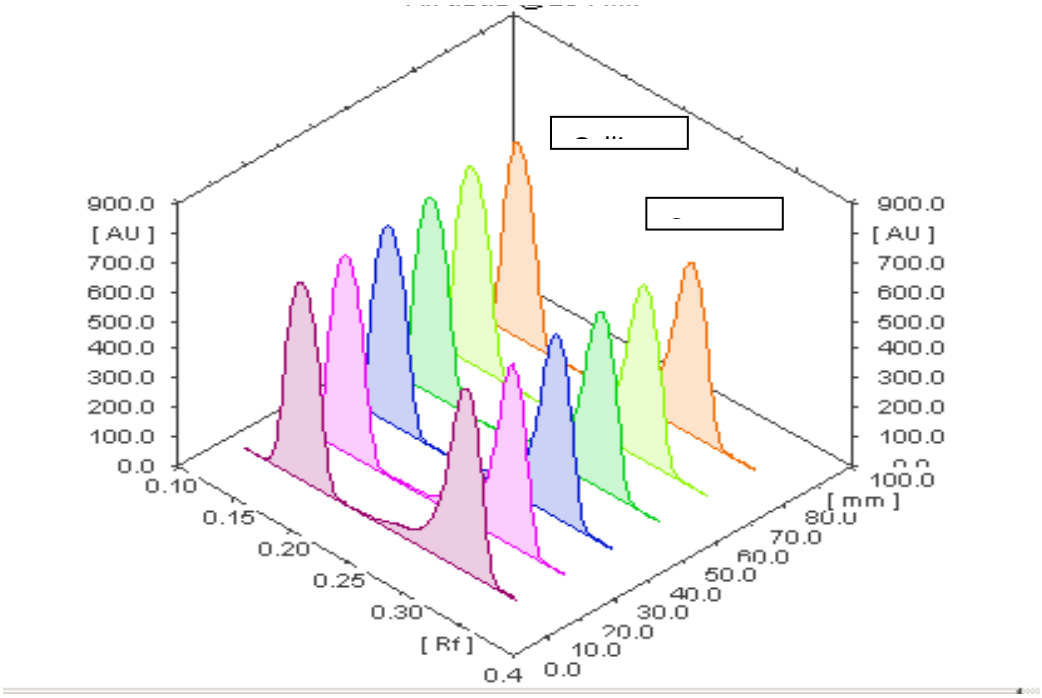


Figure 9. 3D densitogram of linearity of gallic acid and quercetin

System precision and method precision was carried out using standard Gallic acid, Quercetin and Herbal blend and % relative standard deviation was calculated. The repeatability of sample application and measurement of the peak area was expressed in terms of % RSD. The % RSD found was in the acceptable limit that is less than 2.0 which indicates that the method has an acceptable level of precision. Data is shown in Table 2 and 3 for system precision and method precision.

Table 2

System precision

Sr. no.	Peak area (AU)	
	Gallic acid	Quercetin
1	15740.21	16010.02
2	15589.45	15680.20
3	15837.19	15848.01
4	15674.14	15828.97
5	15904.31	15986.17
6	15447.08	15749.17
SD	166.8	129.3
% RSD	1.06	0.82

Table 3

Method precision

Sr. no.	Peak Area of Herbal blend (30 µl)	
	Gallic acid	Quercetin
1	9009.75	14126.60
2	9021.30	14128.14
3	9029.00	14120.39
4	9015.10	14126.02
5	9020.12	14122.22
6	9014.62	14122.90
SD	6.69	2.98
% RSD	0.074	0.02

The accuracy levels were checked at three levels of 80 %, 100 % and 120 % using standard addition method by over spotting herbal extract with standard. The amount of the standard recovered were within acceptable limits as per ICH guidelines. The percent recovery was found to be 98.52–101.04 % for gallic acid and 98.92–101.04 % for quercetin. Data represented in Table 4 and 5 is of recovery obtained for gallic acid and quercetin respectively by standard addition method.

