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Original Article

SIMULTANEOUS RP-HPLC ANALYSIS OF QUERCETIN AND KAEMPFEROL IN DIFFERENT PLANT PARTS OF CISSUS QUADRANGULARIS

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ABSTRACT

Objective: To develop a simple, rapid and specific reversed phase High-Performance Liquid Chromatography (HPLC) method for comparative analysis of flavonoids, quercetin and kaempferol, in different plant parts (leaves, stem and roots) of *Cissus quadrangularis* Linn.

Methods: The HPLC method which can be used effectively for separation of components from plants has been developed to perform a comparative analysis of flavonoids, quercetin and kaempferol, in different plant parts (leaves, stem and roots) of *Cissus quadrangularis* Linn. An endcapped C18 column at 370 nm, and water: acetonitrile (45:55) containing 0.1% o-phosphoric acid as mobile phase was used.

Results: Quercetin and kaempferol were well resolved at about 5 min and 7 min respectively. Calibration curves for quercetin and kaempferol were linear in the range of 1-10 µg/ml (R^2= 0.999) and 0.5-10 µg/ml (R^2= 0.999) respectively. The sensitivity of the method was found to be higher, with a limit of detection (LOD) values of 0.42 µg/ml for kaempferol and 0.48 µg/ml for quercetin and limit of quantification (LOQ) values for kaempferol and quercetin 1.27 µg/ml and 1.47 µg/ml respectively. Percentage recovery values for kaempferol were found to be 99.18, 99.03 and 98.32 for the leaves, stem and root respectively, and for quercetin, they were 99.77, 100.12 and 100.54 for the leaves, stem and root respectively.

Conclusion: The developed HPLC method for the analysis of flavonoids has enabled rapid, accurate and reproducible determination. The method can be applied successfully for analysis of quercetin and kaempferol in various plant parts of *C. quadrangularis*.

Keywords: *Cissus quadrangularis,* HPLC, Kaempferol, Quercetin

INTRODUCTION

Since ancient time medicinal plants have been considered as main ingredients in Ayurvedic formulations for the treatment of various diseases and they have never lost their importance with the emergence of modern science. Plants possess pharmacological properties due to the presence of bioactive molecules, and this had led to more interest in developing simple and accurate methods of analysis of these bioactive markers.

Cissus quadrangularis is one of such important medicinal plants rich in steroids, flavonoids, and polyphenols. The extracts and powder of cissus have been used for many years to promote bone healing, as an analgesic and anabolic [1, 2]. So far, quite a few approaches have been developed for the ascertainment of the bioactive constituents from *C. quadrangularis*, but there is no systematic study on the simultaneous determination of flavonoids in different parts of *C. quadrangularis*. The purpose of this work is to analyze quercetin and kaempferol content in stems, leaves and roots of *C. quadrangularis* by HPLC, which would be useful for quality control applications of cissus and other plants associated with these components.

Cissus quadrangularis Linn. (Vitaceae family) is frequently distributed through out the hotter parts of India and Sri Lanka and is known as *asthisanhara* in Sanskrit. The plant is useful for the treatment of bone fracture, diarrhoea, skin disorders and scurvy [3-5]. The plant is described to contain high amounts of dietary antioxidants that includes vitamin C, carotenoids, and polyphenols. Tetracyclic triterpenoid (7-oxo, onocer-8-ene-3β, 21α-diol), ketosteroids, pentacyclic triterpenoids (δ-amyrin and *δ*-amyrone), β-sitosterol, stillbene derivatives and lipids have been reported from aerial parts, specifically from stems [6-9]. From the *C. quadrangularis* stems aliphatic acid hexadecanoic acid, triterpene δ-amyrin acetate and stilbene glucoside trans-resveratrol-3-O-glucoside were isolated for the first time. Long chain aliphatic hydrocarbons have also been reported from the hexane extract of leaves of *C. quadrangularis* [10, 11]. Other important constituents reported in the plant are flavonoids (quercetin, kaempferol), quadrangularis A, B, C, resveratrol, piceatanon, pallidol and perthenocissin [12-14].

