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Validation of the method for the simultaneous estimation of bioactive marker gallic acid and quercetin in *Abutilon indicum* by HPTLC

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ABSTRACT

Objective: To establish and validate an simultaneous estimation of the two biomarker compounds gallic acid (GA) and quercetin (QE) from methanolic extract of *Abutilon indicum* (AI). **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 GA and 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 ($\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 0.69% ± 0.01% and 0.57% ± 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 AI.

1. Introduction

Herbal medicines have stood the test of time for their safety, efficacy, cultural acceptability and lesser side effects. They are believed to have better compatibility with the human body. Some of the herbal plants traditionally used in formulations as antidiabetic, antioxidant[1]. Flavonoids are a group of polyphenolic compounds, which are widely distributed throughout the plant kingdom. To date about 300 varieties of flavonoids are known[2]. Many have low toxicity in mammals and some of them are widely used in medicine for maintenance of capillary integrity[3,4]. When consumed regularly by humans, flavonoids have been linked to a reduction in the incidence of diseases such as cancer and heart disease[5-10]. 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 possess astringent activity and quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one). 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

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glycosides may be more bioeffective[11]. Studies have shown that quercetin, which is the major flavonol present in onions, capers, apples, tea and berries[12], exhibits exhibit anti-inflammatory, antihepatotoxic[13], antiulcer[14], antiallergic and antiviral actions and some of them provides protection against cardiovascular mortality[15,16]. Both possess antioxidant activity and antidiabetic reduce low densitylipoproteins oxidation[17]. Quercetin in combination with other flavonoids, inhibits a number of enzymes like bradykinin[18], tyrosine kinase[19], and 5′- nucleotidase activity[20-23]. Quercetins have shown regulatory activity of hormones, such as transport, metabolism and action of thyroid hormones.

Nowadays HPTLC is becoming a routine analytical technique due to its advantages of low operating cost; high sample throughput and need for minimum sample clean up. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis[24-28]. HPTLC chromatogram pattern comparison seems to be promising for fingerprinting the active compounds in plant extracts. TLC and HPTLC are methods commonly applied for the identification, the assay and the testing for purity, stability, dissolution or content uniformity of raw materials (herbal and animal extracts, fermentation mixtures, drugs and excipients) and formulated products (pharmaceuticals, cosmetics, nutriments)[29]. These flexible and cost-effective techniques present the advantage of the simultaneous processing of standards and samples with versatile detection possibilities, including a great variety of post-chromatographic derivatization reagents. Due to 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. 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[30-36].

Abutilon indicum (Linn) Sweet of family Malvaceae is known as, Atibal in Sanskrit and "Thuthi" in Tamil and Kanghi in Hindi is found throughout tropical and sub tropical region in India, particularly in Indo-China where it is claimed to be of therapeutic use as a febrifuge, anthelmintic, antiemetic, anti-inflammatory, and in urinary and uterine discharges, piles, and lumbago[37-41]. The juice from its leaves has been used to formulate into an ointment for quick ulcer healing. Its extract is also used in relieving thirst; in treating bronchitis, diarrhea, gonorrhea, and inflammation of the bladder; and in reducing fever. In addition, it is used in cleaning wounds and ulcers; treating vaginal infections, diabetes, and hemorrhoids; and can also be used as an enema. The leaves are effective in ulcer, for the treatment of diabetes, diuretic infection and gingivitis. Fomentation of plant materials are used to relief body pain. The decoction of the leaf is used in toothache, tender gums and internally for inflammation of bladder. In some places, juice from the leaves in combination with the liquid extract of Allium cepa is used to treat jaundice, and in cases of hepatic disorders[42, 43]. The leaves and seeds are crushed with water to form paste which is applied to penis to cure syphilis[44-46]. In Siddha system of medicine, it used as a remedy for jaundice, piles, ulcer and leprosy[47]. The plant was reported to contain many active and inactive compounds[48,49]. Previous phytochemical investigations showed it to contain saponins, flavonoids, alkaloids, and essential oils[50]. In another investigation gallic acid, β-sitosterol, β-amyrin, eudesmol, eugenol, geraniol, and caryophylline were reported[51,49]. Subramanian and Nair[41] and Sharma and Ahmad[51] reported separately the isolation of gossyptin-7-glucoside, cyanidin-3-rutinoside, and two sesquiterpene lactones named alantolactone and isoalantolactone, in addition to gossypetin-8-glucoside[52].

However, pertaining to our knowledge there is no any hyphenated HPTLC technique available anywhere else for simultaneous estimation of GA and QE in methanolic extract of *Abutilon indicum*. So, the attempt has been made to accept this challenge towards development and validation of GA and QE simultaneously by such a hyphenated technology like HPTLC-UV for the betterment of herbal quality standards.

2. Materials and methods

2.1. Plant material and chemicals

The fresh plant of *Abutilon indicum*(Linn) Sweet were collected from the field area of Saharsa (Bihar), India in the month of April 2009; and the specimens (voucher no: SHC 57/05/2009) were authenticated by Dr. Anjani kumar Sinha (taxonomist), Department of Botany MLT Saharsa College, Bihar. Standard gallic acid (Purity: 98.7% 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 TLC instrumentation and conditions

Chromatography was performed, as described previously[24-28] 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}.

2.3 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.

2.4. Extraction of plant material for analysis

The whole plant *Abutilon indicum* (Linn) Sweet 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 AI 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 17.3% 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 methanolic extracts of *Abutilon indicum* 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 gallic acid and quercetin 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 Selection and optimization of mobile phase (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 LOD and 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 AI 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).

