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Simultaneous Estimation of Antioxidant Biomarkers from Leaves of Cucumis Melo Var. Agrestis by using Validated HPTLC Method

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Abstract

Background: This research focused on HPTLC method for the simultaneous estimation of natural antioxidant markers namely rutin, gallic acid and quercetin from the plant extract of Cucumis melo var agrestis (CME). *Aims and Objectives:* The main aim of this research is to extract and simultaneously quantitate the three antioxidant markers-rutin, gallic acid and quercetin from CME by using normal phase HPTLC. *Materials and Methods:* Separation of compounds was performed on TLC aluminium plates precoated with silica 60 F254 after that detection of rutin, gallic acid and quercetin. Camag TLC scanner 3 prepared with win CATS software was used for densitrometric scanning at 254 nm. This method was additional validated in terms of linearity, precision, accuracy and sensitivity as per the ICH guidelines. *Results:* A good linear relationship was obtained for the calibration plots with R^2 = 0.9957, 0.9909, and 0.9920 for rutin, gallic acid and quercetin respectively. Accuracy was checked by recovery study performed at three different stages with the average recovery between 95% and 98% for all the three markers. *Conclusion:* This developed method of HPTLC can be used for the detection of antioxidant markers in botanicals and herbal formulations.

Keywords: Bioflavonoids; Rutin; Gallic acid; Quercetin; Antioxidant and Developed method of HPTLC.

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Introduction

Antioxidant properties play an important role for cure various diseases because during disease stage oxidative stress and free radicals were produced and scavenging of radicals was important for cure disease [1,2]. Numerous herbals and herbal extracts having a various number of phyto-pharmacological compounds like flavonoids, steroids, terpenoids, glycosides, alkaloids, tannins and phenols. Phenolic and flavonoids has potent antioxidant properties among other phytochemicals [3,4,5,6]. Flavonoids and phenol compounds namely rutin, gallic acid and quercetin (Fig. 2) were commonly used antioxidant markers. Rutin was chemically named as quercetin-3-O-rutinoside, rutoside and sophorin. This was the glycoside of flavonol quercetin and rutinose (disaccharide) [7]. Rutin was one of the greatest flavonoids in the human diet, which has potent antioxidant properties for different oxidizing species like hydroxide and peroxide radicals. Quercetin was chemically known as 3,30,40,5,7-pentahydroxyflavone which was flavonoid and is present in numerous plants.

Quercetin uses antioxidant activities [8]. Gallic acid was one of the phenolic compound which chemically named as 3,4,5 trihydroxybenzoic acid. Most of the gallic acid derivatives present naturally in plant and having potent antioxidant properties. *Cucumis melo var agrestis* is belongs family of cucurbitaceae, this plant is widely distributed in rural and coastal areas (Fig. 1). This plant is commonly called as wild musk melon, kachari, small guard [9].

Materials Methods

Chemicals

All chemicals of analytical grade were purchased from Merck Specialities Private Limited, Mumbai. Antioxidant markers-rutin (98.5% purity), gallic acid (98% purity) and quercetin (97% purity) were procured from Sigma Aldrich Chemical Company, (Steinheim, Germany).

Preparation of Plant material

Fresh leaves of *Cucumis melo var agrestis* was collected from village of Pungavarnatham, Thoothukudi district, Tamil Nadu, India month of December 2017 and authenticated from Government Siddha Medical College Chennai, voucher specimen no. GSMC/MB-87/18. Collected leaves were dried under sunshade in dark room after drying of leaves was powdered by using mechanical grinder. After size reduction of leaves were sieved in sieve no. 40 and sieve no. 60, stored in airtight container at room temperature.

Preparation of Standard

Accurately weighed 100 mg of each Rutin, gallic acid and quercetin were dissolved separately in 100 ml standard flask with methanol (stock solution of 1 mg/ml). Prepared stock solutions were filtered under whatman filter paper no. 1 and sonicated for 10 minutes then stored in amber colored container [10,11].

Extraction of plant material and preparation of sample solution

Accurately weighed 100g of powder of CME was extracted in 500 mL of 60% methanol (methanol: water- 60:40) under soxhlet apparatus for 48h. [12] Obtained extract was concentrated under distillation and dried at room temperature until get solid mass of extract, and stored at 4^0C under refrigerator for further studies. Accurately weighed 100 mg of plant extract was dissolved in a 100 ml standard flask with methanol (stock solution of 1 mg/ml). Prepared stock solution was filtered under what man filter paper no. 1 and sonicated for 10 minutes then stored in amber colored container.

