Validated High-Performance Thin-Layer Chromatographic Method for the Simultaneous Determination of Quercetin, Rutin, and Gallic Acid in *Amaranthus tricolor* L.

Sayan Biswas, Ranjit K. Harwansh, Amit Kar, and **Pulok K. Mukherjee***

Key Words:

***Amaranthus tricolor* L.**
Quercetin
Rutin
Gallic acid
High-performance thin-layer chromatography
Densitometry
Validation

Summary

A simple, rapid, quantitative, and validated high-performance thin-layer chromatographic (HPTLC) method has been developed for the simultaneous estimation of quercetin (QU), rutin (RU), and gallic acid (GA) in the methanolic extract of *Amaranthus tricolor* L. aerial parts. Densitometric analysis of QU, RU, and GA were carried out in the absorbance mode at 254 nm. The method gave spot at R_F 0.49, 0.14, and 0.28 corresponding to quercetin, rutin, and gallic acid, respectively. The limit of detection (22.31, 14.12, and 16.24 ng per spot) and limit of quantification (67.80, 55.32, and 52.54 ng per spot), respectively, were confirmed with the mobile phase toluene–ethyl acetate–formic acid in a ratio of 7:5:1 (*v/v*). Linear regression analysis data for the calibration plot for QU, RU, and GA showed a good linear relationship with a correlation coefficient $r > 0.9955$ in the concentration range of 200–800 ng per spot, 200–500 ng per spot, and 200–600 ng per spot, respectively. The method was validated for linearity, accuracy, precision, detection, and quantification limits, specificity, and robustness as per the International Conference on Harmonization (ICH) guidelines. The proposed validated high-performance thin-layer chromatographic method provides a novel approach for the quality control and standardization of *A. tricolor* L.

1 Introduction

The genus *Amaranthus* is an important source of essential nutrients and bioactive compounds such as phenolics. Besides the major nutrients, the secondary metabolites of *Amaranthus* plants have also been shown to possess many therapeutic properties [1]. *Amaranthus tricolor* L. (family: Amaranthaceae), commonly known as "Red amaranth" or "Joseph's coat", is a widely cultivated plant of this genus found in Southeast Asia and many tropical countries including India [2]. *A. tricolor* L. has a rich antioxidant property and has been used traditionally for the treatment of a variety of ailments like coughs, throat infections, toothache, eczema, piles, diarrhoea, leucorrhoea, etc. [3]. *A. tricolor* L. is an erect tropical annual herb with long-stemmed leaves rounded at the tips. The flowers are whitish-green in color, and the seeds are very small, black or red-brown, and relatively large [4]. The phytochemical analysis of this plant showed the presence of betacyanins A and B, amaranthin, isoamaranthin, and various sterol compounds like spinasterol, cholesterol, campestrol, 24-methylene cholesterol, stigmasterol, *β*-sitosterol, fucosterol, and isofucosterol [3]. Phenolics such as quercetin (QU, **Figure 1a**), rutin (RU, **Figure 1b**), gallic acid (GA, **Figure 1c**), vanillic acid, syringic acid, *p*-hydroxybenzoic acid, ellagic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, *m*-coumaric acid, sinapic acid, etc. have also been obtained from this plant species [1, 5]. The plant has been reported for its various therapeutic properties, which include the prevention of complications during postmenopause [6], cardioprotective [2], anti-diarrheal [7], vision enhancer [8], anti-diabetic [9], anti-ulcer [10], anti-bacterial [11], hepatoprotective [12], antinociceptive [3] and inhibitory action on cyclooxygenase and tumor cell proliferation [13].

High-performance thin-layer chromatography (HPTLC) is a useful technique because of the advantages of ease of use, consistency, and fast quantitative determination of phytoconstituents in herbs [14]. The development of proper standardization parameters and the estimation of marker profile is highly important for maintaining the quality of botanicals [15]. As literature review revealed no report of standardization of this nutritionally and medicinally important plant with suitable marker compounds, a simple HPTLC method for the quantitative estimation of QU, RU, and GA simultaneously is proposed for *A. tricolor* L. The proposed method has been validated by different validation parameters according to the International Conference on Harmonization (ICH) guidelines [16].