The thorough screening of literature available on *C. quadrangularis* depicted an interesting fact that though the plant is a well-known restorative for a variety of ailments and a range of formulations have been marketed, little effort has been made to quantify the bioactive constituents present in different plant parts through scientific screening. The aim of the present proposed study is the qualitatively analyze quercetin and kaempferol in different plant parts of *C. quadrangularis* using HPLC, with the prime advantage that allows the possibility to enquire into one or more component without their previous isolation and purification.

MATERIALS AND METHODS

Plant material

Plant material (leaves, stem, and roots of *Cissus quadrangularis*) was procured from in-house medicinal plant garden maintained at Oriental College of Pharmacy, Sanpada, Navi Mumbai and was authenticated by a botanist (Specimen No. VJ/CQL/1).

Chemicals and reagents

All the chemicals used were of HPLC grade and analytical grade solvents were used for HPLC analysis was procured from Thomas Baker, Mumbai. Standard flavonoids i. e kaempferol and quercetin of HPLC grade (purity>95%) were purchased from Yucca Enterprises, Mumbai (MH).

Extraction

All the different plant parts (leaves, stem, and roots) were dried separately and powdered for further extraction. Accurately one gram of each sample was extracted with methanol used in small portions. A clear solution was obtained by filtering the sample solution. The stock solution after suitable dilutions was used for further analysis.

Method development

Instrument

Execution of HPLC using Phenomex C18 column (250*4.5 mm, 0.5 µ) on LC-20 AD Prominence liquid chromatograph (Shimadzu, Japan) attached to Spd-20A/20AV prominence SPD-20A prominence UV/Vis detector. An electronic weighing balance (1 mg sensitivity, Contech CA 123) and a sonicator (Expo-Hi-tech, Sr. No. 2K810011) were used.

Optimized chromatographic conditions

Quantitative determination of kaempferol and quercetin was performed for different plant part of the crude drug by HPLC as per standard procedure cited in the literature with some modification [15-18]. The optimized chromatographic conditions were as follows: volume injected 20 µl, flow rate 1.0 ml/min; detector was set at a wavelength of 370 nm for both quercetin and kaempferol. The mobile phase comprises of water: acetonitrile (45:55) containing 0.1 % o-phosphoric acid. The retention time of kaempferol obtained was 6.490±0.2 min and for quercetin, it was 5.083±0.2 min.

HPLC method validation [19]

System suitability test

The system suitability test was done by injecting six replicates of kaempferol and quercetin standard solution of 10 µg/ml concentration and observed the parameters viz. theoretical plate, tailing factor and percentage relative standard deviation (% RSD) of the area of the peak. As the acceptance criteria were ≤2% relative standard deviation (RSD) for peak areas, more than 2000 theoretical plates and less than 2 of the tailing factor, the results got were all within the acceptable limits.

Linearity and range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. It was determined by analyzing the area of the peak as a function of the concentrations of the analytes.

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The Calibration curve was found in the concentration range of 1-10 µg/ml for kaempferol and 0.5-10 µg/ml for quercetin. The linearity of this method was measured by linear regression analysis.

Quantification determination of kaempferol and quercetin by HPLC

Quantitative determination of kaempferol and quercetin was performed for each plant part of the crude drug by HPLC as per the optimized chromatographic conditions. The retention time of the peak in the samples was compared with the standard used. The amount of quercetin and kaempferol in crude drug extracts were determined from the linear regression equation of the calibration graph of quercetin and kaempferol. The method was validated for linearity, accuracy, precision and robustness of both the analytes.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. The accuracy of the method was evaluated carefully by running three replicate analyses at three different concentrations (within the working range).

LOD and LOQ

The LOD of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily constituted as an exact value. The LOQ of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ were established using the calibration curve parameters viz. slope and standard deviation. They are expressed as

$$LOD = 3.3 \frac{\sigma}{S}$$

$$LOQ = 10 \frac{\sigma}{S}$$

Where σ = standard deviation and S = slope of the curve

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision of the method was determined by the intraday precision (repeatability) and interday precision (intermediate precision) of kaempferol and quercetin solutions. Precision was determined in 3 replicates of both kaempferol and quercetin standard solution of 3 different concentrations on the same day (intra-day precision) and 3 injections of each concentration over a period of 3 d (inter-day precision).