High-Performance Thin-Layer Chromatographic Analysis Instrumentation and operating conditions

Instrumental parameters were shown in Table 1. A CAMAG HPTLC system equipped with Linomat V Automatic Sample Spotter (Camag Muttenz, Switzerland) and CAMAG TLC Scanner III with winCATS planar chromatography manager software version 1.4.3 was used for the analysis. Samples were applied on TLC plates precoated with silica gel 60 F254 (E. Merck#5554) of 0.2 mm thickness with aluminium sheet support in 7 mm bands at 10 mm from the bottom, 15 mm from the sides and 7 mm space between two bands. Plates were developed in a twin trough chamber pre-saturated with mobile phase of Toluene: Ethyl acetate: Formic acid: Methanol (3:6:1.6:0.4) for 30 min to a height of 8.5 cm from the base. After development quantitative evaluation of the plate was carried out in the reflectance mode at 254 nm. with tungsten lamp in conjunction with WINCATS software [13].

Method Validation

International Conference on Harmonisation (ICH) guidelines were followed for the validation of developed analytical method [14]. The parameters evaluated were linearity, precision, specificity, accuracy, sensitivity and ruggedness [15].

Specificity

Specificity of the technique was established by comparing the bands of the sample solutions with that of the particular reference standards in terms of Rf value.

Sensitivity

Sensitivity was determined to limit of detection (LOD) and limit of quantification (LOQ). Different dilutions (1-5 µg/ml) of standard solutions of rutin, gallic acid and quercetin were applied on TLC plates along with methanol as blank. LOD was determined at S/N of 3:1 and LOQ at S/N of 10:1.

Calibration and quality control samples

The calibration curves were prepared from the stock solutions to get desired concentrations in the

quantification range. The employed standards in the range of 1-5 µg/ml, 1-5 µg ml, 1-5 µg/mL for rutin, gallic acid and quercetin, respectively, were applied on TLC plate for obtaining a 5 point linear calibration curve [16,17].

Precision Instrumental precision

Instrumental precision was checked by repeated scanning (n = 3) of 5 µg/ml of rutin, gallic acid, quercetin and additional expressed as relative standard deviation (% RSD).

Repeatability

The repeatability of the method was confirmed by analyzing 5 µg mL–1 of all the three markers rutin, gallic acid and quercetin on a HPTLC plate (n = 3) and expressed as % RSD [18].

Inter- and intra-day precision

Variability of the method was studied by spotting the quality control samples of rutin, gallic acid and quercetin on the same day (intra-day precision) and on different days (inter-day precision) and the result was expressed as % RSD.

Accuracy

The accuracy was measured by spiking the quality control samples of rutin, gallic acid and quercetin in plant matrix.

Assay

The content of all the antioxidant biomarkers from the extract of CME was determined by applying the samples (10 µL) in triplicate along with pure standards of rutin, gallic acid and quercetin [19,20].

Results

The three bioflavonoids of antioxidant mark rutin, gallic acid and lupeol was eluted under various solvent systems consisting of Toluene: Ethyl acetate: Formic acid: Methanol (3:6:1.6:0.4) were tried on normal phase HPTLC, effectively resolved the antioxidant markers rutin (Rf = 0.18), gallic acid (Rf = 0.78) and quercetin (Rf = 0.87) and allowed their simultaneous estimation from plant extract of CME. The plate was visualised in 254 nm (Fig. 3). The method developed was found to be selective with good baseline resolution of each compound (Fig. 4). The established method was validated of specificity, precision, sensitivity, ruggedness and accuracy. Specificity of the method was confirmed by matching Rf of the bands from sample CME with that of the reference antioxidant markers. The absence of interfering bands confirms that the method was specific. The five point calibration curves for three standard reference compounds found to be linear in the range of 1-5, 1-5 and 1-5 µg/ml for rutin (Fig. 5), gallic acid (Fig. 6) and quercetin (Fig. 7), respectively. Regression equation and coefficient for the reference standard compounds were: $y = 3131.6x + 1564.3$, $R^2=0.9957$ for rutin, $y = 4264.4x - 248.72$, $R^2= 0.9909$ for gallic acid and $y = 4309.7x – 3715$, $R^2=0.9920$ for quercetin (Table 2). The LOD and LOQ values obtained were 1 µg/ml and 5 µg/ml for rutin, 1 µg/ml and 5 µg/ml for gallic acid and 1 µg/ml and 5 µg/ml for quercetin indicating that the developed method was more sensitive for rutin as compared to gallic acid and quercetin (Table 3). The % RSD of rutin, gallic acid and quercetin was found to be 1.03, 1.16 and 1.28 respectively which was less than 2% when minute thoughtful changes were made in the mobile phase composition and spotting volume of the samples stating that the method was rugged. Intra-day and inter-day precision were in the range of 0.9-2% representing good precision and repeatability. Good recoveries were obtained from the standard reference markers. rutin, gallic and quercetin were simultaneously estimated from the hydro alcoholic extract of CME. Rutin was present maximum amount in CME compared with other antioxidant markers. Amount of rutin, gallic acid and quercetin were present in extract was found to be 18.83 mg/g, 6.78 mg/g and 9.72 mg/g respectively (Table 4).