S. Biswas, R.K. Harwansh, A. Kar, P.K. Mukherjee, School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, India.
*E-mail: naturalproductm@gmail.com;
pulok.mukherjee@jadavpuruniversity.in

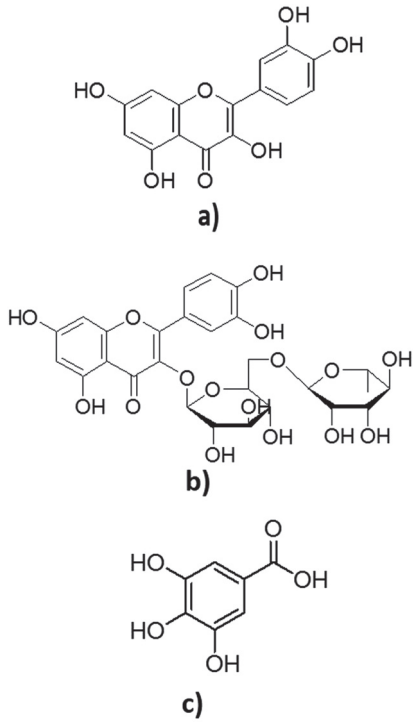

Figure 1

The chemical structures of QU (a), RU (b), and GA (d).

2 Experimental

2.1 Chemicals and Solvents

QU (≥95%), RU (≥94%), and GA (≥97.5%) were procured from Sigma Aldrich (St. Louis, MO, USA). Toluene, ethyl acetate, and formic acid of analytical grade were procured from Merck (Mumbai, India). A Whatman syringe filter (NYL 0.45 µm) was used for filtration of the samples and the standard.

2.2 Plant Material

Fresh aerial parts of *A. tricolor* L. were collected from a local market of Jadavpur, Kolkata, India, and authenticated. Voucher specimens of the plant material have been retained in the School of Natural Product Studies (SNPS/2012/2098), Jadavpur University, Kolkata, India, for further studies.

2.3 Extraction Procedure

The aerial parts were kept for drying, and the dried part was crushed to powder. About an amount of 500 g of the powdered material was macerated with methanol for 72 h, and the extract was filtered. This process was repeated consecutively three times, and the resultant filtrate was combined. The filtrate containing the extract was concentrated by a rotary