Robustness

The robustness of an analytical procedure are a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The robustness of the method was determined by a small variety of conditions, including changes of flow rate and mobile phase ratio.

RESULTS AND DISCUSSION

System suitability

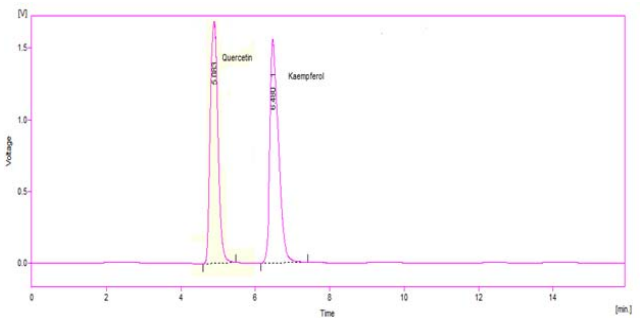

Fig. 1: HPLC chromatogram for simultaneous estimation of quercetin and kaempferol

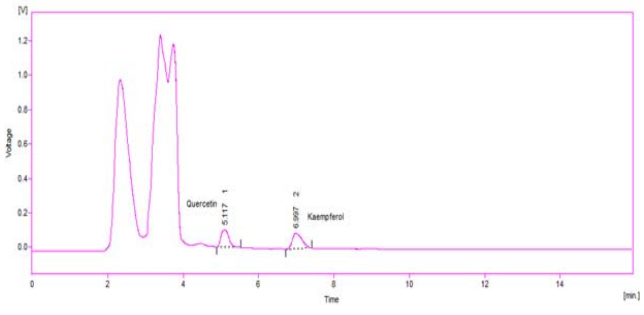

Fig. 2: HPLC chromatogram of *C. quadrangularis* leaves extract

Linearity

The graph was plotted with concentration (in µg/ml) and was found to be linear in the concentration range of 1-10 µg/ml (1.0, 2.0, 3.0, 5.0, 10.0 µg/ml) for kaempferol and 0.5-10 µg/ml (0.5, 1.0, 2.0, 5.0, 10.0 µg/ml) for quercetin. The regression equation obtained were $y = 49.598x - 6.9134$ $(r^2 = 0.999)$ and $y = 42.932x - 13.049$

($r^2 = 0.999$) for kaempferol and quercetin respectively, where y=peak area, x=solution concentration, and r^2=square of the correlation coefficient, which was determined. The result indicates that the method was linear over the concentration range plotted.

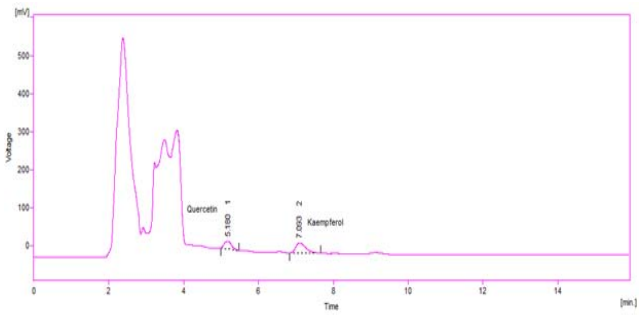

Fig. 3: HPLC chromatogram of *C. quadrangularis* stem extract

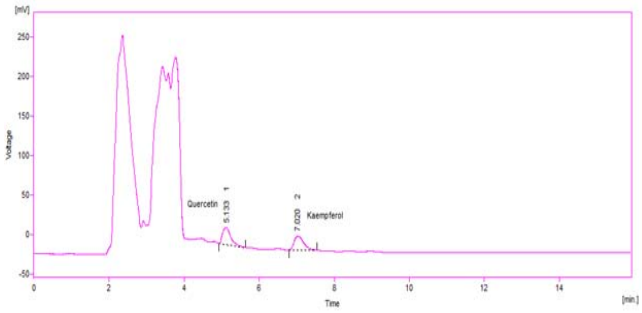

Fig. 4: HPLC chromatogram of *C. quadrangularis* root extract

Quantification of kaempferol and quercetin in stem leaves and roots of *C. quadrangularis*