Table 1: Instrumental parameters for HPTLC analysis

S.No	Instrumental parameters	Value
1.	Number of sample	6
2.	Position of first track X	10.00 mm
3.	Distance between tracks	10.0 mm
4.	Scan start position Y	5.00 mm
5.	Scan and position	75.0 mm
6.	Slit dimension	6 mm X 0.45 mm
7.	Optimize optical system	light
8.	Scanning speed	20 mm/S
9.	Data resolution	100 µm/steps
10.	Detector mode	automatic
11.	Application port	Linomat V
12.	Detector	CAMAG TLC scanner

Table 2: Calibration parameters for examined Antioxidant biomarkers

Antioxidant Marker	Linear working range (µg/ml)	Regression equation	Coefficient of determination (R2)
Rutin	1-5	y = 3131.6x + 1564.3	0.9957
Gallic acid	1-5	y = 4264.4x - 248.72	0.9909
Quercetin	1-5	y = 4309.7x - 3715	0.9920

Values are obtained from standard curves of Rutin, Gallic acid and Quercetin

Table 3: Method validation parameters for Rutin, Gallic acid and Quercetin

S. No	Parameters	Rutin	Gallic acid	Quercetin
	Rf value	0.18	0.78	0.87
	LOD (ng/spot)	2.5	1.0	2.0
	LOQ (ng/spot)	5.0	5.0	5.0
	Instrumental precision (% RSD), n=3	1.03	1.16	1.28
	Intraday precision (% RSD)	0.96	1.02	1.18
	Interday precision (% RSD)	1.13	1.08	1.20
	Recovery (%), n=3	99.78	99.90	99.92
	Specificity	Specific	Specific	Specific
	Ruggedness	Rugged	Rugged	Rugged

LOD – Limit of detection; LOQ – Limit of quantification; % RSD – Relative standard deviation

Table 4: Content of Rutin, Gallic acid and Quercetin in HALEC

Name of Antioxidant biomarker	Amount of anitioxidant biomarker present in HALEC (mg/g)	Percentage of biomarker present in HALEC
Rutin	18.83	1.255 %
Gallic acid	6.78	0.4522 %
Quercetin	9.72	0.6613 %