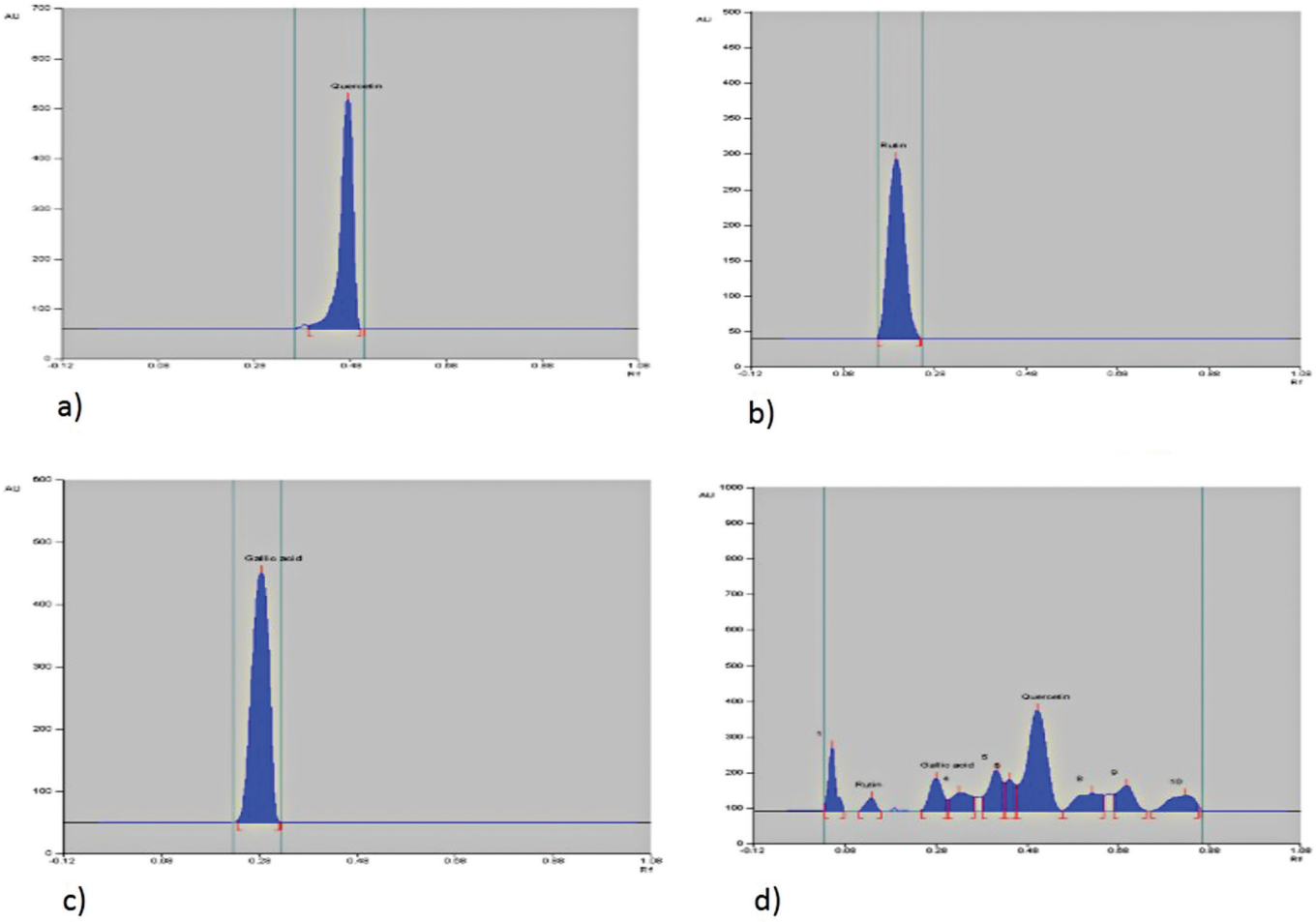

Figure 2

Chromatograms of the standards (a, QU; b, RU; c, GA) and the extract (d, ATME).

vacuum evaporator (IKA, RV 10, Staufen im Breisgau, Germany) at 45°C, and the concentrated extract was lyophilized. The resultant crude extract was weighted as 50 g for *A. tricolor* methanolic extract (ATME). The percentage yield of ATME was 14% *w/w*. The plant extract was stored at 4°C prior to analysis.

2.4 Chromatographic Conditions

The CAMAG (Muttenz, Switzerland) HPTLC system – consisting of winCATS software, a Linomat V automatic sample applicator, a scanning densitometer CAMAG Scanner 3, and photodocumentation apparatus CAMAG Reprostar 3 – was used. The stationary phase used was aluminum-backed silica gel plate 60 F_{254} (Merck, Mumbai, India) with dimensions of 20 cm × 10 cm. The solvents used were of analytical grades. A 100-µL syringe (Hamilton, Bonaduz, Switzerland) was used for sample application on HPTLC plates. QU, RU, and GA were used as marker compounds, and a working standard (100 µg mL^{-1}) was prepared for each. An amount of 10 mg of the extract was weighted and dissolved in 1 mL methanol to obtain a 10 mg mL^{-1} extract solution. The mobile phase containing toluene, ethyl acetate, and formic acid in a ratio of 7:5:1 (*v/v*) was found to give the desired separation. Standard (2–10 µL) and plant extract (12–16 µL) solutions were applied as an 8-mm bands onto a TLC plate using a TLC sampler Linomat V (CAMAG). The distance between the bands was kept at 1.5 cm each. The plate was then dried and developed to a distance of 8.5 cm in a twin-trough glass chamber kept at a temperature of 25°C, previously saturated with toluene, ethyl acetate, and formic acid. After development, the plate was dried and scanned in a CAMAG TLC Scanner 3 at a wavelength of 254 nm.