Kaempferol and quercetin content were determined in different plant parts (leaves, stem, and roots) of *C. quadrangularis* using optimized chromatographic conditions. Kaempferol content in *C. quadrangularis* leaves, stem and roots were found to be 0.038, 0.017 and 0.013 (mg/g) respectively as calculated using linear regression equation. While the quercetin content was found to be 0.0478, 0.021 and 0.022 (mg/g) in leaves, stem and roots of *C. quadrangularis* respectively.

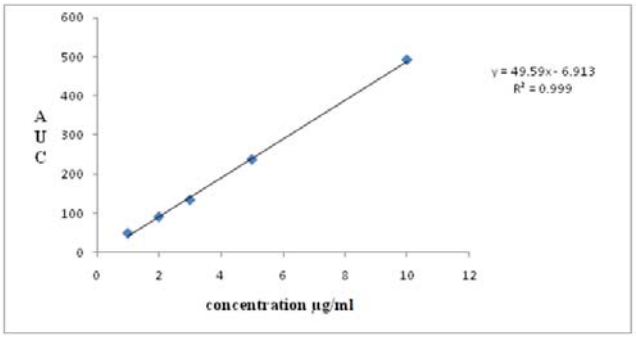

Fig. 5: Calibration curve of kaempferol by HPLC

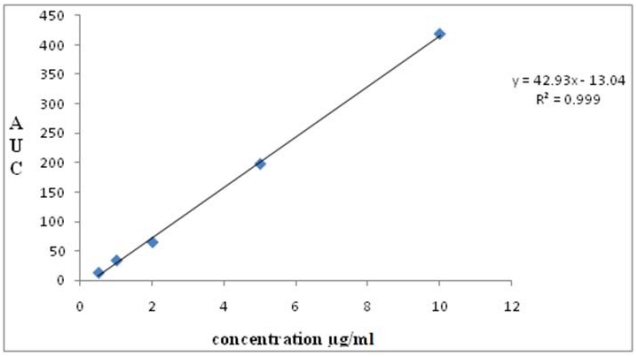

Fig. 6: Calibration curve of quercetin by HPLC

Accuracy

The mean percentage recovery values were found within the acceptable limits i.e. 98-102%, thereby proving the accuracy of the method.

Table 1: Results for accuracy for kaempferol

Plant part	Amount of kaempferol found (µg)	Amount of kaempferol added (µg)	Total amount of kaempferol (µg)	Amount of kaempferol found (µg) Mean (n=3)	% Recovery	Mean % Recovery
Leaves		2	40	39.6	99.00	99.18
	38	5	43	43.1	100.23	
		10	48	47.2	98.33	
Stem		2	19	19.2	101.05	99.03
	17	5	22	21.3	96.81	
		10	27	26.8	99.25	
Roots		2	15	14.9	99.33	98.32
	13	5	18	17.7	98.33	
		10	23	22.3	96.95	

Table 2: Results for accuracy for quercetin

Plant part	Amount of quercetin found (µg)	Amount of quercetin added (µg)	Total amount of quercetin (µg)	Amount of quercetin found (µg) Mean (n=3)	% Recovery	Mean % Recovery
Leaves		2	49	49.4	100.81	99.77
	47	5	52	51.5	99.03	
		10	57	56.7	99.47	
Stem		2	23	23.3	101.30	100.12
	21	5	26	26.1	100.38	
		10	31	30.6	98.70	
Roots		2	24	24.5	102.08	100.54
	22	5	27	26.8	99.25	
		10	32	32.1	100.31	

LOD and LOQ

LOD value for kaempferol was found to be 0.42 µg/ml and for quercetin it was found to be 0.48 µg/ml. And the LOQ value for kaempferol and quercetin were found to be 1.27 µg/ml and 1.47 µg/ml respectively.

Precision

The precision was performed and the results were expressed as % RSD of the measurements. The % RSD was found less than 2%, hence the method was found to be precise.

