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Original article

Recent progress in simultaneous estimation of rutin, quercetin and liquiritin in *Cocculus hirsutus* by HPTLC

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Abstract

Background and objectives: Rutin, quercetin and liquiritin are polyphenol flavonoids which have shown anti-inflammatory, antihepatotoxic, antiulcer, antiallergic, antidiabetic, antiviral and antioxidant activities. They are found in many herbal plants, one of them is *Cocculus hirsutus*. The objective of this research was to develop and validate a new, accurate, precise and economic HPTLC method for simultaneous estimation of rutin, quercetin and liquiritin in ethanol extract of *Cocculus hirsutus* leaves. **Methods:** The simultaneous estimation of rutin, quercetin and liquiritin has been done by HPTLC on silica gel 60 F_{254} TLC plate using *n*- butanol: acetic acid: water: formic acid (7:1:1:0.25) as the mobile phase and was quantified by densitometric scanning at 254 nm. The method was validated as well. **Results:** Rutin, quercetin and liquiritin were satisfactorily resolved with R_f values of 0.47±0.03, 0.63 ±0.03 and 0.82±0.02, respectively. The linearity was found to be 1500-4000, 500-3000 and 100-700 ng per spot for rutin, quercetin and liquiritin, respectively. The inter-day RSD values were always less than 2, accuracy was 99.25% ±5% for rutin, 99.29% ±5% for quercetin, and 94.04 ±6% for liquiritin. The LOD was found to be 310.234, 346.8421 and 11.5571 ng per spot and LOQ was found to be 940.1032, 451.037 and 35.0213 ng per spot for rutin, quercetin and liquiritin, respectively. **Conclusion:** The statistically validated results indicated that the proposed new method has good accuracy and precision. Thus this new HPTLC method could be successfully applied for simultaneous determination of rutin, quercetin and liquiritin in herbal plants and their product.

Keywords*: Cocculus hirsutus*, HPTLC, liquiritin, quercetin, rutin

Introduction

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure and are ubiquitously present in plant world, where they perform several important functions such as antioxidant and chelating activities [1-3]. Flavonoids have the ability to induce human protective enzyme systems. A number of studies have suggested protective effects of flavonoids against many infectious (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, cancers and other age-related diseases [4-6]. Flavonoids also act as a secondary antioxidant defence system in plant tissues

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exposed to different abiotic and biotic stresses. They are located in the nucleus of mesophyll cells and within centers of ROS generation. They also regulate growth factors such as plants auxin [7]. Biosynthetic genes have been assembled in several bacteria and fungi for enhanced production of flavonoids [8]. Flavonoids have a wide range of biological and pharmacological activities [9-10]. Rutin (RUT) is a citrus flavonoid glycoside. Chemically, it is 5,7,3,4-tetrahydroxy flavonol-3-rhamanoglucoside and widely used in medicine for maintenance of capillary integrity. Quercetin (QCT) is 3,5,7,3′,4′-pentahydroxyflavone and liquiritin (LIQ) is 4'-*O*-glucoside of the flavanone liquiritigenin.

Herbal plants are important sources of new compounds leading to drugs in all major disease areas. They represent a source of structures that have been the source of most of the active ingredients of medicines. *Cocculus hirsutus* is one of the important medicinal plants and possesses various therapeutic properties [11]. *Cocculus hirsutus* is a perennial climbing shrub with hairy sepals belonging to Menispermaceae family, found in Sudan, central Asia, China and India (throughout tropical and subtropical regions).

The literature revealed that a few number of papers were reported toward the detection of quercetin in *Cocculus hirsutus* by UV-spectrophotometric method [12]. As per author's best knowledge yet there is no HPTLC method available for estimation of rutin, quercetin and liquiritin in the leaves of *Cocculus hirsutus.* The aim of this research was to reduce the analysis time for the simultaneous estimation of rutin, quercetin and liquiritin in the leaves of *Cocculus hirsutus* by HPTLC.

Experimental

Materials

Plant Material was collected from in October 2014 from the forests of different localities in Aurangabad district, Maharashtra, India. It was authenticated at Botanical Department of Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, Maharashtra, India (authentication No. BOT/2012-13/0552). The ethanol extract of air shade dried material of *Cocculus hirsutus* leaves was obtained by soxhlet extraction technique. Flavanoids standards rutin and quercetin were purchased from Natural remedies, Bangalore, India (purity >97%). Liquiritin was purchased from Sigma Life Science. All other chemicals and reagents were of analytical grade and were purchased from S.D. fine, Mumbai.

