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Hyphenated chromatographic analysis of bioactive gallic acid and quercetin in *Hygrophila auriculata* (K. Schum) Heine growing wildly in marshy places in India by validated HPTLC method

Md. Sarfaraj Hussain[1*], Sheeba Fareed[1], Mohammad Ali[2]

[1]*Faculty of Pharmacy, Integral University, Dasauli, Kursi Road, Lucknow-226026, Uttar Pradesh, India*
[2]*Department of Pharmacognosy & Phytochemistry, Faculty of Pharmacy, Hamdard University, Hamdard Nagar, New Delhi-110062, India*

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ABSTRACT

Objective: A simple, accurate, and rapid high-performance thin-layer chromatographic (HPTLC) method for simultaneous quantification of the two biologically active flavonoidal compounds, gallic acid and quercetin, in *Hygrophila auriculata* (K. Schum) Heine (HA) has been established and validated. **Methods:** Chromatography was performed on aluminium foil-backed silica gel 60 F254 HPTLC plates with the binary mobile phase toluene: ethyl acetate: formic acid (5:4:1, v/v/v). Ultraviolet detection was performed densitometrically at the maximum absorbance wavelength, 270nm. The method was validated for precision, recovery, robustness, specificity, and detection and quantification limits, in accordance with ICH guidelines. **Results:** The system was found to give compact spots for gallic acid (GA) and quercetin (QE) (Rf value of 0.31 and 0.50, respectively). The limit of detection (23 and 41 ng band^{-1}) limit of quantification (69 and 123 ng band^{-1}), recovery (99.4-99.9 and 98.7-99.4%), and precision (i.e $\leqslant$1.98 and 1.97) were satisfactory for gallic acid and quercetin respectively. Linearity range for GA and QE were 100-1000 (r^2= 0.9991) and 150-900 ng band^{-1} (r^2= 0.9956) and the contents estimated as 0.28±0.01% and 0.41±0.01% w/w respectively. **Conclusions:** This simple, precise and accurate method gave good resolution from other constituents present in the extract. The method has been successfully applied in the analysis and routine quality control of herbal material and formulations containing *Hygrophila auriculata* (K. Schum) Heine.

1. Introduction

Nowadays, HPTLC has become a routine analytical technique due to its advantages of reliability in quantitation of analytes at micro and even in nanogram levels and cost effectiveness[1]. It has proved a very useful technique because of its low operating cost, high sample throughput and need for minimum sample clean-up. The major advantage of HPTLC is in reducing analysis time and cost per analysis. Unlike HPLC, for which substantial amounts of mobile phase and long analysis times are required for quantification of multiple samples, HPTLC has the advantage that several samples can be estimated simultaneously using a small quantity of mobile phase. HPTLC also has the advantage of providing visualization of the separated constituents of the sample. It also provides on line identification of the analyte by in-situ spectrum scanning and post chromatographic derivatization, along with Rf comparison with the standard. It requires very little sample clean up since the layer is disposable. Several samples can be run simultaneously using a small quantity of mobile phase, thus reducing the time and cost per analysis. Due to low consumption of solvent the methodology is eco friendly. Another advantage of HPTLC is that several samples can be analyzed simultaneously using a small quantity of mobile phase unlike HPLC. This reduces the time and cost of analysis and possibilities of pollution of the environment. HPTLC also facilitates repeated detection (scanning) of the chromatogram with same or different parameters. Simultaneous assay of several components in a multicomponent formulation is also possible. Due to

*Corresponding author: Md. Sarfaraj Hussain, Faculty of Pharmacy, Integral University, Lucknow (Uttar Pradesh) India. 226026;
E-mail: sarfarajpharma@gmail.com
Mobile no: +91-8604415498, Fax No.: 0522-2890809

several advantages, such as the rapidity, the fewer amounts of sample, and an extremely limited solvent waste, HPTLC has gained widespread interest as a favorable technique for the determination of pharmacologically interesting compounds in biological matrices, such as plants, leaves, and flowers and herbal formulations[2-7]. A number of high performance liquid chromatography (HPLC) which need sample cleanup to remove the interfering constituents in the plant extracts, making the procedure more tedious and unsuitable for screening large number of samples. Recently, high performance thin layer chromatography (HPTLC) has been widely employed for the quantification of secondary metabolites[8-13].