Table 4

Accuracy studies for Gallic acid

Sr. no	Level of recovery	Amount of Herbal blend taken (µg/band)	Amount of Std. Gallic acid Added (µg/band)	Amount of Gallic acid Recovered (µg/band)	% Recovery
1	80 %	1500	3.2	3.153	98.53
		1500	3.2	3.171	99.09
		1500	3.2	3.222	100.68
2	100 %	1500	4.0	3.981	99.52
		1500	4.0	4.038	100.95
		1500	4.0	3.961	99.03
3	120 %	1500	4.8	4.834	100.70
		1500	4.8	4.821	100.44
		1500	4.8	4.85	101.04

Table 5

Accuracy studies for Quercetin

Sr. no	Level of recovery	Amount of Herbal blend taken (µg/band)	Amount of Std Quercetin Added (µg/band)	Amount of Quercetin Recovered (µg/band)	% Recovery
1	80 %	1500	3.2	3.194	99.81
		1500	3.2	3.165	98.92
		1500	3.2	3.182	99.43
2	100 %	1500	4.0	4.031	100.78
		1500	4.0	4.026	100.65
		1500	4.0	4.001	100.25
3	120 %	1500	4.8	4.819	100.39
		1500	4.8	4.826	100.54
		1500	4.8	4.84	101.04

The limits of detection (LOD) were obtained 0.35 & 0.027 µg/ml and limit of quantification (LOQ) 0.106 & 0.082 µg/ml for Gallic acid and Quercetin respectively.

The method was found to be robust and specific.

All the validation parameter results are shown in Table 6

Quantitative estimation have found out that in 1500µg of Herbal blend (Figure 10) 0.065 µg of Gallic acid i.e. 1.648 % and 0.13 µg of Quercetin i.e. 3.165 % was present. Table 7

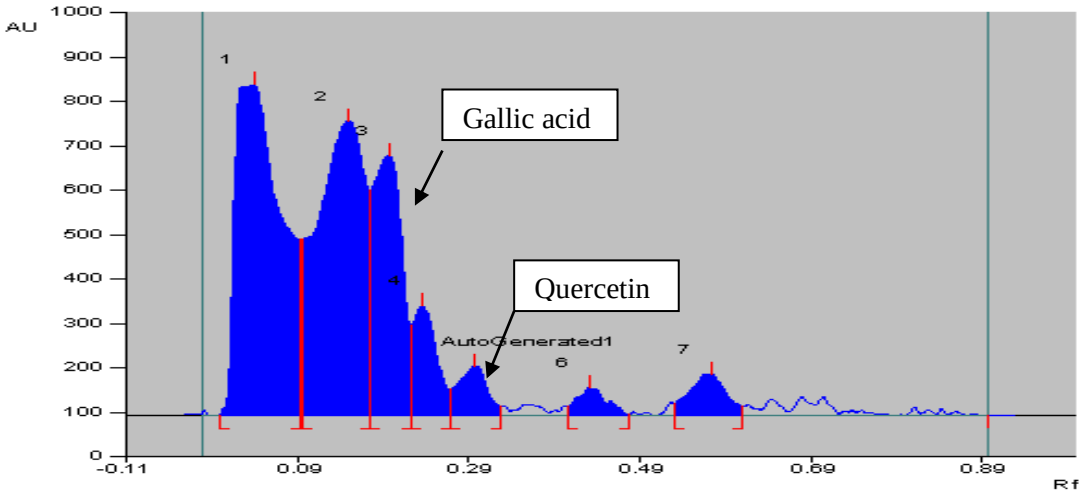


Figure 10. Densitogram of Herbal blend (1500 µg/ml)

Table 6

Validation parameters of gallic acid and quercetin

Parameters	Result	
	Gallic acid	Quercetin
Correlation coefficient	0.993	0.998
Linearity range (ng/band)	2000–6000 ng/band	2000–6000 ng/band
Precision (C.V)		
System Precision	1.06	0.82
Method precision	0.074	0.02
Intra day	0.21	0.14
Inter day	0.45	0.09
Limit of Detection (ng/band)	35	27
Limit of Quantification	106	82
Accuracy (%)	98.52–101.04	98.92–101.04
Specificity	Specific	Specific

Table 7

Quantification of gallic acid and quercetin in Herbal blend

Phytochemical	Stock solution of extract	Concentration of extract spotted on TLC plate	Area	Calculated concentration in extract	% in extract
Gallic acid	(50 mg/ml)	30 µl (1500 µg/band)	13281.05	0.065µg	1.648 %
Quercetin	(50 mg/ml)	30 µl (1500 µg/band)	11402.21	0.13µg	3.165 %

Conclusions

Thus, a rapid, simple, accurate and specific HPTLC method for quantitative estimation of Gallic acid and Quercetin in polyherbal blend comprising of *Emblica officinalis*, *Camellia sinensis* and *Garcinia cambogia* has been developed and validated as per ICH guidelines. The method used in this work resulted in good peak shape with good resolution of Gallic acid and Quercetin from other constituents of the plant mate-

rial. Also it didn't show any interference of any other constituents with Gallic acid and Quercetin proving method specificity. The data could be used as a quality standard method for simultaneous estimation of these phytochemicals in single and polyherbal blend in in-house or marketed formulations. Also the developed method can be used for quantification of gallic acid and quercetin in the herbal mixture. Gallic acid and quercetin are the biomarkers available in most of the herbal plants and have been proved to be an important phytoconstituents responsible for the activity.