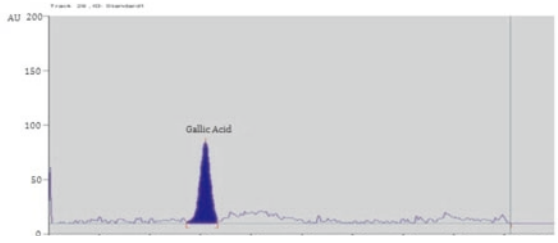

Figure 2. HPTLC chromatogram of standard gallic acid at RF 0.31

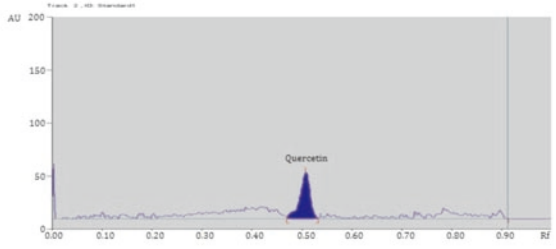

Figure 3. HPTLC chromatogram of standard Quercetin at RF 0.50.

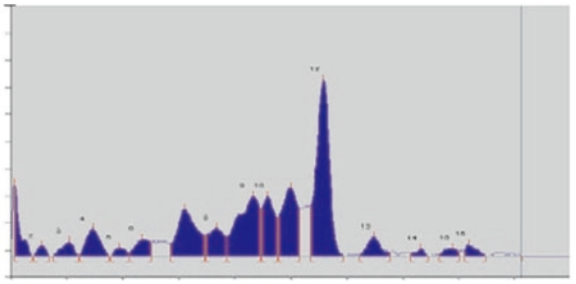

Figure 4. HPTLC chromatogram of methanolic extract of *Abutilon indicum* scanned at 270 nm [peak 1–16; GA (0.31) and QE (0.50)]

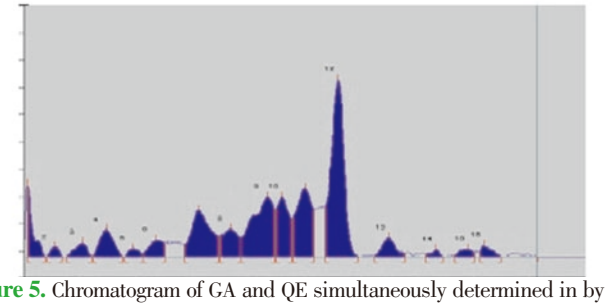

Figure 5. Chromatogram of GA and QE simultaneously determined in by using toluene–ethyl acetate–formic acid (5:4:1, v/v/v) as solvent system scanned at 270 nm [GA (0.31) and QE (0.50)].

As far as we are aware, there is no any HPTLC method reported to quantify GA and QE simultaneously in AI 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 from AI 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 1–16 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 methanolic extract of *Abutilon indicum* at 270 nm.

Plants	Solvent system	Rf value
Abutilon indicum	Toulene: ethyl acetate: formic acid (5:4:1)	0.01, 0.06, 0.10, 0.15, 0.19, 0.23, 0.31, 0.37, 0.43, 0.46, 0.50, 0.56, 0.65, 0.73, 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 (r2=0.9991) for gallic acid and Y=0.033–0.017for quercetin (r2=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 GA and QE

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 ≤1.70% and ≤1.89%, however the inter-day precisions were ≤1.98% and ≤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 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-UV270 nm analysis of bioactive GA and QE in methanolic extract of AI

The content of gallic acid and quercetin was estimated in

Table 3
Precision and accuracy of the method.

Gallic caid				Quercetin			
Nominal centrationa	Obtaineda,b	Precisionc	Accuracyd	Nominal concentrationa	Obtaineda,b	Precisionc	Accuracyd
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	1.79	1.82	1.91	1.65
(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)	1.25	1.77	1.62	0.89
Mobile-phase volume (18, 20, and 22 mL)	1.98	1.63	1.09	1.01
Duration of saturation (10, 20, and 30 min)	1.89	1.78	1.53	1.22
Activation of TLC plates (2, 5, and 7 min)				

Table 5
Recovery studies of GA and QE

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
GA and QE contents estimated in methanolic extract of *Abutilon indicum* roxb by developed method

	Quercetin *		Rutin *	
Abutilon indicum (AI)	Content (ng spot−1)	%RSD	Content (ng spot−1)	%RSD
	69.0	1.08	57.0	1.14

the AI 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 0.69 and 0.57 respectively with RSD. It is for the first time, a simple, accurate and rapid HPTLC method has been developed for the simultaneous quantification of two bioactive compounds in AI.

4. Discussion

A validated HPTLC method has been developed for the simultaneous determination of gallic acid and quercetin in *Abutilon indicum* collected from field area of Koshi river area of Saharsa (Bihar) region of India. The proposed method is simple, precise, specific, accurate, less time consuming and cost effective. Statistical analysis proved that the method is evitable for the analysis of gallic acid and quercetin. The developed HPTLC method will help the manufacturer for quality control and standardization of herbal formulations. Such finger printing is useful in differentiating the species from the adulterant and act as a biochemical marker for this medicinally important plant in the pharmaceutical industry [53, 54].

The presented study clearly gave evidence of the simultaneous bioactive quantitative of GA and QE in methanolic extracts of AI. 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.

Conflict of interest

We declare that we have no conflict of interest.

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Conflict of interest statement

We declare that we have no conflict of interest.

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