Hygrophila auriculata (K. Schum) Heine (HA), a generally occurring wild herb belonging to Acanthaceae family has been advocated for the treatment of variety of diseases including most commonly diabetes and dysentery[14-16]. As per our tradition, roots, seeds, and aerial parts of the plant has been used in the treatment of jaundice, hepatic obstruction, rheumatism, inflammation, urinary infection, gout, malaria and impotence[17]. The plant has been reported to contain flavonoids (apigenin 7-O- glucuronide, apigenin 7-O-glucoside)[18], alkaloids (asteracanthine and asteracanthicine)[19], aliphatic esters (25- oxo - hentricontyl acetate, methyl -8- hexyltetracosanoate)[20], minerals (Fe, Cu, Co)[21], sterols (stimagsterol)[22], triterpenes (lupeol, hentricotane, betulin, luteolin, luteolin -7-O- rutinosides)[20, 23] and essential oils[19]. Earlier scientific investigation showed that the crude extract of HA has anti-nociceptive[24], antitumor[25, 26], antibacterial[27, 28], antioxidant[29, 30], hepatoprotective[31, 32, 33], hypoglycemic[34], haematinic[35], diuretic[36] anabolic and androgenic activities[37]. Flavonoids are the most commonly found phytochemicals, and typically these chemicals help protect the plant against UV light, fungal parasites, herbivores, pathogens and oxidative cell injury[38]. When consumed regularly by humans, flavonoids have been linked to a reduction in the incidence of diseases such as cancer and heart disease[39-41]. There is currently great interest in flavonoid research due to the possibility of improved public health through diet, where preventative health care can be promoted through the consumption of fruit and vegetables. A little information is only available regarding analytical methods for the qualitative and/or quantitative estimation of gallic acid 3,4,5-trihydroxybenzoic acid quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) (Figure 1A &, 1B). Quercetin occurs naturally in plants as conjugated glycosides, with the most common glycosides being quercetin 3,4-diglucoside, quercetin 4-monoglucoside and quercetin 3-monoglucoside. Furthermore, studies have shown that different flavonoid glycosides are preferentially absorbed in the small intestine through various uptake mechanisms, suggesting that certain glycosides may be more bioeffective[42]. Studies have shown that quercetin, which is the major flavonol present in onions, capers, apples, tea and berries[43], exhibits anti-cancer, anti-inflammatory, antiviral activity, and may also prevent cardiovascular disease in humans[44-46].

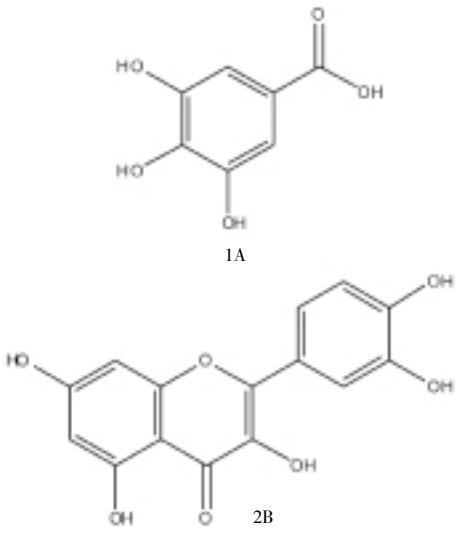

Figure 1. A & B. Chemical structure of gallic acid and quercetin

However, pertaining to our knowledge there is no any hyphenated HPTLC technique available anywhere else for simultaneous estimation of gallic acid and quercetin in HA extract. So, the attempt has been made to accept this challenge towards development and validation of gallic acid and quercetin simultaneously by such a hyphenated technology like HPTLC for the betterment of herbal quality standards.

2. Materials and methods

2.1 Plant material and chemicals.

Hygrophila auriculata (K. Schum) Heine fresh plant were collected from the field area of Saharsa, Bihar, India in the month of January 2009; and the specimens (voucher no: SHC 55/01/2009) were authenticated by Dr. Anjani kumar Sinha (taxonomist), Department of Botany MLT Saharsa College, Bihar. Standard gallic acid (Purity: 99% w/w) and quercetin (purity: 98% w/w) were purchased from Natural Remedies Pvt. Ltd, Bangalore, India. All the solvents used were of chromatography grade and other chemicals used were of analytical reagent (AR) grade. Precoated silica gel 60 F254 HPTLC plates were purchased from *E. Merck*, Germany.