Robustness

As a result of changes in flow rate and mobile phase ratio the % RSD values did not exceed 2.0 %. It was concluded that the method was robust. Hence, the degree of reproducibility of results obtained and the data are presented below.

A number of methods are described in the literature for the analysis of flavonoids. Wang et. al has reported simultaneous HPLC of quercetin and kaempferol using methanol-0.4% phosphoric acid (47:53) as a mobile phase with a flow rate of 1.0 ml/min at 35 °C [17].

Table 3: Results for intraday precision

Conc. (µg/ml)	Precision (% RSD*)					
	2 h## (n=3)		4 h## (n=3)		6 h## (n=3)	
	RT**	PA***	RT**	PA***	RT**	PA***
kaempferol						
2	0.10	1.55	0.11	1.55	0.10	1.55
5	0.11	1.41	0.11	1.43	0.11	1.41
10	0.10	1.40	0.10	1.42	0.11	1.42
quercetin						
2	0.11	1.65	0.11	1.67	0.11	1.63
5	0.10	1.55	0.12	1.54	0.11	1.55
10	0.15	1.42	0.12	1.42	0.13	1.43

*% Relative standard deviation**Retention time***Peak area##Hour

Table 4: Results for inter-day precision

Conc. (µg/ml)	Precision (% RSD*)					
	d# 1 (n=3)		d# 2 (n=3)		d# 3 (n=3)	
	RT**	PA***	RT**	PA***	RT**	PA***
kaempferol						
2	1.11	1.64	1.13	1.62	1.13	1.65
5	1.15	1.95	1.14	1.93	1.16	1.95
10	1.13	1.99	1.14	1.98	1.15	1.95
quercetin						
2	1.10	1.78	1.11	1.80	1.14	1.80
5	1.14	1.66	1.15	1.66	1.14	1.68
10	1.14	1.95	1.13	1.94	1.16	1.96

*% Relative standard deviation**Retention time***Peak area#Day

Table 5: Results for robustness for kaempferol

Parameters	Changes	RT**	PA***
Flow Rate (ml/min)	0.98	6.501	500.14
	1.00	6.490	498.79
	1.02	6.474	495.03
	Mean	6.488	497.98
	% RSD*	0.21	0.53
Mobile Phase Ratio	44:56	6.491	489.67
	45:55	6.509	493.52
	46:54	6.517	493.22
	Mean	6.506	492.14
	% RSD*	0.20	0.44

*% Relative standard deviation**Retention time***Peak area

Table 6: Results for robustness for quercetin

Parameters	Changes	RT**	PA***
Flow Rate	0.98	5.102	425.83
(ml/min)	1.00	5.083	429.12
	1.02	5.077	427.89
	Mean	5.087	427.61
	% RSD*	0.26	0.39
Mobile Phase Ratio	44:56	5.081	430.13
	45:55	5.114	427.22
	46:54	5.193	431.47
	Mean	5.129	429.60
	% RSD*	1.12	0.51

*% Relative standard deviation **Retention time ***Peak area

HPLC analysis of quercetin and kaempferol in *C. quadrangularis* stems is also reported using acetonitrile and phosphate buffer with the elution of markers at 5.7 and 8.4 min respectively [18].

However, the proposed method enables a rapid, precise and accurate analysis of quercetin and kaempferol using isocratic elution. The present method offers the advantage of comparative analysis in different plant parts of *C. quadrangularis* without any interference from other components. The result also indicates a higher quercetin and kaempferol content in the leaves of *C. quadrangualris* as compared to the stems and roots of the plant.

CONCLUSION

In the present study kaempferol and quercetin content were determined in different plant parts (leaves, stem, and roots) of *C. quadrangularis*. The developed RP-HPLC method for analysis of flavonoids has enabled rapid, accurate and reproducible analysis of two flavonoids namely quercetin and kaempferol present in different plant parts of *C. quadrangularis.* The developed method can be used for quantitative analysis and quality control of extracts and commercial samples of other species containing flavonoids.

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CONFLICT OF INTERESTS

Declared none

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