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С.П. Ганди, А.Р. Гавейн, С.Д. Капсе, Д. Нагоре, С.С. Читланге

Көпшөпті қоспадағы галл қышқылы мен кверцетинді бір уақытта бағалау және сандық анықтау үшін расталған жоғарыөнімді жұқа валидацияланған хроматография әдісі

Қарапайым, сезімтал, жоғарыөнімді жұқа қабатты хроматография әдісі үйде өндірілген көпшөпті қоспадағы галл қышқылы мен кверцетиннің құрамын бағалау үшін әзірленді және расталды. Талдау үшін *Emblica officinalis*, *Camellia sinensis* және *Garcinia cambogia* қоса, шөп қоспасының метанолды ерітіндісі пайдаланылды. Бөлу 254 нм сканерлеу толқын ұзындығында G60 F_{254} силикагельмен және

толуол : этилацетат : құмырсқа қышқылымен (5:1,5:1 көлем/көлем/көлем) алдын ала қапталған алюминий TLC пластиналарында орындалды. Жүйе сәйкесінше R_f 0,14 және R_f 0,29 шамасында галл қышқылы мен кверцетин үшін жақсы шешілген шыңдарды берді. Әдіс 2–6 мкг/мл диапазонында галл қышқылы үшін 0,9939 және кверцетин үшін 0,9988 регрессия коэффициентін беретін ICH Q2R1 нұсқауларына сәйкес расталды. Галл қышқылы мен кверцетинді алу 98–102 % аралығында, бұл әдістің дәлдігін нақтылайды. Дәлдікті зерттеу (күн аралық және күндізгі) салыстырмалы стандартты ауытқу 2 %-дан аз екенін көрсетті, бұл әдістің жоғары дәлдігін айқындайды. Ұсынылған валидацияланған HPTLC әдісі қарапайым, дәл, нақты және сенімді және күнделікті сапаны бақылау талдауында пайдаланылуы мүмкін. Бұл әдіс көпшөпті қоспадағы галл қышқылы мен кверцетиннің мөлшерін анықтау үшін қолданылды, ол тиісінше 1,648 % масса/масса және 3,165 % масса/масса құрады.

Кілт сөздер: галл қышқылы, кверцетин, синхронды бағалау, жоғарыөнімді жұқа қабат хроматографиясы, валидация, сандық анықтау, көпшөптер қоспасы, шөптер, сығындылар.

С.П. Ганди, А.Р. Гавейн, С.Д. Капсе, Д. Нагоре, С.С. Читланге

Валидированный метод высокоэффективной тонкослойной хроматографии для одновременной оценки содержания галловой кислоты и кверцетина в политравной смеси и их количественная оценка

Был разработан и валидирован простой, чувствительный высокоэффективный метод тонкослойной хроматографии для оценки содержания галловой кислоты и кверцетина в политравной смеси собственного производства. Для анализа использовали метанольный раствор травяной смеси, включающей *Emblica officinalis, Camellia sinensis* и *Garcinia cambogia*. Разделение проводили на алюминиевых пластинах для ТСХ, предварительно покрытых силикагелем G60 F_{254} и смесью толуол : этилацетат: муравьиная кислота (5:1,5:1 об./об./об.) при длине волны сканирования 254 нм. Система дала хорошо разрешенные пики для галловой кислоты и кверцетина при R_f 0,14 и R_f 0,29 соответственно. Метод валидирован в соответствии с рекомендациями ICH Q2R1, что дает коэффициент регрессии 0,9939 для галловой кислоты и 0,9988 для кверцетина в диапазоне 2–6 мкг/мл. Извлечение галловой кислоты и кверцетина находится в пределах 98–102 %, что подтверждает точность метода. Исследование точности (междневной и внутридневной) показало, что относительное стандартное отклонение составляет менее 2 %, что свидетельствует о высокой точности метода. Предлагаемый валидированный метод ВЭТСХ прост, точен, специфичен и надежен, может найти применение в рутинном анализе контроля качества. Данный метод был использован для количественной оценки содержания галловой кислоты и кверцетина в политравной смеси, которое составило 1,648 % масс./масс. и 3,165 % масс./масс. соответственно.

Ключевые слова: галловая кислота, кверцетин, одновременная оценка, высокоэффективная тонкослойная хроматография, валидация, количественная оценка, политравная смесь, травы, экстракты.

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