Instruments

Camag HPTLC system comprising of Hamilton syringe with a sample applicator Linomat V, Camag twin trough plate development chamber, Camag TLC Scanner 170422 and TLC 4.02, integration software (Switzerland) were used in the study. Pre-coated silica gel $60F_{254}$ aluminium plates (0.2 mm thick) were obtained from E. Merck Ltd., Mumbai (India) as the stationary phase. Shimadzu digital balance was used for weighing.

Preparation of standard solutions

Standard rutin, quercetin and liquiritin (10 mg each) were dissolved separately in methanol and made up to the volume 10 mL in a volumetric flask. These solutions were used as working standard solutions for the analysis.

Method development

After trying several permutations and combinations, the solvent system *n*-butanol: acetic acid: water: formic acid (7:1:1:0.25 v/v/v/v) was found to be the most satisfactory as it had shown good resolution for RUT, QCT and LIQ.

The TLC plates were pre washed with methanol and activated by keeping at 115 °C for about 10 minutes. Solutions of 0.5 µL were applied on the TLC plates as bands of 8 mm thick using Camag Linomat V. Application positions were at least 15mm from the sides and 15mm from the bottom of the plates. In order to reduce tailing effect, mobile phase components were mixed prior use and the development chamber was saturated with

mobile phase vapour for 10 min before each run. Development of the plate was carried out by the ascending technique to a migration distance of 7 cm. Then the plates were dried on a hot plate. Room temperature and relative humidity were always maintained at 20 °C±2 °C and 55% RH ± 5 %RH, respectively.

Densitometric scanning was done in absorbance mode at 254 nm using a Deuterium lamp. The slit dimensions were set at 5 mm×0.45 mm, the scanning speed at 20 mm/s and the data resolution at 100 m/step.

Method validation

Linearity

Standard stock solution (10 µg/mL) RUT, QCT and LIQ were prepared separately using methanol. A volume of 0.5 µL of each solution was applied on the TLC plate to deliver 1500 to 4000 ng of RUT per spot, 500-3000 ng of QCT and of LIQ 100 to 700 ng. This was carried out in triplicate and repeated for three days. For each concentration, the applied spot bands were evenly distributed across the plate to minimize possible variation along the silica layer. The linearity was evaluated visually by observing the calibration curves, of RUT, QCT and LIQ.

Precision

The repeatability and time-different intermediate precision were determined simultaneously. Intra-day assay precision was found by analysis of standard drug three times on the same day. Inter-day assay precision was carried out using three different days and percentage relative standard deviation (%RSD) was calculated. Repeatability of sample application was assessed by spotting 0.5 µL of extract having concentration of 2000, 2500 and 3000 ng per spot of rutin, 1500, 2000 and 2500 ng per spot of quercetin and 400, 500 and 600 ng per spot of liquiritin six times. From the peak areas, the percentage RSD was determined.

Accuracy

The accuracy of the method was assessed by determination of the recovery of the method at 3 different concentrations (80%, 100% and 120%) by addition of known amount of standard to 0.5 µL of original extract. Solutions were prepared in triplicate and analyzed. This procedure was repeated for three consecutive days. Calibration curves were used to estimate the concentration of drug per spot and measured daily on the same plates as the samples. The accuracy was determined and expressed as percentage recovery. Ruggedness of the method was assessed by adding the standard 3 times with different analyst by using the same equipment.

Analysis (assay) of the ethanol extract of Cocculus hirsutus

The method was used for quantization of RUT, QCT and LIQ in ethanol extract of *Cocculus hirsutus*. For sample preparation, methanol was used as solvent for dilution. The ethanol extract of *Cocculus hirsutus* was diluted (10 mg) with methanol, mixed well and filtered through Whattman filter paper no. 41 to obtain the sample stock solution. The test solution (5 µL) was applied on a pre-coated silica gel $60F_{254}$ plate. The amounts of RUT, QCT and LIQ were simultaneously calculated from the peak area using the respective calibration graph. Analysis procedure was repeated three times with the ethanol extract of *Cocculus hirsutus* with the mobile phase *n*-butanol: acetic acid: water and formic acid (7:1:1:0.25) and detection was carried out at 254 nm.