Values obtained from standard Rf values

Fig. 1: Whole plant of *Cucucumismelo var. agrestis*

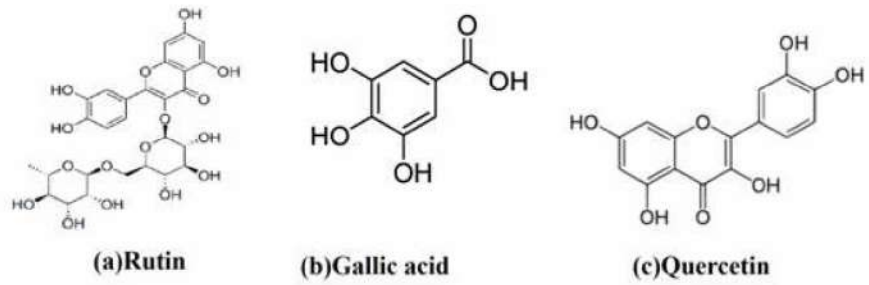

Fig. 2: Structure of (a) Rutin, (b) Gallic acid, and (c) Quercetin

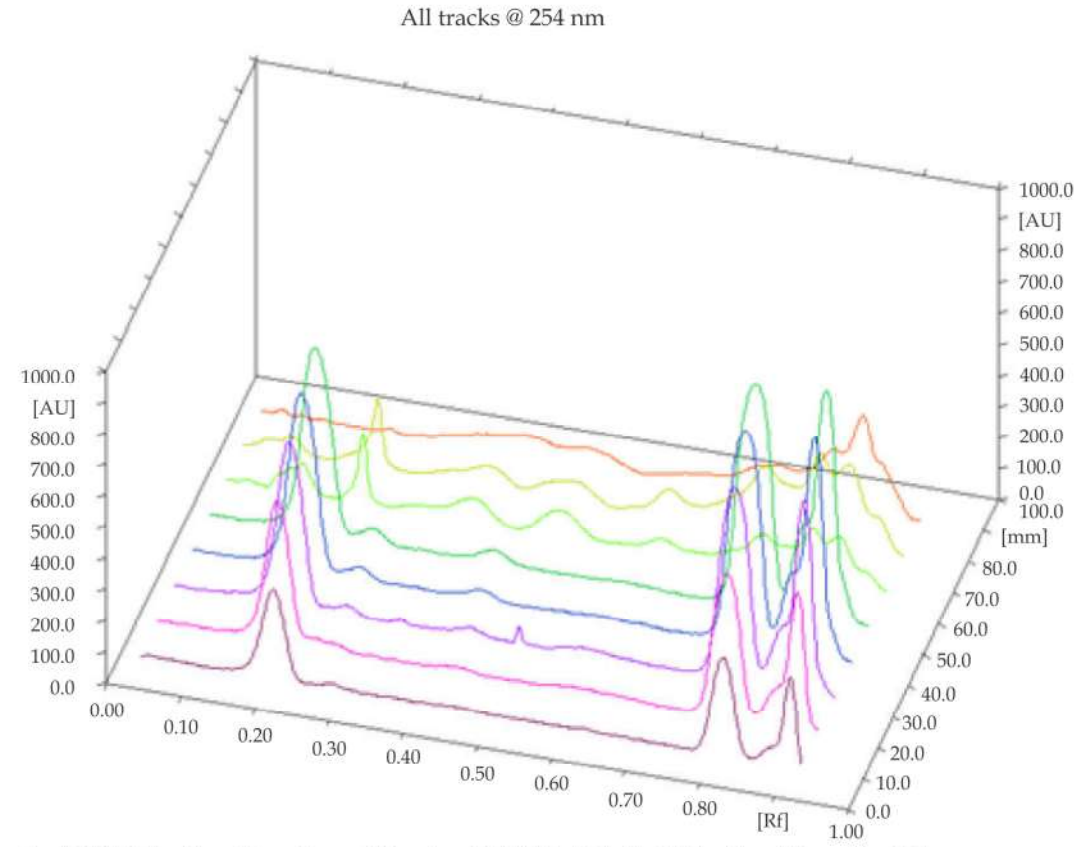

Fig. 3: HPTLC densitometric profile as a 3D overlay of HALEC with Rutin, Gallic acid and Quercetin at 254 nm.