2.5 Validation Studies

Validation of the method was performed as recommended by the ICH guidelines and the Food and Drug Administration (FDA, Silver Spring, MD, USA) guidelines defining linearity, specificity, peak purity, limit of quantification and detection, precision, accuracy, and robustness [16]. The specificity of the method was determined by analyzing the standards and test samples. Peaks were identified by comparison of the R_F value and spectrum of the spot of the standard compounds with those of the extract. Peak specificity was determined by comparing the UV spectrum of the standards and the test sample **(Figure 2).** The limit of detection (LOD) and limit of quantification (LOQ) were calculated by determining the standard deviation (SD) of the response and the slope of the linear equation. LOD and LOQ were calculated using the following formula, LOD = $3.3\sigma / S$, LOQ = $10\sigma / S$, where σ is the standard deviation of the response, and S is the slope of the calibration curve. The slope (S) was determined from the calibration curves of the analytes. LOD and LOQ were determined with a specific calibration curve using sample containing QU, RU, and GA in the range of the detection limit and the quantification limit, respectively. A signal-to-noise ratio of 3 and 10 was considered for the estimation of the detection and quantification limits, respectively. The accuracy of the method was determined by analyzing the percentage recovery of QU, RU, and GA in ATME. The method was studied by performing a standard addition technique and was expressed in terms of % relative standard deviations *(%RSD)* from the mean recovery of the theoretical concentration. The experiment was repeated 6 times. The intra- and inter-day precisions of the method were expressed in terms of *%RSD*. The intra-day precision of the assay was determined by analyzing 6 samples in a single day at different time intervals. Inter-day precision was determined over 6 successive days by analyzing the same concentrations. The robustness for this experiment was studied