2.2 Preparation of standard and quality control (QC) samples

Stock solutions of gallic acid and quercetin (10 mg mL^{-1}) were prepared in methanol, and by appropriate dilution standard solutions were prepared in the concentration range of 0.1 to 1.0 mg mL^{-1}. For calibration, GA standard solution (1-10 μL) was applied to a HPTLC plate to furnish amounts in the range 100-1000 ng $band^{-1}$; however QE standard solution (0.5-5 μL) was applied to furnish amounts in the range 150-900 ng $band^{-1}$. Peak area and amounts applied were treated by linear least-squares regression. Each amount was applied six times. QC samples as low, medium and high at concentration level of 150, 300 and 600 ng $band^{-1}$ were taken for GA and 200, 400 and 800 were considered for QE to carry out validation of the method.

2.3 Chromatography

Chromatography was performed, as described previously

(2-6) on 20 cm×10 cm aluminum Lichrosphere HPTLC plates precoated with 200-μm layers of silica gel 60F254 (E. Merck, Darmstadt, Germany). Samples were applied as bands 6 mm wide and 10 mm apart by means of Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100-μL syringe. The constant application rate was 160 nL s^{-1}. Linear ascending development with toluene: ethyl acetate: formic acid (5:4:1, v/v/v) as mobile phase was performed in a 20 cm×10 cm twin-trough glass chamber (Camag) previously saturated with mobile phase for 15 min at room temperature (25 ±2℃) and relative humidity 60±5%. The development distance was 8 cm (development time 10 min) and 20 mL mobile phase was used. The plates were dried at room temperature in air and warmed (at 75℃for 5 min) to identify compact bands. Densitometric analysis was performed at 270 nm in reflectance mode with a Camag TLC scanner III operated by WinCATS software (Version 1.2.0). The slit dimensions were 5 mm×0.45 mm and the scanning speed of 20 mm s^{-1}.

2.4 HPTLC fingerprinting and image analysis

The plants were air-dried and pulverized. 500 g of the powdered material were packed in muslin cloth and subjected to soxhlet extractor for continuous hot extraction with methanol for 72 hrs. Thereafter methanolic extracts of HA were filtered through Whatman paper no. 42 and the resultant filtrates were concentrated under reduced pressure and finally vacuum dried. The yield of the methanolic extract was 13.2 % w/w. The protocol for preparing sample solutions was optimized for high quality fingerprinting and also to extract the marker compounds efficiently. Since the marker compounds were soluble in methanol, therefore methanol was used for extraction. The fingerprinting of HA extracts were executed by spotting 10 μL of suitably diluted sample solution of the methanolic extract on a HPTLC plate. Each amount was applied six times. Peak area and amounts applied were treated by linear least-squares regression. The plates were developed and scanned as same discussed above. The peak areas were recorded and the amount of GA and QE was calculated using the calibration curve.

2.5 Method validation

Validation of the developed method has been carried out as per ICH guidelines for linearity, range, precision, accuracy, limits of detection (LOD) and quantification (LOQ), and recovery.

2.5.1 Precision and accuracy

Precision (inter and intraday) and accuracy of the assay were evaluated by performing replicate analyses (n=6) of QC samples at low, medium and high QC levels of 150, 300 and 600 ng $band^{-1}$ for gallic acid and 200, 400 and 800 ng $band^{-1}$ for quercetin, respectively. Inter-day precision and accuracy were determined by repeating the intra-day assay on three different days. Precision was expressed as the coefficient of variation (CV, %) of measured concentrations for each calibration level whereas accuracy was expressed as percentage recovery [(Drug found/drug applied)×100].

2.5.2 Robustness

Robustness was studied in triplicate at 400 ng $band^{-1}$ by making small changes to mobile phase composition, mobile phase volume, and duration of mobile phase saturation and activation of TLC plates, the effect on the results were examined by calculation of RSD (%) and SE of peak areas. Mobile phases prepared from toluene: ethyl acetate: formic acid (5:4:1, v/v/v) in different proportions (5.5:3.5:1, v/v/v, 5:4.5:0.5, v/v/v, 5.5:4:0.5, v/v/v and 6:3:1, v/v/v) were used for chromatography. Mobile phase volume and duration of saturation investigated were 20 ±2 mL (18, 20, and 22 mL) and 20±10 min (10, 20, and 30 min), respectively. The plates were activated at 60±5℃for 2, 5, and 7 min before chromatography.