Results and Discussion

The different published methods [13-16] utilized different mobile phases such as methanol : water: formic acid (40:57:3), Ethyl acetate : formic acid: acetic acid: water (10:1.1:1.1:0.6), Toluene: ethyl acetate : methanol (5:3:2), Toluene : ethyl acetate:methanol:formic acid (8:8:3:1), with R_f 0.07 and 0.17, 0.03 and 0.76, 0.17 and 0.65, 0.13 and 0.75 for the separation of rutin and quercetin respectively at λ max 254 nm. The other published methods [17-18] utilized Ethyl acetate: dichloromethane:formic acid:glacial acetic acid:

water (10:2.5:1:1:0.1) as mobile phase with R_f 0.13 and 0.93 at λ max 366 nm and Ethyl acetate : glacial acetic acid : formic acid : water (100:11:11:25) with R_f 0.34 and 0.98 at λ max 366 nm and 280 nm for the separation of rutin and quercetin respectively. But till date no HPTLC method was available for the simultaneous estimation of rutin, quercetin and liquiritin. During the stage of method development, different mobile phases were tried and the mobile phase comprising of *n*-butanol: acetic acid: water: formic acid (7:1:1:0.25) was confirmed for the simultaneous estimation of rutin, quercetin and liquiritin at λ max 254 nm. The aim of achieving an optimum separation between spots (Rs ≥1.5) and a migration of spots with R_f values between 0.47, 0.63 and 0.82 in order to ensure separation reproducibility (figure 1). The chromatogram represented better resolution of RUT, R_f = 0.47±0.03, QCT, R_f = 0.82±0.02 and LIQ, R_f = 0.63±0.03 in ethanol extract of *Cocculus hirsutus* (figure 2).

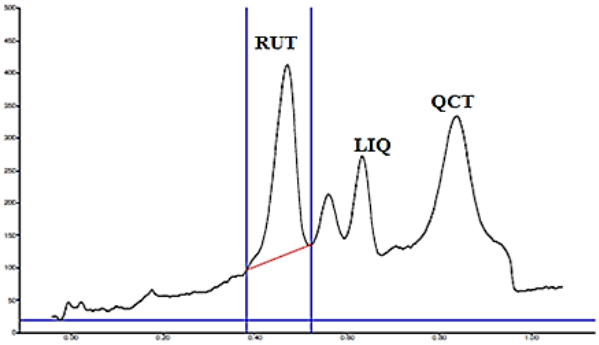

Figure 1. Chromatogram of ethanol extract of *Cocculus hirsutus* with separation of rutin (R_f 0.47), quercetin (R_f 0.82) and liquiritin (R_f 0.63)

A good linear relationship was obtained over the concentration range 1500-4000 ng/spot, 500-3000 ng/spot and 100-700 ng/spot for rutin (RUT), quercetein (QCT) and liquiritin (LIQ), respectively with regression coefficient of 0.999. The LOD with signal/ noise ratio were found to be 310.234, 346.842 and 11.557 ng/spot for rutin, quercetin and liquiritin, respectively. The LOQ with signal/noise ratio was found to be 940.103 ng/spot, 451.037 ng/spot and 35.021 ng/spot for rutin, quercetin and liquiritin, respectively.

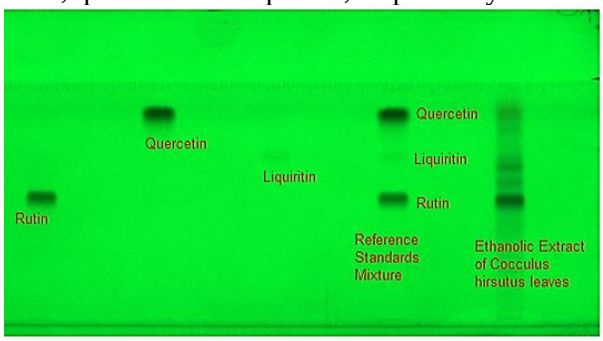

Figure 2. Chromatogram showing resolution of rutin, quercetin and liquiritin in ethanol extract of *Cocculus hirsutus*

Table 1. Evaluation of intra-day and inter-day precision of RUT

RUT taken (ng/Spot)	Intraday precision		Interday precision	
	RUT (ng/spot)	RSD %	RUT (ng/spot)	RSD %
2000	2002.50	0.5477	2002.67	0.8207
2500	2501.17	0.5171	2499.50	0.7857
3000	2997.83	0.4546	2994.50	0.5676

Table 2. Evaluation of intra-day and inter-day precision of QCT

QCT taken (ng/Spot)	Intraday precision		Interday precision	
	QCT found (ng/spot)	RSD %	QCT found (ng/spot)	RSD %
1500	1502.833	0.5822	1498.500	0.6548
2000	2001.000	0.5145	1999.000	0.6568
2500	2497.333	0.4009	2501.833	0.5838

Table 3. Evaluation of intra-day and inter-day precision of LIQ.