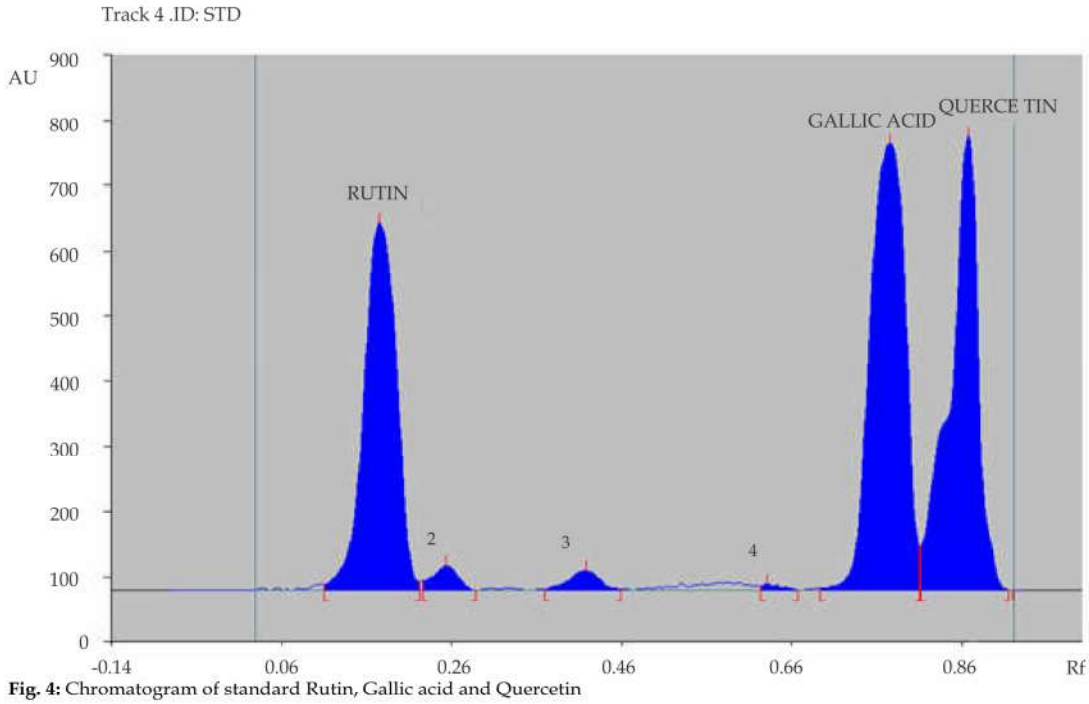

Fig. 4: Chromatogram of standard Rutin, Gallic acid and Quercetin

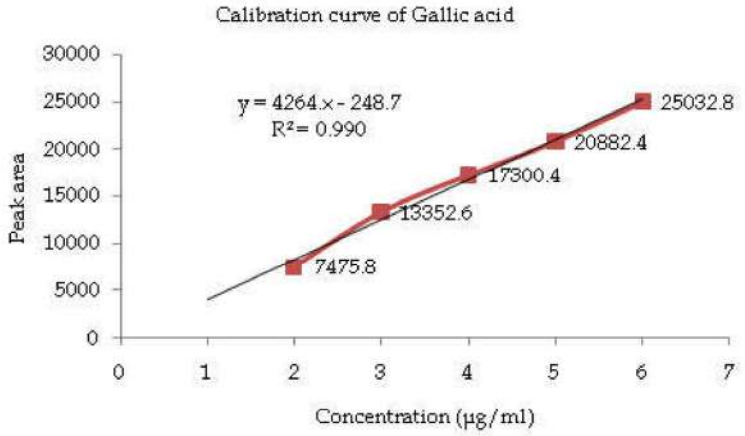

Fig. 5: Calibration curve for Rutin

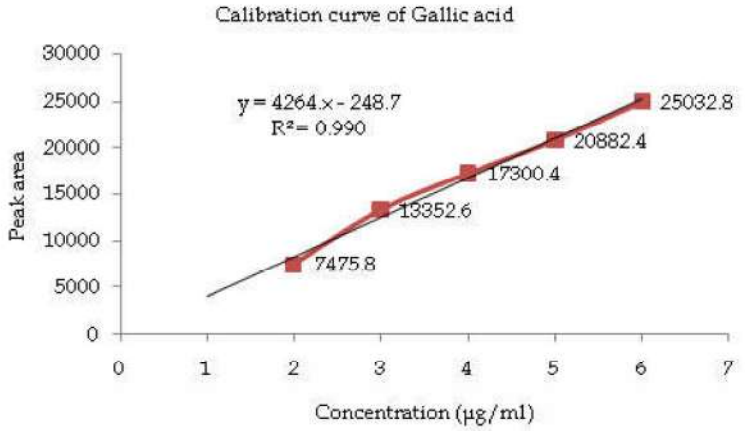

Fig. 6: Calibration curve for Gallic acid

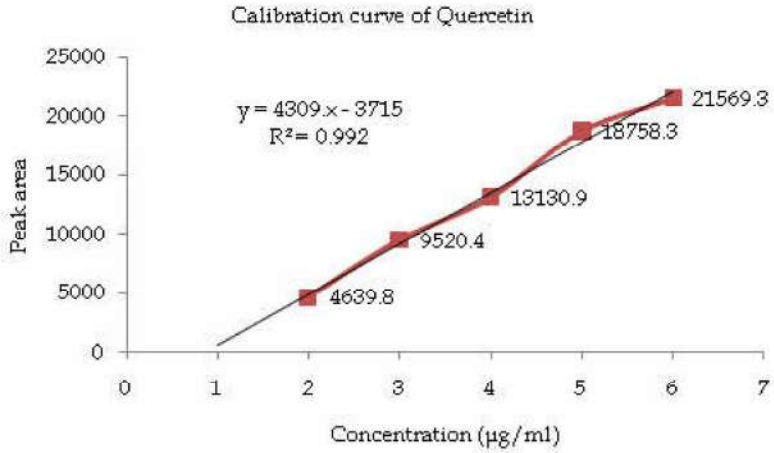

Fig. 7: Calibration curve for Quercetin

Discussion

Antioxidant properties play an important role for cure various diseases because during disease stage oxidative stress and free radicals were produced and scavenging of radicals was important for cure disease. In literature rutin, quercetin and gallic acid were reported as good antioxidant properties. These antioxidant biomarkers were simultaneously estimated in *Cucumis melo var agrestis*.

Conclusion

The developed validated HPTLC method was initiative simultaneous estimation of rutin, gallic acid and quercetin from *Cucumis melo var agrestis*. This method will be used for the simultaneous quantification of these three antioxidant markers (rutin, gallic acid and quercetin) from any other herbal and herbal extracts.

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