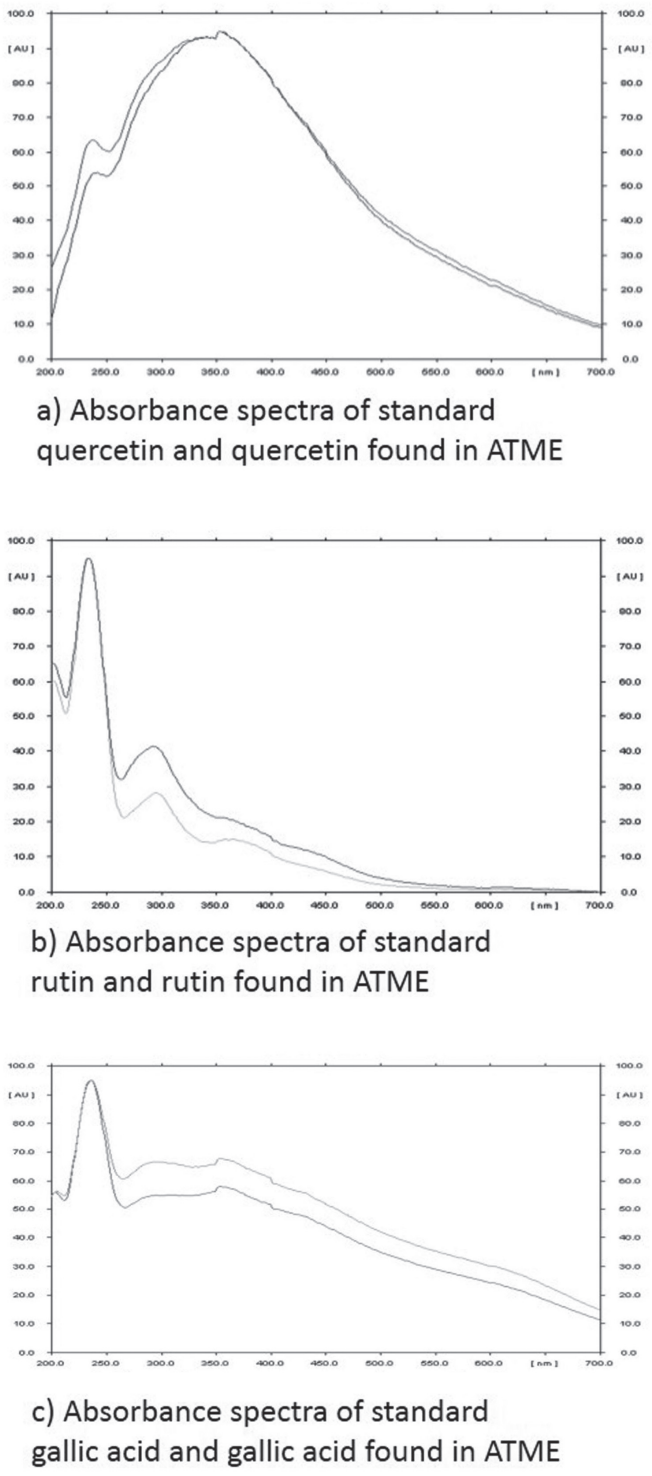

Figure 3

***In situ* absorbance spectrum of the standards (a, QU; b, RU; and c, GA) with the extract (ATME).**

through evaluation of the effect of small but deliberate variations in the chromatographic conditions. The composition of the mobile phase was varied, for example, 8:2:1, 6.5:3.5:1.5, 5.8:4.2:0.5, and 7:3:1 (*v/v*) for petroleum ether–ethyl acetate–formic acid, and chromatograms were developed. The time from spotting to chromatography and from chromatography to scanning was varied by 10 min and analyzed. The chamber was saturated for different time intervals. To verify the specificity of the method, QU, RU, and GA standard and sample solutions were applied on the plate and developed, followed by scanning. The peak purity of QU, RU, and GA were measured by evaluating the spectra of the marker compounds at the peak apex, peak start, and peak end positions of the spot.

3 Results and Discussion

The HPTLC chromatograms of QU, RU, and GA with the R_F values of 0.49, 0.14 and 0.28 are shown in Figures 2a–c. The chromatogram of ATME is shown in Figure 2d; the presence of QU, RU, and GA in the plant extract was confirmed by the corresponding R_F value. To ascertain the purity of the peak in the test sample, the *in situ* absorbance spectrum was compared with that of the standard QU, RU and GA, and both spectra were found to be super imposable, thus confirming the peak purity as observed in **Figures 3a–c.** The calibration curve for QU was linear in the range of 200–800 ng per spot with a correlation coefficient (*r*) of 0.9956, which indicated a good linear dependence of the peak area on the concentration for QU. Similarly, the calibration curve for RU (Figure 3b) and GA (Figure 3c) were linear in the range of 200–500 ng per spot (r = 0.9956) and 200–600 ng per spot (r = of 0.9962), which indicated a good linear dependence of the peak area on concentration. The result for the recovery of QU, RU, and GA in the extract is shown in **Table 1.** The average recoveries indicate good accuracy of the method. The LOD and LOQ values were reliable when compared with the signal-to-noise ratio. There was not much variation in the inter-day and intra-day precisions **(Table 2)** with an RSD of <1%, which indicated that the experimental procedure was found to be in the range of acceptability, as there was not much deviation. During deliberate variation in the chromatographic parameters, it was observed that the *%RSD* values of the peak areas for QU, RU, and GA in the analysis were <1%. This low value suggested the robustness of the proposed method. The specificity of the proposed method was evaluated by assessing the peak purity of QU, RU, and GA by comparing their respective spectra at the peak apex, peak start, and end positions of the peak. A good correlation ($r^2 > 0.9998$) was obtained for all the spectra. This confirms the specificity of the proposed method. The amounts of QU, RU, and GA present in the test extract were found to be 0.167%, 0.13%, and 0.27% (*w/w*), respectively. Photodocumentation of the plate at 254 nm along with a 3D chromatogram view is provided in **Figure 4.** The summary of all validation parameters for the developed HPTLC method for the simultaneous estimation of QU, RU, and GA are listed in Table 1. The present method has been found to be simple, accurate, specific, precise, and reproducible. It has a wide scope for herbal drug development, quality control, and standardization.

Table 1

Results of validation parameters for the simultaneous estimation of QU, RU, and GA in ATME.

Parameters	Results		
	QU	RU	GA
Wavelength [nm]	254	254	254
Retardation factor	0.49	0.14	0.28
Linearity range [ng]	200–800	200–500	200–600
Correlation coefficient (*r*)	0.9956	0.9956	0.9962
LOD [ng per spot]	22.31	14.12	16.24
LOQ [ng per spot]	67.80	55.32	52.54
Average recovery (%)	99.01–99.98	98.43–99.32	99.12–99.84
Specificity	Specific	Specific	Specific
Robustness	Robust	Robust	Robust

Table 2

Intra-day and inter-day precisions of QU, RU, and GA.