LIQ taken (ng/Spot)	Intraday precision		Interday precision	
	LIQ found (ng/spot)	RSD %	LIQ found (ng/spot)	RSD %
400	406.8333	0.9251	406	1.0501
500	501.6667	0.9321	504	1.1223
600	603.8333	0.4732	601	0.7214

The intraday and inter-day RSD valves for precision are reported in table1, table 2 and table 3 which showed excellent RSD % values less than 2% after six applications.

Table 4. Recovery data of RUT, QCT and LIQ

Level	Amount added (ng)			Amount found (ng)			% Recovery		
	RUT	QCT	LIQ	RUT	QCT	LIQ	RUT	QCT	LIQ
80%	1280	1600	240	1268.03	1588.5	223	99.0651	99.28	93.91
100%	1600	2000	300	1581.45	1994.3	280	98.8411	99.72	93.33
120%	1920	2400	360	1917.05	2373.6	338	99.8465	98.9	94.88

Table 5. Effect of variation of condition (composition of mobile phase and temperature) for optimizing the HPTLC method

Mobile phase composition and temperature	*n*-butanol: AA:water: FA 6.5:1.5:1:0.25	Temp. (23 °C)	*n*-butanol: AA:water: FA 7:1:1:0.25	Temp. (25 °C)	*n*-butanol: AA: water: FA 7.5:0.5:1:0.25	Temp. (27 °C)	Acceptance criteria
Rutin (R_f)	0.46	0.46	0.47	0.45	0.45	0.47	
Quercetin (R_f)	0.82	0.83	0.81	0.80	0.83	0.82	0.5-2.0
Liquiritin (R_f)	0.66	0.63	0.67	0.65	0.64	0.66	

Table 6. Ruggedness study by different analysts to validate the HPTLC method

Analyst	Amount taken of RUT (µg/µL)	Amount found of RUT (µg/µL± S.D)	Amount taken of QCT (µg/µL)	Amount found of QCT (µg/µL± S.D)	Amount taken of LIQ (µg/µL)	Amount found of LIQ (µg/µL ± S.D)		Percent difference between two analysts
Analyst 1	10	9.78	10	9.69	5	4.61	RUT	QCT	LIQ
Analyst 2	10	9.83	10	9.71	5	4.72	0.5%	0.3%	2.2%

The recovery was 99.06%, 98.84% and 99.84% for rutin, 99.28%, 99.71% and 98.89 % for quercetin and 93.91%, 93.33% and 94.88 % for liquiritin at 80% 100% and 120% levels (table 4). Assay results show 46.06 % for Rutin, 17.09 % for Quercetin and 0.029 % for Liquiritin.

Table 5 shows that RSD and asymmetry factor for RUT, QCT and LIQ standards were found to be within a limit of 0.5 to 2.0 for variation in composition to mobile phase (*n*-butanol: acetic acid: water and formic acid) and temperature.

Thus, the proposed method was robust for small variations in the test method.

This study signified the ruggedness of the method under varying condition of performance. Studies were carried out only for parameters like different times, different days and different analysts. Results of estimation by proposed method were very similar under variety of conditions shown in table 6.

The proposed method is new, accurate, precise and less time consuming for simultaneous determination of rutin, quercetin and liquiritin as compared with published methods. The percent estimation of rutin, quercetin and liquiritin were found to be 46.06%, 17.09% and 0.029% in ethanolic extract of *Cocculus hirsutus* by proposed method. The method was successfully validated for linearity, precision and accuracy (table 7); however, the new developed HPTLC method is could be suitable as a quality control parameter in simultaneous determination of rutin, quercetin and liquiritin in herbal plants.