2.5.3 Sensitivity

To estimate the limits of detection (LOD) and quantification (LOQ), blank methanol was applied six times and the standard deviation (σ) of the analytical response was determined. The LOD was expressed as 3 σ /slope of the calibration plot and LOQ was expressed as 10 σ /slope of the calibration plot.

2.5.4 Recovery studies

Recovery was studied by applying the method to drug samples to which known amounts of marker corresponding to 50, 100, and 150% of the GA and QE had been added. Each level was analyzed in triplicates. This was to check the recovery of GA and QE at different levels in the extracts. Recovery of the markers at different levels in the samples was determined.

3. Results

3.1 Chromatography

Chromatogram was developed for both gallic acid and quercetin under chamber saturation conditions using toluene: ethyl acetate: formic acid (5:4:1, v/v/v) as mobile phase or solvent system (Figure 2 & 3). The same mobile phase has been also employed for the separation of HA methanolic extracts (Figure 4). The optimized saturation time was found to be 10 min. UV spectra measured for the spots showed maximum absorbance at about 270 nm therefore Densitometric analysis was performed at 270 nm in the reflectance mode as HPTLC. Compact bands as sharp, symmetrical and with high resolution were obtained at RF 0.31±0.02 and 0.50±0.04 for gallic acid and quercetin respectively (Figure 5).

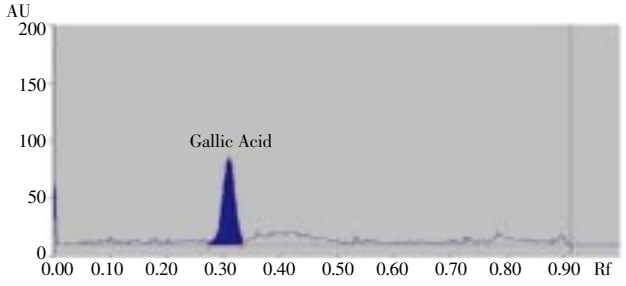

Figure 2. HPTLC chromatogram of standard gallic acid at RF 0.31

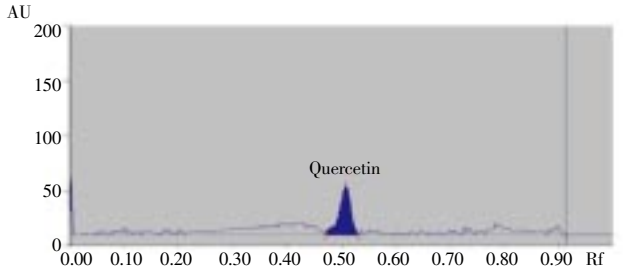

Figure 3. HPTLC chromatogram of standard Quercetin at RF 0.50

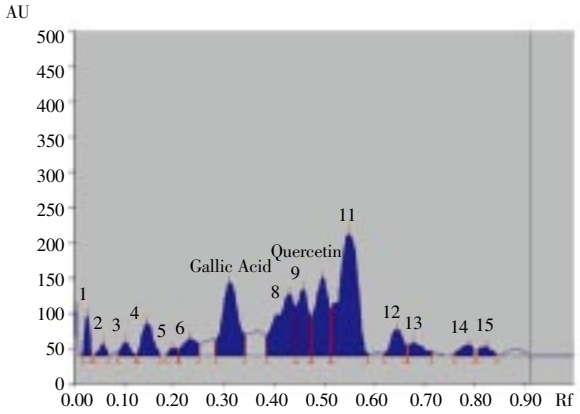

Figure 4. HPTLC chromatogram of methanolic extract of HA scanned at 270 nm [peak 1–15; GA (0.31) and QE (0.50)]

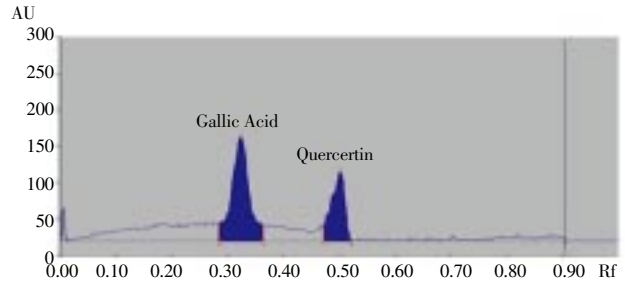

Figure 5. Chromatogram of GA and QE simultaneously determined in by using toluene–ethyl acetate–formic acid (5:4:1, v/v/v) as solvent system scanned at 270 nm