	Analyte	Amount	Mean [ng per spot] ± SD	*%RSD*
Intra-day precision	QU	300	292.67 ± 1.32	0.45
		500	493.56 ± 1.72	0.35
		700	695.21 ± 1.54	0.22
	RU	200	195.32 ± 1.43	0.73
		300	296.43 ± 1.65	0.56
		400	395.54 ± 0.98	0.25
	GA	200	193.26 ± 1.33	0.69
		400	395.23 ± 0.87	0.22
		500	482.12 ± 1.45	0.30
Inter-day precision	QU	300	294.56 ± 2.23	0.76
		500	496.21 ± 2.12	0.43
		700	696.32 ± 2.14	0.31
	RU	200	196.43 ± 1.18	0.60
		300	296.54 ± 1.75	0.59
		400	397.21 ± 2.15	0.54
	GA	200	195.43 ± 1.88	0.96
		400	396.21 ± 2.54	0.64
		500	494.27 ± 1.98	0.40

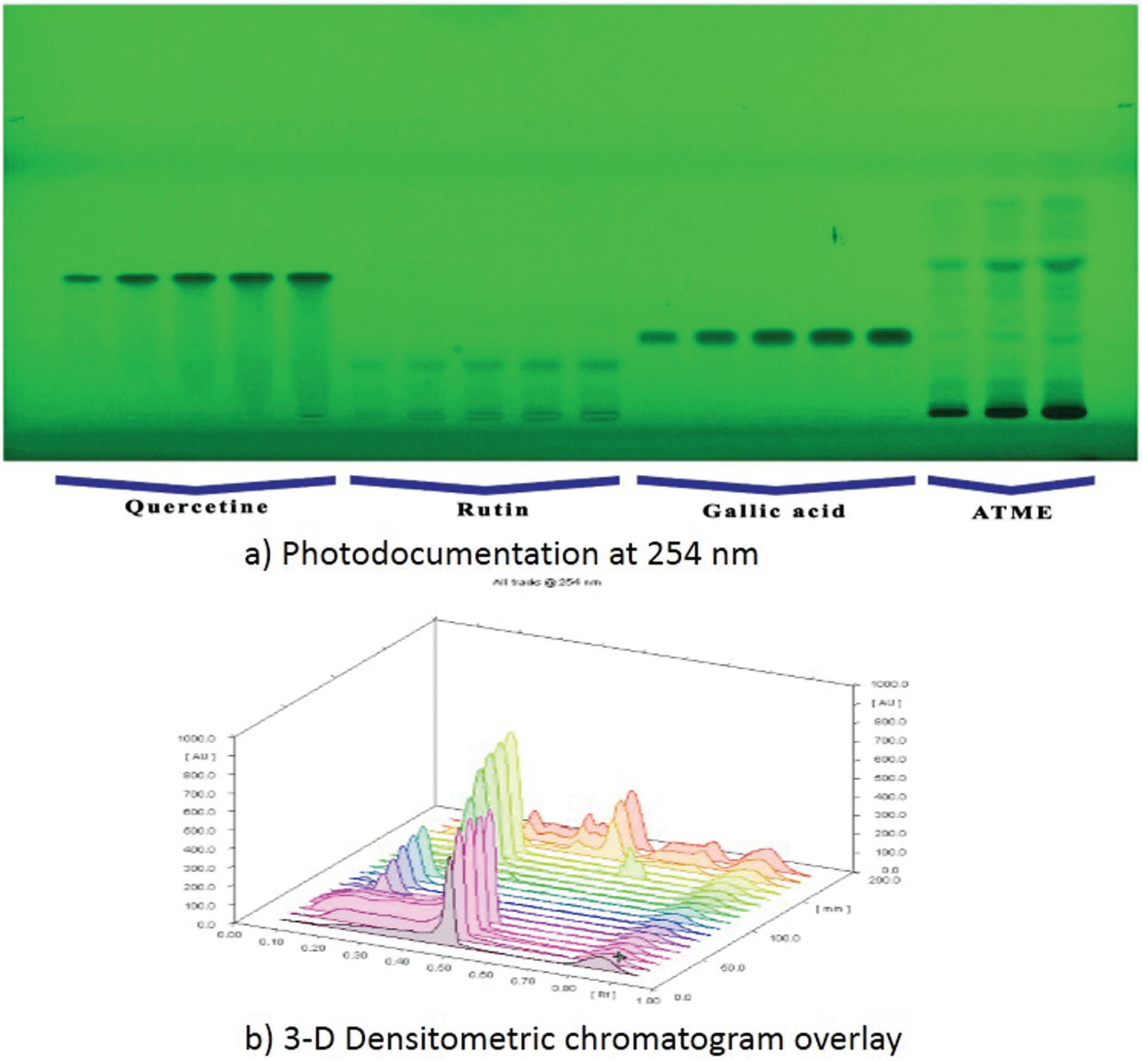

Figure 4

Photodocumentation (a) and 3D densitometric overlay (b) of the plate at 254 nm.

4 Conclusion

Quercetin (QU), rutin (RU), and gallic acid (GA) are important marker compounds present in ATME. A simple validated HPTLC–densitometric method for the simultaneous identification and quantification of these compounds in the aerial part of this plant has been developed and validated. This method may be useful for the development of quality control and marker analysis profile of this medicinal plant.

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Authors' Contributions

R. K. H. contributed to collecting the plant sample and identification and extraction. S. B. and A. K. performed the laboratory work, analysis of the data, and drafted the paper. P. K. M. designed the study, supervised the laboratory work, and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of Interest

The authors declare no conflicts of interest.

References

[1] *H. Li, Z. Deng, R. Liu, H. Zhu, J. Dravis, M. Marcone, Y. Sung, R. Tsao,* J. Food Compos. Anal. **37** (2015) 75–81.