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Table 7. Summary of validation parameters

Mobile Phase	Rutin	Quercetin	Liquiritin
	n-butanol: acetic acid: water and formic acid (7:1:1:0.25 v/v/v/v)		
Wavelength (nm)	254 nm		
R_f Valve	0.47	0.82	0.63
Linearity Range (ng/spot)	1500-4000	500-3000	100-700
Accuracy (%)	99.2509	99.2995	94.04
Intraday Precision (%RSD)	0.5064	0.4992	0.7768
Interday Precision (%RSD)	0.7737	0.6318	0.9646
Limit of Detection (LOD,ng /spot)*	310.234	346.8421	11.5571
Limit of Quantification (LOQ, ng /spot)*	940.1032	451.037	35.0213

*Based on SD of response and slope of regression curve

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

References

[1] Middleton EM, Teramura AH. The role of flavonol glycosides and carotenoids in protecting soybean from ultraviolet-B damage. *Plant Physiol*. 1993; 103: 741-752.

[2] Hein KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: chemistry, metabolism and strcture-activity relationships. *J Nutr Biochem.* 2002; 13(10): 572-584.

[3] Bors W, Heller W, Michel C, Saran M. Flavonoids as antioxidants: determination of radical-scavenging efficiencies. *Method Enzymol*. 1990; 186: 343-355.

[4] Dixon RA, Dey PM, Lamb CJ. Phytoalexins: enzymology and molecular biology. *Adv Enzymol RAMB.*1983; 551-136.

[5] Rice-Evans CA, Miller NJ, Bolwell PG, Broamley PM, Pridham JB. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Res.* 1995; 22(4): 375-383.

[6] Agati G, Azzarello E, Pollastri S, Tattini M. Flavonoids as antioxidants in plants: location and functional significance. *Plant Sci.* 2012; 196: 67-76.

[7] Du F, Zhang F, Chen F, Wang A. Advances in microbial heterologous production of flavonoids. *Afr J Microbiol Res*. 2011; 5(18): 2566-2574.

[8] Middleton EJ. Effect of plant flavonoids on immune and inflammatory cell function. *Adv Exp Med Biol.* 1998; 439: 175-182.

[9] Kumar S, Pandey AK. Chemistry and biological activities of flavonoids: an overview. *Scientific World J.* 2013; 2013:1-16.

[10] Nijveldt RJ, Nood EL, Van Horn DEC, Boelens PG, Van Norren K, van Leeuwen PAM. Flavonoids: A review of probable mechanisms of action and potential applications. *Am J Clin Nutr.* 2001; 74(4): 418-425.

[11] Patil V, Devdhe S, Angadi S. Medicinal values of *Cocculus hirsutus* (L.) Diels: a comprehensive review. *Inventi Impact: Planta Activa*. 2013; (4): 138-144.

[12] Patil V, Angadi S, Devdhe S. Determination of quercetin by uv spectroscopy as quality control parameter in herbal plant: *Cocculus hirsutus*. *J Chem Pharm Res.* 2015; 7(1): 99-104.

[13] Jain A, Lodhi S, Singhai AK. Simultaneous estimation of quercetin and rutin in *Tephrosia purpurea* Pers by high performance thin layer chromatography. *Asian J Tradit Med.* 2009; 4(3): 104-109.

[14] Pawar NP, Salunkhe VR. Development and validation of HPTLC method for simultaneous estimation of rutin and quercetin in hydroalcoholic extract of

Triphala Churna. *Int J Pharm Tech Res.* 2012; 4: 1457-1463.

[15] Srinivasa RA. Simultaneous estimation of quercetin and rutin in ethanolic extract of *Catharanthus roseus* Linn. leaves by HPTLC method. *Global Res Anal.* 2013; 2: 155-157.

[16] Prajapati B, Savai J, Pandita N. Method development and validation for simultaneous estimation of quercetin and rutin in bark of *Saraca asoca* and herbal formulation by HPTLC. *Indo American J Pharmaceut Res.* 2013; 3: 3233-3245.

[17] Jayaveera KN, Tripathi SM, Satish KV. Estimation of gallic acid, rutin and quercetin in *Terminalia chebula* by HPTLC. *Jordan J Pharmaceut Sci*. 2010; 3: 63-68.

[18] Sajeeth CI, Manna PK, Manavalan R, Jolly CI. Quantitative estimation of gallic acid, rutin and quercetin in certain herbal plants by HPTLC method. *Der Chem Sinica*. 2010; 1(2): 80-85.