As far as we are aware, there is no any HPTLC method reported to quantify GA and QE simultaneously in HA herb or extracts. Therefore we have attempted to develop and validate a cost effective simple and sober hyphenated HPTLC technique to quantify bioactive marker components in this herb. Gallic acid and quercetin were well resolved at RF 0.31 and 0.50 respectively (Figure 2 & 3) from HA methanolic extract sample in the solvent system as same used in case of standards. The plates were visualized at two different wavelengths 254 and 270 nm as the compounds were found to absorb at variable spectrum range. In addition, this helped in the generating a better fingerprint data whereby species could be well differentiated on enhanced visual identification of individual compounds. The method developed here was found to be quite selective with good baseline resolution of each compound. The identity of the bands of compounds 1–15 in the sample extracts was confirmed by overlaying their UV absorption spectra with those of the standards at 270 nm (Table 1).

Table 1
TLC fingerprints of HA extracts at 270 nm

Plants	Solvent system	Rf value
Hygrophila auriculata	Toulene: ethyl acetate: formic acid (5:4:1)	0.03, 0.06, 0.10, 0.14, 0.20, 0.23, 0.31(Gallic acid), 0.43, 0.46, 0.50(Quercetin), 0.56, 0.65, 0.68, 0.79, 0.82

3.2 Calibration

Linearity of compounds (gallic acid and quercetin) was validated by the linear regression equation and correlation coefficient. The six-point calibration curves for gallic acid and quercetin were found to be linear in the range of 100–1000 ng band^{-1} and 150–900 ng band^{-1}. Regression equation and correlation coefficient for the reference compound were: Y=0.0048X+0.012 (r^2=0.9991) for gallic acid and Y=0.033–0.017for quercetin (r^2=0.9941), which revealed a good linearity response for developed method and are presented in Table 2. The mean values (±sd) of the slope were 0.0048±0.0003 and 0.033±0.008 and intercept were 0.012±0.007 and 0.017±0.002 respectively for gallic acid and quercetin. No significant difference was observed in the slopes of standard plots (ANOVA, $P > 0.05$).

Table 2
RF, linear regression data for the calibration curve and sensitivity parameter for Gallic acid and Quercetin.

Parameter	Gallic acid	Quercetin
RF	0.31	0.50
Linearity range (ng band^{-1})	100–1000	150–900
Regression equation	Y=0.0048X+0.012	Y=0.033–0.017
Correlation coefficient (r2)	0.9991	0.9956
Slope±sd	0.0048±0.0003	0.033±0.008
Intercept±sd	0.012±0.007	0.017±0.002
Standard error of slope	0.00017	0.0046
Standard error of intercept	0.0040	0.0011
LOD	23	41
LOQ	69	123

3.3 Method validation

3.3.1. Precision and accuracy

Table 3 presents intra-day and inter-day precision (as coefficient of variation, %CV) and accuracy of the assay for GA and QE at three QC levels (150, 300 and 600 ng band^{-1}). Intra-day precisions (n = 6) for GA and QE were i.e ≤1.70% and i.e ≤1.89%, however the inter-day precisions were i.e ≤1.98% and i.e ≤1.97% respectively, which demonstrated the good precision of proposed method. Intra-day accuracy gallic acid and quercetin were 98.8–100.0% and 98.7–100.1%, however inter-day accuracy for gallic acid and quercetin were 99.4–99.7% and 98.8–99.8% respectively. These values are within the acceptable range, so the method was accurate, reliable, and reproducible.

3.3.2. Robustness

The SD and % RSD was calculated for GA and QE. The low value of SD and % RSD (□2) obtained after introducing small deliberate changes in the method indicated that the method was robust (Table 4).

3.3.3. Sensitivity

LOD values for GA and QE were 23 and 41 ng band^{-1} respectively; however LOQ values were 69 and 123 ng band^{-1} respectively (Table 2), indicating adequate assay sensitivity. The LOD and LOQ were determined from the slope of the lowest part of the calibration plot. This indicated that the proposed

method exhibits a good sensitivity for the quantification of above compounds.

3.3.4. Recovery studies

Good recoveries were obtained by the fortification of the sample at three QC levels for GA and QE. It is evident from the results that the percent recoveries for both markers after sample processing and applying were in the range of 99.4–99.9% (gallic acid) and 98.7–99.4% (quercetin) for as shown in Table 5.