[2] *K. Nahar, F. Kabir, P. Islam, Md. M. Rahman, Md. A.A. Mamun, Md. Faruk, N. Subhan, G.M.S. Rahman, H.M. Reza, Md. A. Alam,* Biomed. Pharmacother. **103** (2018) 1154–1162.

[3] *M. Rahmatullah, M. Hosain, S. Rahman, S. Rahman, M. Akter, F. Rahman, F. Rehana, M. Munmun, M.A. Kalpana,* Afr. J. Tradit. Complement. Altern. Med. **10** (2013) 408–411.

[4] *Tharun, K.N. Rao, S.K. Padhy, S.K. Dinakaran, D. Banji, H. Avasarala, S. Ghosh, M.S. Prasad,* Asian J. Chem. **24** (2012) 455–460.

[5] *U.K.S. Khanama, S. Obab, E. Yanaseb, Y. Murakami,* J. Funct. Foods **4** (2012) 979–987.

[6] *S. Kushwaha, P. Chawla, A. Kochhar,* J. Food Sci. Technol. **51** (2014) 3464–3469.

[7] *P. Rawat, P.K. Singh, V. Kumar,* Biomed. Pharmacother. **96** (2017) 1453–1464.

[8] *R. Routray, M. Kar, R.K. Sahu,* Int. J. Pharm. Pharm. Sci. **5** (2013) 232–235.

[9] *A.C. Clemente, P.V. Desai,* Trop. J. Pharm. Res. **10** (2011) 595–602.

[10] *V.C. Devaraj, B.G. Krishna,* Chin. J. Nat. Med. **11** (2013) 0145–0148.

[11] *S. Pulipati, P.S. Babu, L. Narasu,* Afr. J. Microbiol. Res. **9** (2015) 1381–1385.

[12] *S. Aneja, M. Vats, S. Aggarwal, S. Sardana,* J. Ayurveda Integr. Med. **4** (2013) 211–215.

[13] *B. Jayaprakasam, Y. Zhang, M.G. Nair,* J. Agric. Food Chem. **52** (2004) 6939–6943.

[14] *P.K. Mukherjee, A. Wahile, V. Kumar, S. Rai, K. Mukherjee, B.P. Saha,* Drug Inf. J. **40** (2006) 131–139.

[15] *S. Pandit, P.K. Mukherjee, A. Gantait, S. Ponnusankar, S. Bhadra,* J. Planar Chromatogr. **24** (2011) 541–544.

[16] ICH Validation of analytical procedures: text and methodology, Q2 (R1), International Conference on Harmonization, Geneva, November, 2005.

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