3.3.5. HPTLC analysis of bioactive gallic acid and quercetin in HA extract

The content of gallic acid and quercetin was estimated in the HA methanolic extract by the proposed method and the results obtained are summarized in Table 6. The percentage of gallic acid and quercetin obtained in the extract were 0.28 and 0.41 respectively with RSD. It is for the first time, a simple, accurate and rapid HPTLC method has been developed for the

Table 3
Precision and accuracy of the method

Gallic caid				Quercetin			
Nominal concentration[a]	Obtained[a,b]	Precision[c]	Accuracy[d]	Nominal concentration[a]	Obtained[a,b]	Precision[c]	Accuracy[d]
Intraday batch							
150	148.3	1.70	98.8	200	197.5	1.73	98.7
300	299.1	1.65	99.7	400	398.6	1.89	99.6
600	600.4	1.55	100.0	800	801.3	1.37	100.1
Interday batch							
150	149.2	1.98	99.4	200	197.7	1.94	98.8
300	299.8	1.76	99.9	400	396.9	1.97	99.2
600	598.3	1.70	99.7	800	798.8	1.95	99.8

aConcentration in ng band^{-1}
bMean from six determinations (n=6)
cPrecision as coefficient of variation (CV, %) = [(standard deviation)/(concentration found)]×100
dAccuracy (%) = [concentration found)/(nominal concentration)]×100

Table 4
Robustness of the method

Optimisation condition	Gallic acid		Quercetin	
	SD	%RSD	SD	%RSD
Mobile phase				
(Toulene: ethyl acetate:formic acid; 5.5:3.5:1, v/v/v, 5:4.5:0.5, v/v/v, 5.5:4:0.5, v/v/v and 6:3:1, v/v/v)	1.79	1.82	1.91	1.65
Mobile-phase volume (18, 20, and 22 mL)	1.25	1.77	1.62	0.89
Duration of saturation (10, 20, and 30 min)	1.98	1.63	1.09	1.01
Activation of TLC plates (2, 5, and 7 min)	1.89	1.78	1.53	1.22

Table 5
Recovery studies of Gallic acid and Quercetin

Concentration added to analyte (%)	Theoretical (ng)	Added (ng)	Detected (ng)	Recovery (%)	RSD (%)
		Gallic acid			
50					
300	200	497.3	99.4	1.92	
100		400	698.6	99.8	1.51
150		600	899.1	99.9	1.49
		Quercetin			
50					
100	100	197.5	98.7	1.74	
100		200	298.2	99.4	1.92
150		300	397.4	99.3	1.27

Table 6
Gallic acid and Quercetin contents estimated in HA extracts by developed method

Hygrophilla auriculata (HA)	Gallic acid*		Quercetin*	
	Content (ng spot−1)	%RSD	Content (ng spot−1)	%RSD
	28.0	1.11	41.0	1.08

*Volume applied in each replicate was ten microlitres

simultaneous quantification of two bioactive compounds in HA.

4. Discussion

HPTLC is useful as a phytochemical marker and also a good estimator of genetic variability in plant populations. The presence or absence of chemical constituent has been found useful in the placement of the plant in taxonomic categories. HPTLC profile differentiation is such an important and powerful procedure which has often been employed for this purpose. HPTLC fingerprinting is proved to be a liner, precise, accurate method for herbal identification and can be used further in authentication and characterization of the medicinally important plant[47]. The HPTLC method can be used for phytochemical profiling of plants and quantification of compounds present in plants, with increasing demand for herbal products as medicines and cosmetics there is an urgent need for standardization of plant products. Chromatographic fingerprint is a rational option to meet the need for more effective and powerful quality assessment to traditional system of medicine throughout the world[48]. The optimized chromatographic finger print is not only an alternative analytical tool for authentication, but also an approach to express the various patterns of chemical ingredients distributed in the herbal drugs and to preserve such "database" for further multifaceal sustainable studies. HPTLC finger print analysis has become the most of its simplicity and reliability. It can serve as a tool for identification, authentication, qualitative, quantitative analysis and quality control of herbal drug[49-50].

The presented study clearly gave evidence of the simultaneous bioactive quantitative of gallic acid and quercetin in HA extracts. The developed hyphenated HPTLC method for the simultaneous quantification of above marker compounds is simple, precise, specific, sensitive, and accurate. Further, this method can be effectively used for routine quality control of herbal materials as well as formulations containing any or both of these compounds.

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